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Selective labeling of peptides, metalloproteins, and proteolytic enzymes with Pt(II) complexes and electron-transfer reactions of native and cross-linked metalloproteins

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Selective labeling of peptides, metalloproteins, and proteolytic enzymes with Pt(II) complexes and electron-transfer reactions of native and cross-linked metalloproteins

by

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ABBREVIATIONS

AAPA	Ala-Ala-Pro-Ala
AAPF	Ala-Ala-Pro-Phe
AAPNA	acetyl alanine *p*-nitroanilide
Ac	acetyl
Arg	arginine
Asn	asparagine
Asp	aspartic acid
$[Au(trpy)Cl]Cl_2$	chloro(2,2':2",2-terpyridyl)gold(III) chloride
BAPNA	benzoyl arginine *p*-nitroanilide
BTEE	benzoyl tyrosine ethyl ester
Cys	cysteine
cyt *c*	cytochrome *c*
DIFP	diisopropylfluorophosphate
DSS	sodium salt of 3-(trimethylsilyl)-1-propane sulfonic acid
DST	disuccinimidyl tartarate
EDC	1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride
EDTA	ethylene diamine tetraacetic acid

EGS	ethyleneglycolbis(succinimidyl succinate)
Gln	glutamine
Glu	glutamic acid
GSH	reduced glutathione
HET	hydroxyethanethiolate
His	histidine
Im	imidazole
Lys	lysine
MB	methylene blue
Met	methionine
MLCT	metal-to-ligand charge transfer
NMR	nuclear magnetic resonance
pc	plastocyanin
Phe	phenylalanine
PMSF	phenylmethane sulfonic fluoride
Pro	proline
[Pt(trpy)Cl]Cl	chloro(2,2':2",2-terpyridyl)platinum(II) chloride
Ser	serine
Thr	threonine
TPCK	tosylphenylalanine chloroketone

Trp	tryptophan
trpy	2,2',2',2-terpyridyl
Tyr	tyrosine

GENERAL INTRODUCTION

The use of transition-metal complexes as bioconjugation reagents has not been extensively investigated. The only well-known application is the use of heavy atoms as labels in protein crystallography. For a general overview, see references 55 and 56. This dissertation focuses on the use of preformed transition-metal complexes as probes of protein structure and function. Such an approach provides an alternative method in the study of biomolecules with bioconjugation reagents. An overview of popular bioconjugation reagents is discussed in references 52, 53, and 54. Platinum(II) reagents were chosen for this study since they are thermodynamically stable and kinetically inert, and, therefore, when complexed with biomolecules, the metal tags will be stable. The chemistry of platinum(II) is well known. A comprehensive overview of platinum chemistry may be found in reference 136.

Gold(III) complexes, electronically and structurally similar to the platinum(II) complexes studied, were studied as well. The only gold complexes commonly used as bioconjugation reagents are those of gold(I) (see references 50 and 51 for examples). This dissertation extends the use of gold as a label for biomolecules to the +III oxidation state of the metal.

A second topic discussed is that of electron transfer between metalloprotein systems and their reactivity with external redox reagents. Many such electron-transfer systems have been studied by spectroscopic, chromatographic, kinetic, and modelling techniques. Many of these studies deal with the electron transfer system of cyt c and pc. Relevant studies may be found in references 184 through 193. One approach in studying electron transfer is to cross-link proteins to avoid association and dissociation processes (see reference 184 and references therein). In the current study, cyt *c* and pc are cross-linked with organic

spacers to determine how a flexible tether, rather than a tight cross-link, affects electron transfer between the two metalloproteins. In addition, the reactivity of such cross-linked complexes toward external reagents such as monomeric proteins is investigated.

Lastly, blue dyes are investigated as protein photoreductants. No systematic quantitation has been done on their reactivity toward proteins. The blue dyes present a new class of photosensitizers which can be used to study protein redox chemistry in much the same way that flavins have been used. See references 215, 216, and references therein concerning flavin photochemistry.

Explanation of Dissertation Format

Section I deals with the preparation and study of model complexes of the reagent $[Pt(trpy)Cl]^+$ with bioligands. The bulk of this work is part of two publications. See references 36 and 132. Section II deals with the reactivity of the same platinum reagent toward nonbiological ligands and is relevant to the platinum chemistry of Section I.

Section III deals with the preparation and study of model complexes of the reagent $[Au(trpy)Cl]^{2+}$ with bioligands and is unpublished work.

Sections IV, V, and VI address the reactivity of the $[Pt(trpy)Cl]^+$ complex toward three different protein systems. These works have already been published. See references 36, 132, and 182, respectively. Section VII addresses the reactivity of a related complex, $[Pt(sbpaphy)Cl]^-$ toward cyt c and was published. See reference 183.

Sections VIII and IX deal with the electron-transfer aspect of the thesis. Section VIII addresses electron transfer reactions

between the two metalloproteins cyt c and pc, and Section IX discusses reactions of blue dyes with cyt c. These works are unpublished.

References, tables, and figures are sequentially numbered throughout the thesis beginning with Section I.

SECTION I.

AMINO ACID AND PEPTIDE MODEL COMPLEXES OF PT(TRPY)CL+

INTRODUCTION

The transition-metal reagent, chloro(terpyridine)platinum (II) is a potentially useful bioconjugation reagent. Prior to work in this laboratory, the only biological application of the [Pt(trpy)Cl]Cl reagent has been its use as a synthetic template for intercalation reagents of DNA.[1,2] In this study the coordination chemistry of the platinum complex is used as a means to covalently attach the reagent to nucleophilic side chains of amino acids in proteins.

In order to understand its reactivity with large biomolecules, such as proteins, it is necessary to test the reactivity of [Pt(trpy)Cl]Cl with appropriate models of proteins. The monomeric unit of a protein, an amino acid, is a reasonable choice for such a model. Of course, a continuum of models from the simplest case to that which better resembles the protein is desired. A simpler model than an amino acid would be the isolated reactive group of an amino-acid side chain. A more complex model would be a peptide which includes a reactive amino acid in its sequence. This continuum of models--reactive group, amino acid, and peptide--is shown to be a useful first step in understanding the reactivity of $[Pt(trpy)Cl]^+$ with proteins.

EXPERIMENTS

Materials and Methods

The amino acids, amino-acid derivatives, peptides, and (*p*-hydroxymercurio)-benzene sulfonic acid were obtained from Sigma Chemical Co. Chloro (2,2':6',2"-terpyridine)platinum (II) chloride dihydrate, $[Pt(trpy)Cl]Cl \cdot 2H_2O$, and (2-hydroxyethanethiolate)(2,2':6',2"-terpyridine)platinum (II) nitrate, $[Pt(trpy)HET]NO_3$, were purchased from Strem Chemicals.[3] Microanalysis was done by Galbraith Laboratories.

The ^1H NMR spectra were recorded in D_2O solutions with a Nicolet NT 300 spectrometer at 22° C. The residual water signal, at 4.79 ppm versus DSS, was used as an internal reference. The ^{195}Pt NMR spectra of 15-30 mM solutions of $[Pt(trpy)L]^{2+}$ complexes were recorded with a Bruker WM 300 spectrometer at 64.4 MHz, using a 20-mm broad-band probe. The chemical shifts are expressed with respect to $PtCl_4^{2-}$ [4,5]. Absorption spectra were recorded with an IBM 9430 UV-vis spectrophotometer equipped with a two-grating monochromator.

Molecular orbital calculations were done by Mr. Longgen Zhu using the Fenske-Hall method.[6] The dimensions of the $Pt(trpy)^{2+}$ fragment[7] and of the $Pt\text{-}S(CH_3)_2$[8,9] and platinum-imidazole units[10,11] were taken from actual structures. The standard basis sets were used[12].

Survey of Amino Acids

Of the twenty common amino acids, every amino acid containing a heteroatom in the side chain was incubated with [Pt(trpy)Cl]Cl at room temperature for several days; the incubations were effected by addition of 1.0 mL of 5 mM solution of amino acid to 1.0 mL of

5 mM solution of [Pt(trpy)Cl]Cl. Water (purified with a Barnstead Nanopure system and with a resistivity greater than 18 ohm cm) was the medium. The following amino acids caused no change in the UV-vis spectrum after one day: Lys, Trp, Arg, Asp, Asn, Glu, Gln, Pro, Thr, Ser, Tyr, and Met. With cysteine (Cys), the color changed immediately. With histidine (His), the color change developed over an hour. Arg does react under more forcing conditions.[13]

Complexes $[Pt(trpy)L]^{2+}$ with Histidine and its Homologs

A solution containing 15.5 mg (0.1 mmol) of free base L-histidine in 1.0 mL of water was added dropwise to a stirred solution containing 53.2 mg (0.1 mmol) of $[Pt(trpy)Cl]Cl \cdot 2H_2O$ in 4.0 mL of water, and the mixture was kept at 50° C. Although the color soon changed from dark orange to pale yellow and the absorbance quotient (A_{342}/A_{328}) became constant in less than an hour, the heating was continued for 1 day. A concentrated aqueous solution containing 68.4 mg (0.2 mmol) of sodium tetraphenylboron was added dropwise to the stirred reaction mixture. After cooling, the yellow precipitate was filtered off, washed with cold water, and dried overnight in air: yield was 82 mg or 65%. Anal. Calcd. for $[Pt(trpy)His](BPh_4)_2 \cdot H_2O$: C,65.87; H, 5.13; N, 6.68. Found: C, 65.25; H, 4.91; N, 6.53.

Ligands imidazole (Im), Nα-acetyl-histidine (AcHis), L-histidyl-L-histidine (His-His), L-histidyl-L-lysine (His-Lys), and glycyl-L-histidyl-glycine (Gly-His-Gly) were similarly treated with [Pt(trpy)Cl]Cl. After the quotient A_{342}/A_{328} became constant, the complexes in solution were characterized by UV-vis and ^1H NMR spectroscopy. The Job's plot[2] showed that Gly-His-Gly and $Pt(trpy)Cl^+$ react in the ratio of 1:1.

Complexes $[Pt(trpy)L]^{2+}$ with Cysteine and its Homologs

Ligands 1-hyrdroxyethanethiol, homocysteine, and reduced glutathione (GSH) were treated with [Pt(trpy)Cl]Cl under the same conditions as Cys (*vide supra*). These reactions were also done in 85 mM phosphate buffer at pH 7.0. The reaction of $[Pt(trpy)Cl]^{+}$ with glutathione in 0.1% CF_3COOH was done similarly.

Stability of $[Pt(trpy)L]^{n+}$ Complexes

The reaction of $[Pt(trpy)His]^{2+}$ with CysH and the reaction of $[Pt(trpy)Cl]^{+}$ with $CysHgC_6H_4SO_3^-$ were effected by mixing together equimolar amounts of the two reactants at room temperature; in a typical experiment, 1.0 mL of a 5.0 mM solution of each reactant in 100 mM phosphate buffer at pH 7.0 was used. Compound $CysHgC_6H_4SO_3H$ was prepared by the treatment of cysteine (250 µl of a 5.0 mM solution, 1.3 µmol) with *p*-$HOHgC_6H_4SO_3H$ (5.0 mg, 13 µmol) for 2 h; the reaction of $[Pt(trpy)Cl]^{+}$ with $CysHgC_6H_4SO_3H$ was then effected by addition of 200 µl of a 5.0 mM solution of $[Pt(trpy)Cl]^{+}$ to the mixture. Both of these reactions caused a change from yellow to red within seconds. The reaction of $[Pt(trpy)Cys]^{+}$ with $HOHgC_6H_4SO_3^-$, of $[Pt(trpy)His]^{2+}$ with $CysHgC_6H_4SO_3^-$, and of $[Pt(trpy)His]^{2+}$ with $HOHgC_6H_4SO_3^-$ were attempted in a similar manner. These latter three reactions failed to cause any color change even after three days.

Unreactivity of Thio-ether and Disulfide Ligands

Compound [Pt(trpy)Cl]Cl was treated with DL-methionine, N-acetyl-DL-methionine, N-acetyl-L-methionine amide, L-methionine methyl ester hydrochloride, S-methyl-L-cysteine, tetrapeptide Trp-Met-Asp-Phe hydrochloride, cysteine, and oxidized glutathione. First,

[Pt(trpy)Cl]Cl was incubated with an equimolar amount of each ligand at the concentrations of 0.5, 1, 2, 5, 10, or 20 mM, and the mixtures were heated at 50°, 60°, 80°, 90°, or 100° C for 10 h. Next were incubations with tenfold excess of N-acetylmethionine for several hours at 90° C and with 100-fold excess of methionine for 75 days at room temperature. Finally, compound [Pt(trpy)Cl]Cl was treated with $AgNO_3$, AgCl was removed by centrifugation with a Beckman Model TJ-6 centrifuge at 4000 rpm, and an equivalent amount of the ligands was added. None of the experiments caused significant changes in the UV-vis and ^1H NMR spectra of the starting complex of the added ligands. Cation-exchange chromatography of the mixtures on CM-52 cellulose permitted virtually full recovery (*ca.* 95%) of [Pt(trpy)Cl]Cl.

Kinetic Measurements

The relatively slow reactions of [Pt(trpy)Cl]Cl with Im, His, and Gly-His-Gly were followed by a Varian Cary 19 spectrophotometer. The relatively fast reactions of [Pt(trpy)Cl]Cl with 1-hydroxyethanethiol, Cys, and GSH were monitored with an IBM 9430 spectrophotometer equipped with a thermostated rapid-kinetics accessory SFA-11 from Hi-Tech Scientific. All the solutions were made in 85 mM phosphate buffer at pH 7.0, thermostated at 25° C, and the reactions monitored at 343 nm. Pseudo-first-order conditions were achieved with 20 μM $[Pt(trpy)Cl]^+$ and 2 mM entering ligands. The observed rate constants, k_{obsd}, were calculated from the absorbances by a nonlinear least-squares method.

RESULTS AND DISCUSSION

General Reactivity toward Amino Acids

The $[Pt(trpy)Cl]^+$ complex does not react with the α-carboxylate or α-amino groups on the amino acids. The unreactivity of $[Pt(trpy)Cl]^+$ toward many of the amino acids demonstrates this since all amino acids have these two functional groups. The ancillary terpyridyl ligand prevents chelation of the platinum by the α-carboxylate and α-amino groups. Such chelation is well known in $PtCl_4^{2-}$ complexes[14] and other similar square planar complexes[15,16] where more than one site is open to coordination.

Since any side chain with a heteroatom is deemed a potential nucleophile, tests for reactivity with all such amino acids were performed. Platinum is a class b, soft metal and is expected to react with soft bases, such as sulfur. In addition to this, pK_a values of potential ligands have to be considered as a factor in reactivity. From the first criterion, the amino acids methionine (a thioether) and cysteine (a thiol) are expected to be good nucleophiles. Thioethers and thiols and thiolates are known to be good nucleophiles toward platinum.[17] As expected, cysteine is reactive toward the platinum complex. Reactions with thiol and thiolate ligands are discussed below. Surprisingly, methionine was unreactive toward the platinum complex. This too is discussed in detail below.

Ligands with nitrogen atoms as donors are relatively good nucleophiles toward platinum.[17] The reactivity of nitrogen-containing ligands towards platinum depends on the hybridization and pK_a of the nitrogen atom. Hybridized atoms with more s character will have larger overlap integrals than ones with less s character and will form stronger bonds with another atom. Protonation and deprotonation (the pK_a value) will free or shield a

pair of basic lone pair electrons and hence alter the reactivity. The $[Pt(trpy)Cl]^+$ is unreactive toward the ε-amino group on lysine since it has a high pK_a value (10.5) and is also sp^3 hybridized. These same properties of the α-amino groups explain their unreactivity toward platinum. Only when a reaction is aided by the entropic effect of chelation, will such a nitrogen atom coordinate to platinum. The pyrrole-type nitrogen atom in tryptophan is also sp^3 hybridized and has an estimated pK_a of 14 (based on the pK_a for the nitrogen atom in pyrrole).[18] The imino group on arginine also has a high pK_a (12.5), but the N atom is sp^2 hybridized, and it does show reactivity toward $[Pt(trpy)Cl]^+$ under more forcing conditions. The α-nitrogen in proline, although not a side chain atom, has a high pK_a (10.6) and is sp^3 hybridized. The amide nitrogens on glutamine and asparagine have high pK_a values and are also sp^3 hybridized. The Nε2 nitrogen in the imidazole ring of the side chain of His has a relatively low pK_a value of 6 and is sp^2 hybridized. Both of these factors favor reactivity toward His. Histidine is reactive toward $[Pt(trpy)Cl]^+$ and is discussed in detail below.

The amino acids with oxygen atoms as potential donors are unreactive toward the platinum complex. Oxygen atoms are classed as hard bases and this unreactivity is not surprising. Although several complexes of platinum with oxygen donors are known, the hardness, in conjunction with the high pK_a values of serine, threonine, and tyrosine (all alcohols), make them poor nucleophiles toward platinum. Carboxylate oxygen atoms appear unable to coordinate to platinum in a monodentate fashion. Although the low pK_a values of carboxylate oxygens (pK_a 3-4) favors reactivity, the electron lone pair is held tightly against the electronegative oxygen atom and disfavors reactivity toward platinum. It would be an interesting study to test the reactivity of platinum complexes toward activated alcoxides at physiological pH.

Complexes of Histidine and Cysteine and their Derivatives

The model complexes $[Pt(trpy)L]^{n+}$ are shown in Scheme I. Imidazole, histidine, and its homologs and 2-hydroxyethanethiol, cysteine, and its homologs displace the Cl^- ligand from $[Pt(trpy)Cl]^+$ as shown in Scheme I. The UV-visible properties of all of the resultant complexes are shown in Table I. The bands in the region 300-350 nm can be attributed to

Table I. Absorption spectra of $[Pt(trpy)L]^{n+}$ complexes, models for tags on proteins

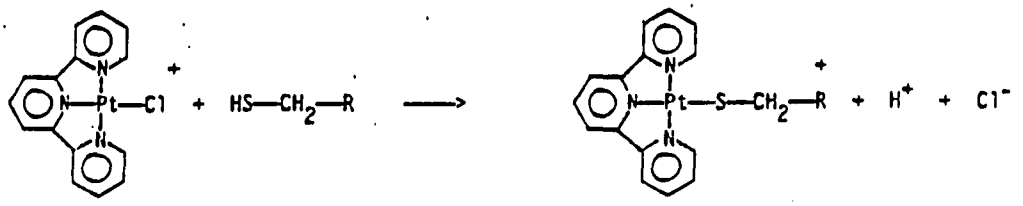
		absorptivity, ε, of 10 μM aqueous solution, $M^{-1}cm^{-1}$					
L	n[a]	342[b]	328	311	278	270	242
$-SCH_2OH$	1	12800	11200	11200	19500		27800
Cys	1	14200	12400	12100	21500		30300
GSH[c]	0	14100	12600	12500	21800		30900
Im	2	16000	10400			21200	31800
His	2	14600	9400			19600	28700
GHG[d]	2	13700	10400			22000	30800

[a] When the thiol, imidazole, and carboxyl groups are deprotonated and the amino groups are protonated.

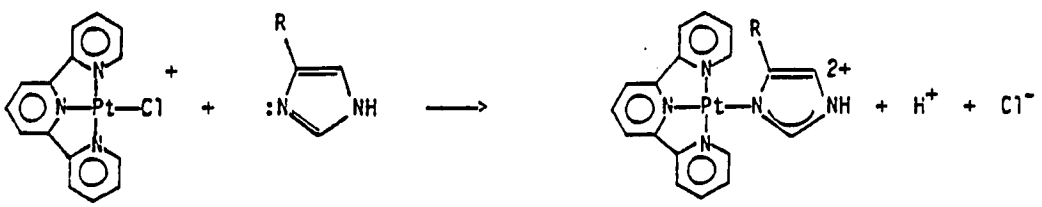
[b] wavelength in nm.

[c] Glutathione.

[d] Gly-His-Gly.



Ligand	−R
2-hydroxy-1-ethanethiol	$-CH_2-OH$
Cys	$H_3\overset{+}{N}-\underset{\displaystyle CH_2}{\underset{|}{CH}}-COO^-$
Glu-Cys-Gly	$H_3\overset{+}{N}-\underset{\displaystyle COO^-}{\underset{|}{CH}}-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-NH-\underset{\displaystyle CH_2}{\underset{|}{CH}}-\overset{O}{\overset{\|}{C}}-NH-CH_2-COO^-$



Ligand	−R
Im	H
His	$H_3\overset{+}{N}-\underset{\displaystyle CH_2}{\underset{|}{CH}}-COO^-$
Gly-His-Gly	$H_3\overset{+}{N}-CH_2-\overset{O}{\overset{\|}{C}}-NH-\underset{\displaystyle CH_2}{\underset{|}{CH}}-\overset{O}{\overset{\|}{C}}-NH-CH_2-COO^-$

Scheme I.

metal-to-ligand charge-transfer (MLCT) transitions because of their high intensity and their dependence on ligands L and on other species in solution.[2,19] The bands below 300 nm are due to transitions in the aromatic terpyridyl ligand. The relative intensities of the bands, especially of those at 342 and 328 nm, are nearly identical among the complexes with similar ligands L, but markedly different between the complexes with different ligands, such as imidazole and thiolate. Job's plots[2]show that each of the tripeptides γ-glutamyl-cysteinyl-glycine (glutathione) and glycyl-histidyl-glycine has only one group that is reactive toward $[Pt(trpy)Cl]^+$--thiol and imidazole, respectively. The terdentate trpy ligand obviously prevents multidentate binding of other ligands.

For the imidazole complexes--although all the complexes contain imidazole as the fourth ligand--their $\varepsilon_{342}/\varepsilon_{328}$ quotients differ. The main chain, attached to the imidazole ring, evidently affects the chromophore by direct interaction or by altering the solvation of the complex. The relative band intensities proved diagnostic of the labels differently located on proteins, as will be discussed below. Since all of the $[Pt(trpy)L]^{n+}$ complexes, where L is an imidazole-type ligand, yield virtually identical ^1H NMR spectra (see Table II) , all of the ligands must be bonded solely through the imidazole ring. Since the pyrrole-type nitrogen atom is highly basic (pK_a 14.5 in imidazole),[20] it remains protonated in the experimental conditions used in this study. Evidently, the ligating atom is the pyridine-type nitrogen, whose pK_a value is 6.0-6.5.[20]

The ^1H NMR signals were assigned on the basis of NMR titrations of $[Pt(trpy)Cl]^+$ with the ligands and of pH titrations of formed complexes. The assignments are shown in Table II. These assignments agree with previous analyses of (terpyridine)platinum

Table II. ^1H NMR of $[Pt(trpy)L]^{n+}$ complexes and of free imidazole-containing ligands L

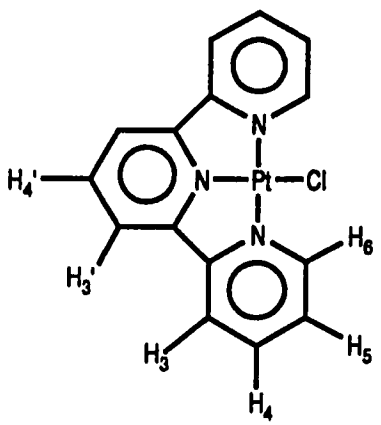
	^1H NMR Chemical shift [a], ppm											
	Pt(trpy)				Im		aliphatic					
L	H4,4'	H3,3'	H6	H5	H2	H5	H7	H8	H9	H10,11	H12	H13
Cl-	8.08	7.88	7.82	7.37								
	m,3H	d,4H	m,2H	m,2H								
Im	8.33	7.85	8.45	7.67	8.5	7.58						
	m,3H	m,4H	m,2H	m,2H	s,1H	s,1H						
AcH	8.32	7.82	8.45	7.66	8.50	7.44	4.55	3.18	1.96			
	m,3H	m,4H	m,2H	m,2H	s,1H	s,1H	t,1H	d,2H	s,3H			
GHG [b]	8.41	7.84	8.43	7.73	8.52	7.55				3.82	3.46	[c]
	m,3H	m,4H	m,2H	m,2H	s,1H	s,1H				m,4H	m,2H	
Free Ligand												
Im					7.73	7.09						
					s,1H	s,1H						
AcH					7.67	6.90	4.40	3.00	1.94			
					s,1H	s,1H	t,1H	d,2H	s,3H			
GHG [b]					7.82	7.01				3.75	3.13	4.70
					s,1H	s,1H				m,4H	m,2H	t,1H

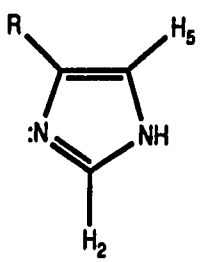
[a] For solutions in D_2O at 22° C.

[b] Glycyl-histidyl-glycine.

[c] Signal of H13 is buried under the HDO peak.

complexes[7] and of the free amino acids and peptides.[23-25] Coordination is invariably accompanied by a large movement downfield of the H^6 signal and by slight such movements of the other trpy signals. Similar changes, observed in $[Pt(trpy)SR]^+$ complexes, were attributed to the magnetic anisotropy of the Pt-S bond,[7] and it is possible that the new Pt-N bond may have a similar effect. The imidazole signals move downfield because of the electron-attracting power of the Pt(II) complex.[26,27] The aliphatic H atoms in the main chain, distant from the donor atom, are not affected significantly. No further investigation of the NMR spectroscopic properties of the thiolate complexes was done, since these data are well documented.[7] The numbering of the hydrogen atoms for Table II are shown below.





In addition, the spectrophotometrically determined overall rate constant, k_{obsd}, for the reaction between 20 μM [Pt(trpy)Cl]Cl and 0.40 mM imidazole at pH values between 5.5 and 8.6, increases with increasing pH, indicating a deprotonation process occurring in this pH range. The reaction of $[Pt(trpy)Cl]^+$ with imidazole is shown in equations (1) and (2) below.

$$ImH^+ \underset{<\!\!-k_{-1}-}{\overset{-k_1\!\!\rightarrow}{}} Im + H^+ \quad (1)$$

$$Pt(trpy)Cl^+ + Im \xrightarrow{k_2} Pt(trpy)Im^{2+} + Cl^- \quad (2)$$

If a preequilibrium is considered, then equation (3) is true. The

$$k_{obs} = \frac{K_a k_2 [Im]_t}{K_a + [H^+]} \quad (3)$$

increase in reaction rate observed with increasing pH is what is expected upon examination of equation (3).

The ^{195}Pt chemical shifts for $[Pt(trpy)Cl]^+$, $[Pt(trpy)Im]^{2+}$, and $[Pt(trpy)His]^{2+}$ are -1080, -1150, and -1146 ppm with respect to K_2PtCl_4. These shifts fall in the region characteristic of $Pt(II)N_4$ complexes.[21] The signals appear about 70 ppm upfield from that of $Pt(trpy)Cl^+$, as expected upon displacement of the Cl^- ligand by a nitrogen donor.[22]

Kinetics of Displacement Reactions

The reactions shown in Scheme I were followed under pseudo-first-order conditions, with a 100-fold excess of entering ligands. The rate constants in Table III show that thiol ligands react approximately 300-times faster than imidazole ligands under identical conditions. A natural consequence of this difference in rate is the complete selectivity of $[Pt(trpy)]Cl^+$ for γ-Glu-Cys-Gly over Gly-His-Gly. When the two tripeptides were allowed to compete for the platinum complex, both the rate constant k_{obsd} and the absorption spectrum characteristic of the glutathione complex were obtained. The observed selectivity is consistent with the large difference between thiolate and imidazole ligands in their

nucleophilicity toward Pt(II) complexes; the respective n_{pt} parameters are 7.17 and 3.44.[17] The selectivity remains although, at pH 7.0, the concentration of deprotonated imidazole (pK_a 6.0 in histidine) is 18 times larger than that of deprotonated thiolate (pKa 8.3 in cysteine).[18] The rate constants for the proteins in Table III are discussed below.

Table III. Pseudo-first-order rate constants for reactions shown in scheme I[a]

ligand	k_{obsd}, s^{-1}
$HSCH_2CH_2OH$	$(2.5 \pm 0.3) \times 10^{-2}$
Cys	$(1.3 \pm 0.3) \times 10^{-2}$
γ-Glu-Cys-Gly	$(5.5 \pm 0.3) \times 10^{-2}$
γ-Glu-*Cys*-Gly and Gly-*His*-Gly[b]	$(1.2 \pm 0.4) \times 10^{-2}$
Im	$(2.5 \pm 0.1) \times 10^{-4}$
His	$(0.85 \pm 0.1) \times 10^{-4}$
Gly-His-Gly	$(3.1 \pm 0.1) \times 10^{-4}$
horse heart cyt *c* [c]	$(3.4 \pm 0.4) \times 10^{-4}$
bakers' yeast cyt *c* [c]	$(3.5 \pm 0.4) \times 10^{-4}$

[a]At 25° C, with 20 μM [Pt(trpy)Cl]Cl and 2 mM entering ligand.
[b]Equimolar amounts of competing ligands.
[c]Discussed in Section IV.

Stability of $[Pt(trpy)L]^{n+}$ Complexes

Success of peptide-mapping experiments (as discussed in Section IV) depends on the stability of the $Pt(trpy)^{2+}$ tags at the sites of their initial attachment to the proteins. Migration of the tags during protein digestion or during chromatography of the resulting

peptides could cause an erroneous identification of the binding sites. The $[Pt(trpy)His]^{2+}$ complex is stable even at pH 3.0, but since the thiolate is far more nucleophilic than imidazole, the displacement reaction in eq (4) is a very facile and rapid one. The symbol H denotes protonation

$$[Pt(trpy)His]^{2+} + CysH \longrightarrow [Pt(trpy)Cys]^{+} + HisH^{+} \quad (4)$$

of the side chain. The reaction in equation (4) is fast even at pH 2.0, at which the concentration of the thiolate form is negligibly low. Standard peptide-mapping procedures proved inadequate for the bakers' yeast cytochrome *c* (see Section IV) because the tag did migrate from the His-containing peptides to the Cys-containing one. This migration was prevented by a method based on the following reactions.

Even in the presence of a large excess of $HOHgC_6H_4SO_3^-$, the platinum complex displaces the organomercurial reagents from the cysteinato compound of the latter, according to eq (5). The reaction is rapid and the UV-vis spectrum

$$[Pt(trpy)Cl]^{+} + CysHgC_6H_4SO_3^- \longrightarrow [Pt(trpy)Cys]^{+} + ClHgC_6H_4SO_3^- \quad (5)$$

showed $[Pt(trpy)Cys]^{+}$ to be the only platinum complex present. The affinity of $[Pt(trpy)Cl]^{+}$ toward cysteine side chain evidently is extremely high, higher even than the well-known affinity of the organomercurial reagents, which are commonly used for cysteine modifications. The reaction in eq (6) does not happen.

$$[Pt(trpy)Cys]^{+} + HOHgC_6H_4SO_3^- \xrightarrow{NRXN} [Pt(trpy)OH]^{+} + CysHgC_6H_4SO_3^- \quad (6)$$

A similar reaction, shown in eq (7), involving $[Pt(trpy)His]^{2+}$,

does not happen. Fortunately, this organomercurial reagent cannot displace the platinum labels from the histidine residues, eq (8).

$$[Pt(trpy)His]^{2+} + CysHgC_6H_4SO_3^- \xrightarrow{NRXN} [Pt(trpy)Cys]^+ + HisHgC_6H_4SO_3^- \quad (7)$$

$$[Pt(trpy)His]^{2+} + HOHgC_6H_4SO_3^- \xrightarrow{NRXN} [Pt(trpy)OH]^+ + HisHgC_6H_4SO_3 \quad (8)$$

These experiments show that the $Pt(trpy)^{2+}$ tag can migrate from histidine to free cysteine (eq 4) but not to mercurated cysteine (eq 7). Treatment of the $Pt(trpy)^{2+}$-modified protein with $HOHgC_6H_4SO_3^-$ prior to tryptic digestion ensures the fidelity of peptide mapping, as will be discussed in Section IV.

Unreactivity of Thio-ether and Disulfide Ligands

Six thio ethers (methionine and its derivatives) and two disulfides (cystine and oxidized glutathione) failed to displace the Cl^- ligand from $[Pt(trpy)Cl]^+$ even under forcing conditions. Their unreactivity is surprising in view of the affinity of the soft, nucleophilic thio-ether ligands toward the soft, electrophilic Pt(II) atom,[17,28-30] especially since the trpy ligand accelerates the displacement of the fourth ligand; for example, $[Pt(trpy)Cl]^+$ is approximately 10^3-10^4 times more reactive than its aliphatic homolog, $[Pt(dien)Cl]^+$, toward various small nucleophiles.[31]

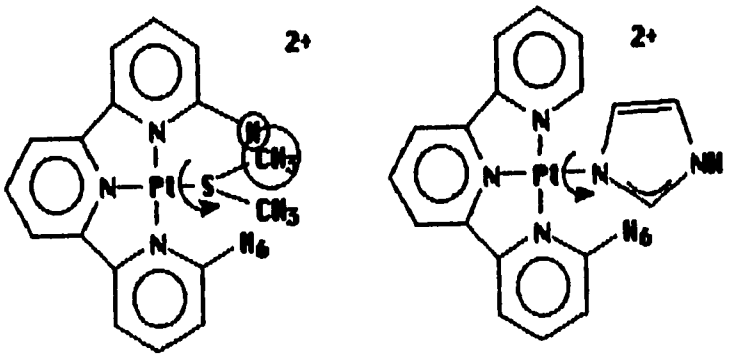
Whereas methionine, S-methylcysteine, and oxidized glutathione are unreactive, homocysteine, cysteine, and reduced glutathione displace the Cl^- ligand fast.[2] These thiols react rapidly even in the neutral and weakly acidic solutions, in which the SH group (pK_a 8.3) is protonated. Although the anionic thiolate is more nucleophilic than the thio-ether or disulfide, the contrast between

reactivity and inertness cannot be ascribed solely to the difference in nucleophilicity. The unreactivity of the biological thio-ethers and disulfides is attributed to the bulkiness of their respective $RSCH_3$ and RSSR functional groups. The only thio-ether reported to react with $[Pt(trpy)Cl]^+$ is tetrahydrothiophene, a five-membered ring with a relatively small bond angle and cone angle at the S atom.[32] Since no details of this reaction were given, it is impossible to consider this reaction in the context of the present study. Since steric effects on the rate of substitution at Pt(II) metal centers are known,[33] molecular models were examined. The pentacoordinate $[Pt(trpy)Cl(SMe_2)]^+$, representing the putative intermediate or the transition state of the reactions that failed to occur, does not seem to be prohibitively crowded, but the expected product, $[Pt(trpy)SMe_2]^{2+}$, seems to be. The origin of this steric hindrance was examined in a quantum-chemical study which is summarized below.

Electronic Structure and Bonding

Molecular orbital calculations of $[Pt(trpy)SMe_2]^{2+}$ were performed with Pt-S torsion angles of 0°, 22.5°, 45°, 67.5°, and 90° . In each case total overlap population between the Pt and S atoms is 0.44 to 0.47 e, corresponding to a normal metal-ligand bond. Regardless of the torsion angle, however, the contacts between a methyl group of the thio ether and an ortho H atom of a pyridine ring are far shorter than the sum of the van der Waals radii; see Scheme II. These repulsions between the filled C-H orbitals, a quantum mechanical equivalent of steric crowding, outweigh the Pt-S attraction and cause net repulsion between the $Pt(trpy)^{2+}$ and the thio ether. On the contrary, net attraction seems to exist between the $Pt(trpy)^{2+}$ and imidazole fragments at all Pt-N torsion angles

although the planar conformation exhibits some crowding. Calculations of $PtCl_3(SMe_2)^-$ and $PtCl_3Im^-$ complexes reveal an absence of steric interactions because the Cl^- ligands are sufficiently small.



SCHEME II

SECTION II.

COMPLEXES OF [PT(TRPY)CL]CL WITH NONBIOLOGICAL LIGANDS

INTRODUCTION

The inorganic chemistry of the [Pt(trpy)Cl]Cl complex is still largely undeveloped. This platinum complex provides a new template on which a variety of chemical structures can be built. Of interest is the interaction of stacked aromatic systems, such as the eclipsing of two $Pt(trpy)^{2+}$ groups. Also of interest is the possibility of placing two $Pt(trpy)^{2+}$ groups close enough together such that there may be some metal-metal interaction. Since sulfur and nitrogen atoms are good donors toward platinum, ligands with multiple potential binding sites of sulfur or nitrogen were chosen for study. The strong spectroscopic properties of the $Pt(trpy)^{2+}$ chromophore may serve as an indicator of new interactions, such as Pt-Pt interaction or π-stacking.

EXPERIMENTS

Chemicals

Dithiooxamide, cyanuric acid, and pyridazine were obtained from Aldrich Chemical Co. Dithiobiuret was obtained from Fairfield. Dithiobiurea was obtained from Alfa Products. [Pt(trpy)Cl]Cl was obtained from Aldrich. Sodium tetraphenylboron was obtained from J. T. Baker Chemical Co.

Reactions of [Pt(trpy)Cl]Cl with Dithiocarbonyl Ligands

The solubility of dithiooxamide in water at 25° C is very low. A solution of dithiooxamide was saturated by heating at 60° C for 1 hour and then cooling back to room temperature. Four mL of this solution was then placed in a preweighed vessel (12.6124 g) and the solution was evaporated and the vessel reweighed (12.6153 g). The net weight was 2.9 mg. The solubility of dithiooxamide (M.W.=120.2 g/mol) is approximately 6 mM. A second trial indicated a solubility of 4.7 mM.

A quantity of 3.7 mg of [Pt(trpy)Cl]Cl was dissolved in 1.380 mL (5 mM) and divided into 2 aliquots of 690 μL each. The molarity of the dithiooxamide was assumed to be 5.4 mM *(vide supra)*. An aliquot of 640 μL of dithiooxamide solution is required for a 1:1 reaction with one of the platinum aliquots. The [Pt(trpy)Cl]Cl was added dropwise with stirring to an equal volume of the dithiooxamide solution. The reaction mixture immediately turned a brownish-red color and then a heavy brown precipitate fell out of solution in approximately one minute. The experiment was repeated in the dark and yielded identical results.

A 12.1 mg quantity of [Pt(trpy)Cl]Cl was dissolved in 4.520 mL of H_2O and divided into 6 aliquots of 750 μL each (5 mM in

[Pt(trpy)Cl]Cl). Dithiobiuret (10.6 mg) was dissolved in 4170 μL of H_2O. A 200 μL aliquot of the dithiobiuret solution was added dropwise with stirring to one of the $[Pt(trpy)Cl]^+$ aliquots to make a 1:1 reaction mixture. Dithiobiurea (7.9 mg) was dissolved in 2780 μL of H_2O and 200 μL of this solution was added to another $[Pt(trpy)Cl]^+$ aliquot to make a 1:1 reaction mixture. Each of these reactions was divided into two aliquots. To one aliquot was added another equivalent of $[Pt(trpy)Cl]^+$. The addition of the second equivalent of $[Pt(trpy)Cl]^+$ caused a darkening of the solutions. The volume of the 2:1 mixture was adjusted such that the platinum concentrations were equal in both the 1:1 and 2:1 mixtures. These reaction mixtures were studied with UV-vis spectrophotometry and then heated at 80° C for 1.5 hours. After standing at room temperature another 3.5 hours, UV-vis spectra were again recorded. A similar prep was done with dithiooxamide and a 2:1 reaction mixture of dithiooxamide:platinum was prepared.

As a control, a 1:1 thiourea:Pt(trpy)Cl solution was prepared in the same manner as the 1:1 reactions discussed above. Furthermore, a reaction mixture of 300:1 urea:Pt(trpy)Cl+ was prepared in water. The concentration of the platinum complex was 5 mM.

Precipitation of Dithiobiurea$(Pt(trpy))_2$ complex

A 10 mM sodium tetraphenyl borate (M. W. = 342.24 g/mol) solution was prepared by dissolving 25.9 mg in 7.570 mL of water. Four mL of the 2:1 reaction mixture of [Pt(trpy)Cl]Cl:dithiobiurea was treated with 8 mL of the tetraphenylborate solution. A red-purple solid immediately precipitated. The solid in its mother liquor was cooled to 4° C. The precipitate was isolated by filtration and was washed with several small aliquots of water on the filter and then allowed to air dry at room temperature. The precipitate

was then dried at 110° C for 2 hours to remove water of crystallization. The solid (25.9 mg) was recovered with a yield of 56.7%. A 24.2 mg sample was sent for elemental analysis. Anal. Calcd. for [(Pt(trpy))$_2$ dithiobiurea](BΦ_4)$_4$·2H$_2$O: C, 66.27; H, 4.69; N, 6.04. Found: C, 62.74; H, 4.36; N, 6.99.

Reaction of [Pt(trpy)Cl]Cl with Pyridazine

A 5 μL aliquot of pyridazine was added to 6.830 mL of H_2O to yield a 10 mM solution. The complex [Pt(trpy)Cl]Cl (5 mg) was dissolved in 935 μL H_2O to yield a 10 mM solution. A 470 μL aliquot of the platinum complex was treated with 235 μL of the pyridazine solution. No immediate color change was observed upon mixing. A similar reaction mixture was prepared that was 1.5 mM in each reactant. A 2:1 $[Pt(trpy)Cl]^+$:pyridazine mixture was prepared by adding 1 mL of 3 mM [Pt(trpy)Cl]Cl to 1 mL of 1.5 mM pyridazine.

Reaction of [Pt(trpy)Cl]Cl with Cyanuric Acid

A 9 mM solution of cyanuric acid was prepared by dissolving 8.0 mg (M. W. = 129.02 g/mol) in 6.890 mL of H_2O. A 3 mM [Pt(trpy)Cl]Cl solution was prepared by dissolving 10.3 mg in 6.430 mL H_2O. A 1:1 [Pt(trpy)Cl]Cl:cyanuric acid reaction mixture was prepared by diluting 1 mL of 9 mM cyanuric acid to 3 mL (3 mM) and adding 1 mL of 3 mM [Pt(trpy)Cl]Cl to 1 mL of 3 mM cyanuric acid. A 2:1 reaction mixture was prepared by adding 1 mL of 3 mM [Pt(trpy)Cl]Cl to 1 mL of 1.5 mM cyanuric acid. A 3:1 reaction mixture was prepared by adding 1 mL of 3 mM [Pt(trpy)Cl]Cl to 1 mL of 1 mM cyanuric acid. All three reactions sat at room temperature, and the platinum complex was added dropwise with stirring in all cases. The 1:1 reaction was heated 6 hours at 50° C. The others were heated 3 hours at 50° C. After

heating, all of these reactions stood at room temperature for 3 days.

Spectroscopy of Reaction Mixtures with Pyridazine and Cyanuric Acid

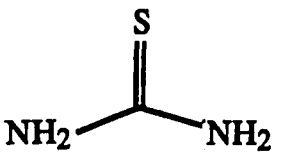
As a basis for NMR studies, ^1H NMR spectra of the ligands (pyridazine and cyanuric acid) were measured. The 300 MHz spectrum of pyridazine showed 2 types of protons as expected. The sample was 17.6 mg of pyridazine in 600 μL D_2O. Cyanuric acid (5.0 mg) in 600 μL D_2O and was heated to 80° C to dissolve the solid. The ^1H NMR of the supernatant showed no peaks as expected.

A reaction of [Pt(trpy)Cl]Cl with pyridazine was done in an NMR tube. The complex [Pt(trpy)Cl]Cl (7.1 mg) was dissolved in 600 μL D_2O. A 2 μL aliquot of pyridazine was placed in 1.250 mL of D_2O. A 300 μL aliquot of the platinum solution was added to 300 μL of the pyridazine solution and allowed to stand at room temperature in the NMR tube. NMR spectra were periodically recorded to monitor the reaction.

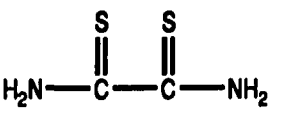
RESULTS AND DISCUSSION

Reaction of [Pt(trpy)Cl]Cl with Thiocarbonyl Compounds

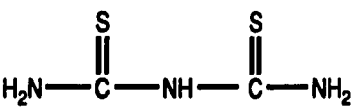
A series of ligands was chosen to provide a continuum of distances between two potential binding sites. Thiourea, with only one potential binding site, served as a model complex. The structures of the ligands used are shown below. The dithio ligands provide a series in which two



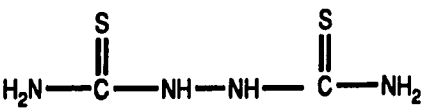
thiourea



dithiooxamide



dithiobiuret



dithiobiurea

$Pt(trpy)^{2+}$ groups can be spaced at different intervals if it is assumed the sulfur atoms are positioned on the same side of the ligand. Of course, different orientations are possible. If this assumption holds, then the S, S distance in each ligand is approximately 1.5, 3.0, and 4.5 Å for dithiooxamide, dithiobiuret, and dithiobiurea, respectively. If the two $Pt(trpy)^{2+}$ groups are spaced close enough together, perhaps there will be some Pt-Pt interaction or $\pi-\pi$ interaction.

The reaction with thiourea is apparently a simple nucleophilic displacement of the chloride by the thiocarbonyl of the ligand. The

color change is from a dark orange to a light yellow. The $[Pt(trpy)Cl]^+$ complex in H_2O has absorption maxima at 332, 280, and 250 nm. Upon the addition of thiourea, the maxima are at 336, 275, and 240 nm. There is no growth of a new band in the 400-700 nm band, as is observed with thiolate ligands and diplatinated arginine-type ligands.[13] These shift in maxima may be due in part to the absorbance of the ligand itself. The proposed reaction is shown below.

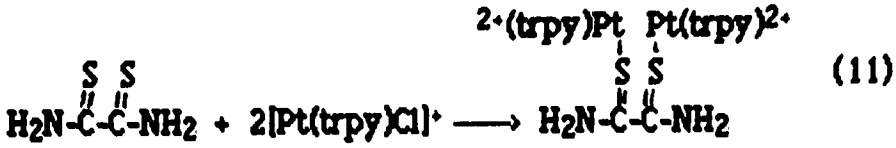
$$[Pt(trpy)Cl]^+ + (NH_2)_2CS \longrightarrow [Pt(trpy)SC(NH_2)_2]^{2+} + Cl^- \quad (9)$$

As a control, $[Pt(trpy)Cl]^+$ was treated with a 300-fold excess of urea. Upon mixing, there was no evidence of reaction. See eq (10). After prolonged heating, changes in the UV-vis spectrum were minimal and may be due to a slight

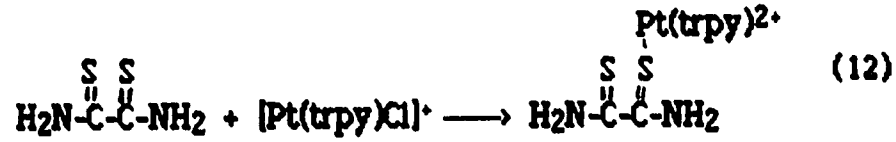
$$[Pt(trpy)Cl]^+ + (NH_2)_2CO \xrightarrow{NRXN} [Pt(trpy)OC(NH_2)_2]^{2+} + Cl^- \quad (10)$$

decomposition of the starting complex. If a reaction does occur, it is slow and marked by a slight shift of the 342 nm band to 340 nm (in phosphate buffer) and a decrease in absorbance in the 300-310 nm region. It is clear that the reaction observed with thiourea is not due to ligation by the amide nitrogens.

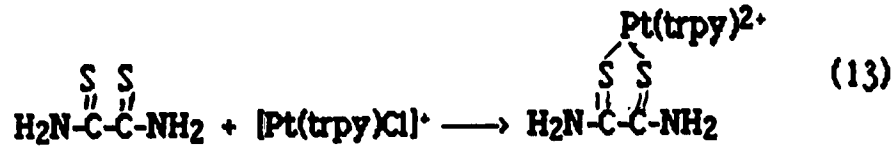
The reaction with dithiooxamide is markedly different. The addition of one equivalent of platinum complex to the ligand produced a dark reddish-brown color, unlike that of the thiourea reaction. Since the solubility of dithiooxamide is low in water, the concentration of the ligand could not be determined accurately. This darkening may indicate a new interaction, perhaps between two platinum groups close to each other as proposed in eq (11). If the concentrations of the reactants were equimolar, then statistically the distribution should be as in eq (12).



(11)



(12)



(13)

The 1:1 reaction exhibited biphasic behavior. Approximately one minute after initiation, a brown precipitate was observed. This is probably due to chelation as shown in eq (13). This chelation is thermodynamically favored over that of the terpyridyl ring because of the stronger Pt-S bond.[34] The entropic effects of ring closure are probably near equivalent in both cases since both rings are five-membered ones.

The preparation of a 2:1 platinum:dithiooxamide solution did not precipitate, probably because platination of both S atoms shuts down the chelation mechanism of eq (13). The 2:1 reaction mixture was darker in color than the 1:1 reaction mixture even though the Pt concentration was equivalent in both cases. The UV-vis spectrum of the doubly-platinated dithiooxamide clearly showed

transitions from the $Pt(trpy)^{2+}$ group, although they were heavily masked by absorbances from the ligand. Peak maxima are at 308, 270, and 238 nm in water. The most notable spectral feature is a new broad band at approximately 465 nm. The extinction coefficient of the 465 nm band is on the order of 1000 $M^{-1}cm^{-1}$.

Analogous reactions with dithiobiuret and dithiobiurea also darkened to a reddish-brown color when the molar ratio of platinum to ligand was equivalent. In all cases the 2:1 platinum complex:ligand reactions appeared darker however. No precipitation was observed with the 1:1 reactions, indicating no displacement of the terpyridyl ligand. Ring formation is less favorable with the larger ligands. With the dithiobiuret ligand, UV-vis spectra were matched for the 1:1 and 2:1 reactions such that the concentration of $Pt(trpy)^{2+}$ in the reactions, whether bound or unbound, was equivalent. The new red band in the spectrum was twice as intense for the doubly-labeled ligand than for the singly-labeled one, indicating that the color change is not simply due to a Pt-S transition (since this is in equivalent amounts in both cases). This however was a qualitative study and warrants a quantitative study for better interpretation. In the reaction with dithiobiurea, the intensity of the red band is nearly equivalent for both 1:1 and 2:1 reaction mixtures. The 2:1 reaction apparently has a slightly higher extinction coefficient. In dithiobiurea, the $Pt(trpy)^{2+}$ groups are far enough apart for interaction between them to be minimal. The new electronic transitions observed for the reaction mixtures are shown in Table IV.

The ^1H NMR spectrum of the reaction mixture of a 1:1 complex with dithiobiurea shows a general downfield shift of the terpyridyl hydrogens. The data are shown in Table V. There was no control in pH in this study; both the control [Pt(trpy)Cl]Cl and the reaction mixture were in D_2O. The resonances from a small amount

Table IV. Electronic transitions observed in the visible region in reactions of [Pt(trpy)Cl]+ with dithio-containing ligands

Ligand	1:1[a]	2:1[a]
dithiooxamide	[b]	450 nm
dithiobiuret	450 nm	450 nm
dithiobiurea	457 nm	455 nm

[a]Ratio of $[Pt(trpy)Cl]^+$ to ligand.
[b]Not detected due to precipitation.

Table V. ^1H NMR chemical shifts (in ppm) of $[Pt(trpy)Cl]^+$ and its reaction mixture with dithiobiurea

	H3,3'	H4,4'	H5	H6
$Pt(trpy)Cl^+$	7.80	8.00	7.20	7.70
$Pt(trpy)Cl^+$ and dithiobiurea	8.35,8.17	8.70	7.60	8.41,8.36
$\Delta\delta$ ppm	0.5	0.7	0.4	0.7

of unreacted [Pt(trpy)Cl]Cl are visible, so the observed shifts in the reaction mixture are clearly not from a pH effect. The general downfield shift is probably due to the electronegativity of the coordinated sulfur atom. There appear to be two sets of H6 and H3,3' resonances, but this phenomenon is not easily explained. The upfield shifts may be due to a small amount of diplatinated

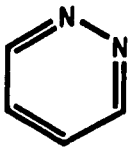
complex. Similar upfield shifts are noted in the diplatinated, canavanine complex in going from [Pt(trpy)CanH]Cl_2 to [$(Pt(trpy))_2$Can]Cl_3.[21] The order of perturbation in that case is H3,3' > H6 = H5 > H4,4'.

Previously in this laboratory, the complex [$(Pt(trpy))_2$Can]Cl_3 has been prepared.[13] This complex is deep burgundy with a broad visible band also in the 450 nm region. This new band is attributed to metal-metal or π-stacking interaction between the two closely spaced platinum groups. In the canavanine compound, the platinum-platinum distance is 3 Å. The canavanine system is also similar to the thiocarbonyl systems in that the singly substituted complex is light yellow, much like the thiourea complex of $Pt(trpy)^{2+}$.

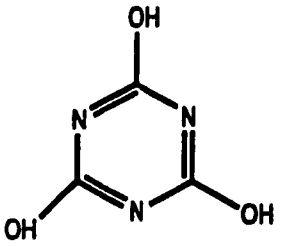
Elemental analysis gave unsatisfactory results. The poor agreement between theoretical and calculated percentages may be from incomplete substitution of both thiocarbonyl groups or contamination of the sample by NaCl.

Reaction of [Pt(trpy)Cl]Cl with Pyridine-Like Compounds

The preparation of multiply platinated ligands with nitrogen donors eliminates the possibility of platinum-sulfur transitions being confused with platinum-platinum transitions. The reactivity of [Pt(trpy)Cl]Cl with imidazole has been studied[35-37] and the chemistry of platinum with pyridine and its derivatives is extensive.[38-41] Two templates used here are pyridazine and cyanuric acid, shown below. The pyridazine template gives another possibility of putting two $Pt(trpy)^{2+}$ groups adjacent to each other. The cyanuric acid template would put three $Pt(trpy)^{2+}$ units proximate to each other, not eclipsed, but in a paddlewheel orientation.



pyridazine



cyanuric acid

UV-vis spectroscopy indicates that there clearly is a reaction between $Pt(trpy)Cl^+$ and pyridazine in a 1:1 ratio. The change in absorbance in the 300 to 350 nm region is similar to that seen in the coordination of imidazole. There appear to be small bands growing in at 365 and 385 nm and at 265 nm. Pyridazine itself has a UV maximum at 338 nm. Peak positions and intensities are given in Table VI.

Table VI. UV-vis spectroscopic properties of $[Pt(trpy)pyridazine]^{2+}$

λ_{max}[a]	386	365	337	324	278	265	242
ε[b]	1600	2000	11100	7700	14600	15000	21300

[a] nm.
[b] $M^{-1}cm^{-1}$.

The addition of a second equivalent of $[Pt(trpy)Cl]^+$ appears to cause no further reaction. The UV-vis spectrum appears as the sum of $[Pt(trpy)Cl]^+$ and $[Pt(trpy)pyridazine]^{2+}$. Proton NMR of a 1:1 reaction mixture also indicates a reaction. There is clear evidence of growth of new peaks at 8.5 and 8.8 ppm-these shifts correspond to the hydrogens meta to the pyridazine N atoms in the coordinated ligand. Due to the complexity of the spectrum in the aromatic region, it is difficult to interpret other chemical shifts. There is a

general downfield shift of the terpyridine resonances. The resonances of the H atoms ortho to the nitrogens are likely buried in the terpyridyl resonances.

Of interest to note is the gradual appearance of peaks in the aliphatic region over the course of several days. This reaction was not characterized but is perhaps due to some activation of the pyridazine ring as a result of metal coordination. One hypothesis is attack on an α-carbon in the pyridazine ring by hydroxide to cause a ring opening. The aliphatic peaks are at 2.31, 1.94, 1.88, and 1.31 ppm. A control ^{1}H NMR experiment with pyridazine in water showed no evidence of impurities or decomposition after heating for 5 hours at 50° C, 2 hours at 90° C, or after standing 6 days at room temperature. This reaction warrants further investigation.

Table VII shows changes in the UV-vis spectra of reaction mixtures of $[Pt(trpy)Cl]^{+}$ with cyanuric acid in different ratios. The platinum concentration in all cases is equivalent. It is clear that there is a reaction in the 1:1 reaction mixture. There is growth of a new band at 346 nm and a shift of a band from 280 nm to 275 nm and from 252 to 248 nm when comparing the UV-vis spectrum of the chloro complex to that of the cyanuric acid complex. The UV-vis spectrum of the 2:1 reaction mixture is nearly identical with that of the 1:1 reaction mixture and it appears that a second $Pt(trpy)^{2+}$ group can be incorporated onto cyanuric acid. It appears that the addition of a third $Pt(trpy)^{2+}$ group was not achieved. The UV-vis spectrum approaches that of unreacted [Pt(trpy)Cl]Cl (essentially the sum of [Pt(trpy)Cl]Cl and $[(Pt(trpy))_2$cyanuric acid$]Cl_4$). The lack of reaction with the third equivalent of platinum may be due to the steric bulk of the disubstituted complex or the tetrapositive charge of the

Table VII. UV-vis spectroscopic properties of $[Pt(trpy)_nCA]^{2n+}$ [a]

Pt:L[b] Ratio	nm	nm	nm	nm
1:1	346	332	275	248
2:1	344	332	277	249
3:1		332	278	251
1:0		334	280	253
sum[c]		334	280	252

[a]CA is cyanuric acid.
[b]Molar ratio of $[Pt(trpy)Cl]^+$ to cyanuric acid.
[c]Sum of the UV-vis spectra of the reactants in a 1:1 ratio.

disubstituted complex. No further characterization of this reaction has been conducted. It can be said that the two platinum groups on cyanuric acid show no evidence of interaction with each other as is expected from simple geometric considerations.

SECTION III.

$[Au(trpy)Cl]Cl_2$ AS A BIOCONJUGATION REAGENT

INTRODUCTION

The use of d^8 transition metals as bioconjugation reagents is not limited to Pt(II) complexes. Complexes of Au(III) are also d^8. Gold is a group VIIIB metal with stable oxidation states of +I and +III. The +III oxidation state of gold complexes has a d^8 electronic configuration. The comparison between the reactivity of Au(III) and Pt(II) toward bioligands is interesting since both have similar coordination geometries but different charges. The radii of the metal centers are essentially the same. Pt(II) has a radius of 74 pm and Au(III) has a radius of 82 pm.[42] Since [Pt(trpy)Cl]Cl worked well as a protein-labeling reagent, $[Au(trpy)Cl]Cl_2$ was chosen as the homologous gold protein-labeling reagent. The reagent $[Au(trpy)Cl]Cl_2$ has been prepared and structurally characterized.[43]

EXPERIMENTS

Materials and Methods

Tetrachloroauric acid, 2,2',2',2 -terpyridine, and amino acids and their derivatives were obtained from Sigma Chemical Co. Deuteriated solvents were obtained from Aldrich Chemical Co. Proton NMR measurements were done on a Nicolet NT 300 MHz spectrometer at 22 °C. Chemical shifts are reported with respect to HDO at 4.79 ppm versus DSS. UV-vis spectra were recorded with an IBM 9430 spectrophotometer.

Synthesis of $[Au(trpy)Cl]Cl_2$

A quantity of 100 mg of $HAuCl_4 \cdot 3H_2O$ (M.W.= 388 g/mol) and 65 mg of terpyridine were placed in 7.73 mL of H_2O. The pH of the reaction mixture was adjusted to 3.0 with 0.1 N NaOH. The reaction mixture was then refluxed for 18 hours. A small amount of insoluble purple solid was seen on the walls of the reaction vessel after refluxing. The deep-orange solution was cooled to room temperature and evaporated to dryness with a water aspirator. The solid was redissolved in H_2O and cooled to attempt recrystallization. This could not be achieved so the solid was again evaporated to dryness. The amorphous dull-orange solid (with a purplish hue) weighed approximately 90 mg.

A second synthesis of $[Au(trpy)Cl]Cl_2$ was performed. A quantity of 95.7 mg of $HAuCl_4$ and 63.1 mg of trpy were prepared as above except the reflux time was increased to 24 hours. Approximately 112 mg of the crude product was recovered which implies a 77% yield.

A few mg of this product was dissolved in 1 mL of 10 mM HCL and was placed on a Sephadex G-25-80 column with 10 mM

HCl as the eluent. Two bands were observed: a major orange band and a retained, minor, purple band. UV-vis spectra of the orange band showed gross $[Au(trpy)Cl]^{2+}$ features but had lost some of its detail. Concentration of the orange band under vacuum caused it to turn pink. The UV-vis spectrum of this pink band was similar to that of the orange band. $[Au(trpy)Cl]Cl_2$ on both Sephadex and DE52 columns appears to react with the resins if allowed to stand overnight; the complex turns the columns purple. The metal complex can not be removed with 2 M NaCl. A concentrated solution of KCN does remove the metal complex from these columns.

A more conventional means of purification seemed to work better. The $[Au(trpy)Cl]Cl_2$ was purified by placing the crude product in acetone. In acetone, the $[Au(trpy)Cl]^{2+}$ becomes a bright orange crystalline solid. The solid was isolated and dried by vacuum filtration on a glass frit. The ^1H NMR spectrum of the purified complex showed no residual contaminants.

Spectroscopic Characterization of $[Au(trpy)Cl]Cl_2$

Molar absorptivities were obtained from this pure $[Au(trpy)Cl]Cl_2$. UV-vis spectra were recorded for 10 μL of a 5 mM solution diluted to 5 ml with water (a 10 μM solution). The UV-vis spectrum showed features similar to that of [Pt(trpy)Cl]Cl but was noticeably different. Even after 68 days of the solid standing at room temperature and in light, the UV-vis spectrum of $[Au(trpy)Cl]^{2+}$ looked the same as it did when freshly prepared. For comparison, 8.4 mg of $HAuCl_4$ (M.W. = 393.83 g/mol) was placed in 4.270 mL of water to yield a 5 mM solution. The pH was 2.36. The UV-vis spectrum of $HAuCl_4$ showed a single band at 207 nm with an extinction coefficient of *ca.* 19300 $M^{-1}cm^{-1}$.

A few mg of the product from the above synthesis were dissolved in D_2O. The ^1H NMR spectrum showed 4 main groups of

peaks. The positions of the groups of peaks were in good agreement with those reported in the literature. [43] Although terpyridine is insoluble in D_2O, an attempt was made to see if any signals from free terpyridine could be detected in the $[Au(trpy)Cl]^{2+}$ solution. As a control, a small quantity of terpyridine was placed in 500 μL of D_2O. The ^1H NMR showed weak resonances at *ca.* 8.65, 8.15, 8.30, and 7.55 ppm.

Survey of Amino Acids

Several amino acids containing a heteroatom in the side chain were incubated with $[Au(trpy)Cl]Cl_2$ at room temperature for several days. The concentrations of both reactants were 5 mM. The following amino acids were tried: His, Met, Arg, Cys, Trp, Asp, N-Ac-His, N-Ac-Met and Cys. With cysteine, a precipitation occurred. With tryptophan, a color change develops almost immediately. After one day, the reaction with tryptophan had completely precipitated a brown solid. The supernatant was clear. With methionine, the solution reddens and with histidine, the solution darkens within hours. The reaction mixtures with N-Ac-His, N-Ac-Met, Arg, and Asp show no immediate sign of reaction.

After 17 days, the reaction mixtures with N-Ac-Met, N-Ac-His, and histidine were lyophilized and the reaction mixtures with Cys, Met, and Trp were centrifuged. Redissolution of the recovered solids from the N-Ac-Met, N-Ac-His, and His reactions failed. The cysteine reaction precipitated a purple solid while the supernatant remained clear and colorless. The following solvents failed to dissolve the purple solid: acetone, CH_2Cl_2, $HCCl_3$, and CCl_4. The reactions with methionine and tryptophan yielded orange supernatants with an orange-brown solid deposited on the walls of the test tube. The following solvents failed to dissolve these deposits: chloroform, acetone, methanol, acetonitrile, and DMF. The

supernatant from the Trp reaction was lyophilized and the recovered solid was redissolved in D_2O. ^1H NMR showed 2 sets of 3 main groups of peaks; apparently, there is unreacted $[Au(trpy)Cl]^{2+}$ also present. A ^1H NMR spectrum was taken of 5 mM tryptophan in D_2O to serve as a reference in the analysis of reaction mixtures involving this amino acid. As a further control for the reaction of tryptophan with $[Au(trpy)Cl]^{2+}$, the reaction of Trp with $[Pt(trpy)Cl]^{+}$ was reinvestigated. Tryptophan (4.1 mg) (M.W. = 204.2 g/mol) was dissolved in 4.020 ml of water to give a 5 mM solution. An equivalent volume of 5 mM [Pt(trpy)Cl]Cl was added to 2 different 1 ml portions of the tryptophan solution to yield 1:1 reaction mixtures. One reaction mixture sat at room temperature while the other was heated at 100 °C. A control tryptophan solution in water was also heated at 100 °C to see if the amino acid decomposes since it had been noticed that tryptophan solutions darken upon standing. The control tryptophan solution and the heated reaction mixture both darkened after two days of heating.

The reaction of $[Au(trpy)Cl]^{2+}$ with tryptophan was investigated further by conducting a reaction with indole, the chemical group on the side chain of tryptophan. The reaction with indole was done in aqueous buffer and in methanol. 2.9 mg of Indole (2.9 mg) (M.W. = 117.2 g/mol) was dissolved in 6.190 ml of 85 mM phosphate buffer. The indole was not readily soluble in the buffer, so it was heated to 90 °C. Another 4 mM solution of indole was made in methanol (4.7 mg in 10 ml). A 4 mM solution of $[Au(trpy)Cl]^{2+}$ was prepared in methanol (5.2 mg in 2.241 ml). Equivalent volumes of indole and $[Au(trpy)Cl]^{2+}$ were mixed together in each solvent. The reaction mixture in methanol slowly faded compared to the reddish blank of $[Au(trpy)Cl]^{2+}$. In water, there was an instantaneous color changer to darker red. The color continued to darken to brown.

The reaction with tryptophan and indole was further

investigated with ^1H NMR. A 10 mM solution of indole and of $[Au(trpy)Cl]^{2+}$ were prepared in d_4-methanol. The ^1H NMR spectra of the reactants were recorded and the reaction mixture was monitored with over time with ^1H NMR.

In order to conduct similar experiments, a series of solubility tests were performed for $[Au(trpy)Cl]^{2+}$ and potential ligands. The $[Au(trpy)Cl]^{2+}$ complex is fairly soluble in methanol and in DMF (methanol > DMF). The complex is orange-red in these solvents and orange in water. $[Au(trpy)Cl]^{2+}$ is soluble in hot acetone and gives a pink solution. After standing, the $[Au(trpy)Cl]^{2+}$ turned a greenish-yellow color in DMF. Cysteine is somewhat soluble in methanol and ethanol (methanol > ethanol). Water is the best solvent for cysteine. Tryptophan is not soluble in DMF, acetone, or hot acetone. It is somewhat soluble in hot DMF. Indole is very soluble in DMF or acetone. The best solvent for indole studies is methanol. Indole is known to be soluble in hot alcohol. [18]

The reaction of $[Au(trpy)Cl]^{2+}$ with pyrrole was also investigated. Pyrrole is sparingly soluble in H_2O. A quantity of 10 mg of pyrrole was dissolved in 37.260 mL of 85 mM phosphate buffer to make a 4 mM solution. A 1:1 2 mM reaction with $[Au(trpy)Cl]^{2+}$ causes a slow darkening to a deep golden color. The color is different from a blank solution of $[Au(trpy)Cl]^{2+}$. A similar reaction with excess pyrrole caused the solution to turn green, then blue, and then a bluish-black precipitate formed. The reaction with pyrrole was followed by ^1H NMR in a 1:1 and a 10:1 pyrrole : $Au(trpy)Cl^{2+}$ 5 mM (in metal complex) reaction mixture in D_2O.

The 1:1 reaction mixture of $Au(trpy)Cl^{2+}$ with pyrrole was extracted with $CDCl_3$ after one day of reaction. The NMR of the extract showed free terpyridyl ligand. The ^1H NMR was also done on the remaining D_2O layer.

Another set of trial reactions with amino acids was done in buffer. N-Ac-Met (M.W.= 191.2 g/mol), N-Ac-His (M.W.= 215.21

g/mol), Trp (M.W. = 204.2 g/mol), Arg (M.W.= 174.2 g/mol), and N-Ac-Arg (M.W. = 216.2 g/mol) were dissolved in 85 mM phosphate buffer of pH 7.0 to make 4 mM solutions. An equivalent volume of 4 mM $[Au(trpy)Cl]^{2+}$ was added to each amino-acid solution to give a 1:1 2 mM reaction mixture. The tryptophan reaction mixture turned red immediately and a slight precipitation occurred. No immediate reaction was observed with the other ligands.

Protein Labeling with $[Au(trpy)Cl]^{2+}$

A 2 mM solution of $[Au(trpy)Cl]^{2+}$ (M.W. = 580 g/mol) was prepared in 100 mM phosphate buffer of pH 7.0. A 200 µL aliquot of that solution was added to approximately 1 ml of 0.5 mM cytochrome *c* from horse heart. The incubation was done at room temperature for one day. A similar reaction was also done in the dark. The unreacted $[Au(trpy)Cl]^{2+}$ was removed by exhaustive dialysis. The protein was chromatographed on a 20 cm x 1 cm CM 52 column equilibrated with 85 mM phosphate buffer of pH 7.0. The incubation procedure was repeated with a 5-fold excess of gold reagent. Purified horse-heart cytochrome c (5 mL of 100 µM) was concentrated down to a small volume (less than 1 ml) and 1 equivalent of $[Co(phen)_3](ClO_4)_3$ was added to oxidize any reduced protein. The cobalt complex was removed by ultrafiltration. A 1.210 mL aliquot of 2 mM $[Au(trpy)Cl]^{2+}$ in the phosphate buffer was added to the protein. The protein concentration was approximately 0.4 mM The reaction was done in the dark at room temperature. The protein was exhaustively dialyzed after one day of incubation. The protein, now entirely brown in color, was chromatographed on a 1.5 x 20 cm column of CM 52 preequilibrated with 85 mM phosphate buffer. Chromatography yielded three bands designated B1, B2, and B3 in order of elution.

B1 was a very broad smear. B2 was a very faint red and B3 was a dark brown band. B3 was eluted by the addition of 100 mM NaCl.

RESULTS AND DISCUSSION

The $[Au(trpy)Cl]^{2+}$ Complex

Synthesis and purification of the gold complex proved easy. The UV-vis spectrum in aqueous solution yields intense bands in the UV and near UV. Band maxima and extinction coefficients are listed in Table VIII. One major difference between the spectrum of the $[Au(trpy)Cl]^{2+}$ complex and the spectrum of the $[Pt(trpy)Cl]^{+}$ is a weak broad band at 541 nm in the gold complex. Such a transition is not seen in the chloro complex of $[Pt(trpy)Cl]^{+}$. There are strong MLCT bands for $[Au(trpy)Cl]^{2+}$ at 350 nm and 368 nm. These bands are at higher wavelengths than the corresponding bands for $[Pt(trpy)Cl]^{+}$. $[Pt(trpy)Cl]^{+}$ has one main band at 331 nm in water. The transitions in the UV, assigned to terpyridyl transitions, appear to be weaker in the gold complex. This weakening of transitions is not easily explained but perhaps has to do with the possibility of a slight change in the coordination environment when replacing Pt with Au. Au(III) is known to weakly coordinate axial solvent ligands to give a pseudo-octahedral complex. [43] Perhaps the symmetry of the terpyridyl orbitals is altered by this type of coordination.

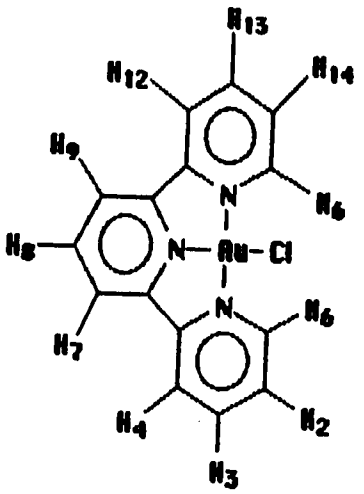
The UV-vis spectrum of tetrachloroauric acid has no strong absorbances in the visible or near UV. There is a maximum at 208 nm and a very weak shoulder at 280 nm. The presence of new bands in $[Au(trpy)Cl]^{2+}$ appear to be a result of metal-ligand interactions.

The ^{1}H NMR spectrum of $[Au(trpy)Cl]^{2+}$ in D_2O agreed well with literature assignments. [43] The product appeared pure. Peak assignments are as follows: H6 (9.29, 9.27 d); H2, H14 (8.19, m); H3-H13 (8.70, m). The numbering scheme is shown below. The resonances from the terpyridyl in the $[Au(trpy)Cl]^{2+}$ spectrum are

Table VIII. UV-vis band maxima and molar absorptivities of $[Au(trpy)Cl]^{2+}$

λ_{max}(nm)	$\varepsilon(M^{-1}cm^{-1})$
541	1100
368	9300
351	8700
275	10000
206	38200

considerably farther downfield than the terpyridyl resonances in the $[Pt(trpy)Cl]^{+}$ spectrum. The tripositive metal center in $[Au(trpy)Cl]^{2+}$ deshields the terpyridyl ring system moreso than the dipositive Pt(II) of $[Pt(trpy)Cl]^{+}$ does.



In comparison, uncoordinated terpyridine ligand in D_2O gives peaks at the following positions: 8.65, m; 8.30, m; 8.25, d; 8.15, m; 7.55 m. These peaks are clearly shifted downfield when coordinated to Au(III). The very low solubility of terpyridine in

water makes exact peak assignment difficult. When $[Au(trpy)Cl]^{2+}$ is placed in d_4-methanol, the ^1H NMR spectrum begins to resemble that of the free ligand. The stability of $[Au(trpy)Cl]^{2+}$ in organic solvents is questionable. The UV-vis spectrum of $[Au(trpy)Cl]^{2+}$ in methanol becomes featureless when compared to the spectrum of the complex in water. The bands above 350 nm have virtually disappeared. Two bands in the UV remain at 272 nm and at 228 nm. These spectra may only reflect a solubility problem, however.

Reactions with Amino Acids

The reaction of $[Au(trpy)Cl]^{2+}$ with cysteine is wholly expected. The homologous complex, $[Pt(trpy)Cl]^{+}$, reacts readily with thiolate ligands. Also, many Au(III) compounds with sulfur are known. [44-46] The $[Au(trpy)Cl]^{2+}$ complex reacts rapidly with cysteine. A pinkish-purple solid precipitates almost immediately. The initial step may be a simple coordination of sulfur to the open coordination site but it is likely followed by formation of complex gold-sulfur clusters, many examples of which are documented. [46,47] The precipitate would not redissolve in water or in acetone, chloroform, methylene chloride, or carbon tetrachloride. The UV-vis spectrum of the turbid reaction mixture in water still showed a broad band at 545 nm but loss of the bands at 368 and 350 nm. The unresolved bands near 275 nm in the chloro complex still could be seen but they were not distinct in the cysteine complex. The very strong band at 205 nm in the chloro complex was not present in the cysteine complex. There is a band in the cysteine complex at 230 nm which appears to be the shoulder seen at this wavelength in the chloro complex.

The UV-vis spectrum of a 12-hour old reaction mixture of $[Au(trpy)Cl]^{2+}$ with histidine indicated that a reaction had occurred. The weak broad band at 537 nm remained. There was a general

loss of features in the 300-340 nm region with a decrease in intensity, but the MLCT bands were still discernible. There appeared to be little change in the UV region except that the band at 206 nm in the chloro complex had shifted farther into the ultraviolet. The reaction mixture with N-Ac-His showed no evidence of reaction after 12 hours. The reaction with methionine showed similar features as the reaction with histidine, but N-Ac-Met showed little evidence of reaction after 12 hours. There were slight differences in the spectrum of the reaction mixture with N-Ac-Met when compared with the unreacted gold complex.. The low broad visible band remained at 540 nm.

The reactions with methionine and histidine are apparently predominated by reactions with the α-amino and α-carboxylate groups of the amino acids, since the N-acetylated derivatives show little or no reactivity. Apparently, the chelated terpyridine is not stable enough to resist chelation by amino acids. The reaction with methionine is interesting in that $[Pt(trpy)Cl]^+$ is wholly unreactive toward thio-ethers. The $[Au(trpy)Cl]^{2+}$ complex is structurally similar to $[Pt(trpy)Cl]^+$ and $[Pt(trpy)HET]^+$. Table IX shows that sterically, $[Pt(trpy)Cl]^+$ would be most favorable in binding methionine. Apparently, the reactivity with methionine is not controlled by sterics alone but perhaps has something to do with the higher charge on the gold complex or the oxidizing ability of the gold complex. The latter explanation is most likely. Perhaps the $Au(trpy)Cl^{2+}$ oxidizes Met to a sulfoxide.

The reaction with tryptophan yields an almost featureless UV-vis spectrum. The MLCT bands have disappeared, apparently along with the broad band in the visible region, and only one maximum at 290 nm remains. This maximum is likely from tryptophan itself. The reaction with tryptophan, indole and pyrrole

Table IX. Crystallographic properties of $[Pt(trpy)Cl]^+$, $[Pt(trpy)SR]^+$, and $[Au(trpy)Cl]^{2+}$

Property	PtCl[a]	PtSR[b]	AuCl[c]
N(1)-M-N(3)[d]	156°	161.4°	162.7°
N(1)-M-L[e]	104.1°	100.4°	98.7°
L-M-N(3)[e]	99.2°	98.1°	98.5°
N(1)-M[f]	2.11Å	2.030Å	2.029Å
N(3)-M[f]	2.01Å	2.023Å	2.018Å

[a]From reference 48.
[b]From reference 7.
[c]From reference 43.
[d]N(1) and N(3) are trans.
[e]L is cis to the nitrogen atom.
[f]N(1) and N(3) are cis to the ligand, L.

is interesting since there is little known coordination chemistry involving isolated pyrrole groups. The pK_a of a pyrrole nitrogen is high (*ca.* 14) [20] and coordination to a metal is unlikely. ^1H NMR shows a shifting of all ligand peaks downfield when $[Au(trpy)Cl]^+$ is added to the tryptophan solution. The presence of ligated terpyridyl peaks in the same region makes peak assignment near impossible.

The ^1H NMR spectrum of indole is easily assigned. Assignments for $[Au(trpy)Cl]^+$ and for indole are shown in Table X.

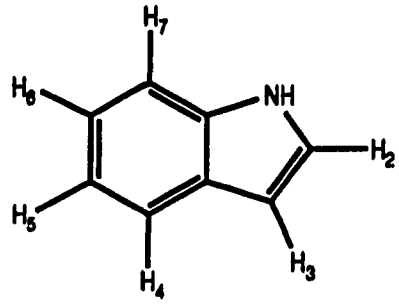
The numbering scheme for indole is shown below. All of the indole peaks disappeared and apparently shifted downfield. The numbering scheme for $[Au(trpy)Cl]^{2+}$ is the same as that for $[Pt(trpy)Cl]^{+}$ in Section I. There were no aliphatic peaks observed.

Table X. ^{1}H NMR assignments for $Au(trpy)Cl^{2+}$ and indole

	Proton	Free Reactant	Coordinated
Indole			
	H2	7.38, d	8.696
	H3	6.58, d	8.584
	H4	7.55, d	[a]
	H5	7.24, m	[a]
	H6	7.14, m	[a]
	H7	7.71, d	8.992
$Au(trpy)Cl^{2+}$			
	H6	9.29, d	9.29
	H5	8.19, m	8.18
	H3,4,3',4'	8.70, m	8.81

[a]Peaks are unresolved.

Apparently, there was no cleavage of the ring to make an aliphatic group; the coordination chemistry is not understood. In methanol, the indole peaks are not shifted when $[Au(trpy)Cl]^{2+}$ is added. The peaks from $[Au(trpy)Cl]^{2+}$ remain unshifted also. Perhaps this lack of reactivity is due to the low solubility of $[Au(trpy)Cl]^{2+}$ in methanol.



The ^{1} H NMR spectra of the reaction with pyrrole are the easiest to interpret. Pyrrole in D_2O has peaks at 6.91 and 6.25 ppm. The terpyridyl resonances of $[Au(trpy)Cl]^{2+}$ in the reaction mixture are slightly shifted from those of the chloro complex. The coordination of terpyridine to the metal center appears to be largely in tact. The H6 hydrogens resonances are shifted downfield *ca.* 0.15 ppm. The H2,14 resonances are shifted downfield by *ca.* 0.07 ppm. In reaction mixtures with a 10-fold molar excess of pyrrole, there is no evidence of free or unreacted pyrrole in the reaction mixture. The complex remaining in D_2O after extraction with $CDCl_3$ is identical to that seen in the reaction mixture in water. The ^1H NMR spectrum of the $CDCl_3$ layer shows a spectrum identical to that of free terpyridine in $CDCl_3$. Either some of the terpyridine ligand is displaced in the reaction with pyrrole and is efficiently extracted into the chloroform layer, or the presence of $CDCl_3$ itself causes loss of the terpyridine ligand. This loss of terpyridine ligand is not quantitative, as evidenced by the ^1H NMR spectrum of the reaction mixture in water.

The reaction of $[Au(trpy)Cl]^{2+}$ with pyrrole, indole, and tryptophan is complex. And it is apparently not a simple ligand substitution. It appears that the pyrrole group is oxidized by the gold complex. This is supported by the loss of terpyridine ligand from $[Au(trpy)Cl]^{2+}$. The gold product is likely a gold (I) species. Gold (I) complexes generally have a coordination number of two. All of the original gold complex is not consumed as evidenced by

the presence of resonances from coordinated terpyridine peaks. Perhaps some of the original $[Au(trpy)Cl]^{2+}$ complex is reduced either to Au(I) or Au(0) and the reduced gold species acts as a catalyst in the oxidation of the pyrrole. The reaction is not stochiometric in two ways. First, all of the $[Au(trpy)Cl]^{2+}$ is not consumed. Second, no unreacted pyrrole remains when present in a 10-fold excess.

Since the organic product or products were not analyzed, it can only be speculated what reactions are occurring. Given the high reduction potential of gold complexes ($Au^{3+} + 2e^- \longrightarrow Au^+$, 1.401 V; $Au^{3+} + 3e^- \longrightarrow Au$, 1.498 V; $AuCl_4^- + 3e^- \longrightarrow Au + 4Cl^-$, 1.002 V),[49] it is not unreasonable to assume that an oxidation of pyrrole occurs. It appears that a ring opening reaction is not occurring since no aliphatic peaks appear in the ^1H NMR spectra of reaction mixtures with any of the pyrrole-containing compounds. Ring opening could be occurring if there is oxidation to an aldehydic or carboxylic group on both sides of the ring in which cases the chemical shifts would be in the 9-12 ppm region. The source of oxygen may be dissolved oxygen or from water. Another possible oxidation reaction is simple formation of a ketone on the closed pyrrole ring to form 2-dihydropyrrole-1-one. Oxidation of the other side would yield succinimide. The hydrogens at the 2-position would resonate in the 2-3 ppm region, but no peaks are observed. Only if the keto-enol equilibrium is heavily in favor of the enol form would these protons be in the aromatic region (15-17 ppm). Allyl cyanide is not a product since no peaks are observed in the allylic region (*ca.* 1.7 ppm). Further investigation of this reaction is required.

Protein Labeling with $Au(trpy)Cl^{2+}$

After protein incubation, the dialysate of unreacted gold complex was studied with UV-vis spectrophotometry. The dialysate was always purple and not orange like the starting complex. The UV-vis spectrum showed the general features of a $[Au(trpy)Cl]^{2+}$ spectrum but was somewhat indistinct. Spectrophotometric analysis of the dialysate of the reaction mixture from two trials indicated that 40% and 57% of the $[Au(trpy)Cl]^{2+}$ originally added was recovered. An extinction coefficient of 10000 $M^{-1}cm^{-1}$ at 282 nm for $[Au(trpy)Cl]^{2+}$ was assumed. In the 1:1 reaction mixture with horse heart cyt *c*, the protein showed no visible signs of denaturation. The protein did have a lower absorbance in the 250-280 nm range than the native cyt *c*. Chromatography of the protein yielded two major bands; the second one was eluted with 100 mM NaCl. The UV-vis spectrum of the protein bands appeared similar to the spectrum of native cyt c. No distinct $Au(trpy)Cl^{2+}$ chromophores were detectable. When the incubation was repeated in the dark, similar results were obtained.

When the incubation procedure was repeated with a 5-fold excess of gold reagent, the protein mixture became dark brown. Again, the dialysate was purple. After dialysis, UV-vis analysis showed no evidence of gold label. The absorbances in the 200-280 nm range were featureless with a rapidly rising slope. There may be evidence of the Au chromophore at 320-330 nm, but it is very slight. Chromatography yielded 3 bands. The UV-vis spectrum of B1 looks much like the spectrum of the native protein except that it slopes up in the ultraviolet region. B2 shows a possible gold label. There are maxima at *ca.* 300 and 270 nm. All 3 bands have a higher absorbance in the UV than in the native protein. This higher absorbance is in contrast to what was observed in the 1:1 reaction mixtures. The differences are probably from denaturation.

The $Au(trpy)Cl^{2+}$ evidently oxidizes some residues on the protein. This is evidenced by the change in color of the dialysate, which may be a gold (I) complex and the absence of $Au(trpy)Cl^{2+}$ spectral features in the modified-protein UV-vis spectra. The gold (I) product may actually bind to the protein. Gold(I) bioconjugation reagents are known.[50,51] The gold probably oxidizes Trp residues and perhaps even Met residues to higher oxidation states of sulfur.

CONCLUSIONS AND PROSPECTS

The reaction of $[Au(trpy)Cl]^{2+}$ with amino acids is far more complicated than its platinum analog. The introduction of a strong oxidant prevents the chemistry from being simple ligand displacement. The chemistry needs to be further understood and reaction products need to be characterized. The potential use of $[Au(trpy)Cl]^{2+}$, or a similar reagent, as a site-selective oxidation reagent for tryptophan and methionine residues on proteins is of interest.

SECTION IV.

LABELING OF CYTOCHROMES *c* WITH [Pt(trpy)Cl]Cl

INTRODUCTION

Covalent modification of amino acid side chains has proved very useful in structural, spectroscopic, and mechanistic studies of proteins. [52-54] Various spin labels, chromophores, fluorescent probes, and radioactive labels developed to date are mostly organic compounds. Except as heavy-atom scatterers in crystallographic studies,[55,56] metal complexes have not been used widely for covalent modification of proteins although their properties are well suited for this purpose. [57,58] Transition-metal complexes can serve as absorption chromophores, as paramagnetic EPR labels, as NMR probes and relaxation agents, and as electron-transfer reagents. Selectivity in binding can be controlled by the oxidation state, hardness or softness, coordination number, ancillary ligands, and charge.

Previous research in this laboratory showed that chloro(2,2':6',2"-terpyridine)platinum(II), $[Pt(trpy)Cl]^+$, reacts specifically with histidine residues in cytochromes c from horse and tuna at pH 5.0. [35] The $Pt(trpy)His^{2+}$ complex, formed by displacement of the Cl^- ligand by the pyridine-like N atom of imidazole, is easily detected and quantitated on account of its characteristic and strong UV-vis absorption spectrum. Because of an interplay between the steric and electronic effects of the terpyridyl ligand, the new reagent is unreactive toward methionine and cystine, which are otherwise very strong ligands, but very reactive toward histidine, which is otherwise a relatively weak ligand. Judiciously chosen ancillary ligands, in this case terpyridyl, evidently can determine selectivity of transition-metal complexes toward biological macromolecules.

Studies with amino acids and peptides showed $[Pt(trpy)Cl]^+$ to be more reactive toward cysteine than toward histidine, as expected of a soft Pt(II) atom. [1,2,59] These model studies are

discussed in detail in Section II. Since neither of the proteins examined before contains a free cysteine residue, the observed selectivity toward histidine seemed fortuitous. In this study, the selectivity of $[Pt(trpy)Cl]^+$ is tested stringently in reactions with two additional cytochromes *c*: the iso-1 form of the protein from bakers' yeast, which contains the highly reactive residue Cys-102, $^{60\text{-}66}$ and the protein from *Candida krusei*, which lacks free Cys residues. Both of these homologous proteins also contain, among other residues, the same set of histidines as potential binding sites.

EXPERIMENTS

Materials and Methods

Iodoacetamide, (*p*-hydroxymercurio)-benzenesulfonic acid, and cytochromes *c* from *C. krusei* (of type VII), bakers' yeast (of type VIII-B), and horse heart (of type VI), were obtained from Sigma Chemical Co. The bakers' yeast protein contained less than *ca.* 5% of the iso-2 form. Chloro(2,2':6',2"-terpyridine)platinum(II) chloride dihydrate, $[Pt(trpy)Cl]Cl{\cdot}2H_2O$, and (2-hydrooxyethanethiolato)(2,2':6',2"-terpyridine)platinum(II) nitrate, $[[Pt(trpy)(SCH_2CH_2OH)]NO_3$,[7] were obtained from Strem Chemicals. Oxidant $[Co(phen)_3](ClO4)_3$ was prepared readily.[67] Deuteriated compounds were from Aldrich Chemicals. All dialyses were done by ultrafiltration with Amicon YM-5 membranes under nitrogen pressure at 4° C.

The ^1H NMR spectra were recorded with a Nicolet NT 300 spectrometer at 22°C. The protein samples were dialyzed, lyophilized repeatedly, and dissolved, all in D_2O. The residual water signal, at 4.79 ppm versus DSS, was used as an internal reference. Absorption spectra were recorded with an IBM 9430 UV-vis spectrophotometer, equipped with a two-grating monochromator. The difference spectra were obtained by subtraction of the spectra of fully oxidized protein samples from each other. The concentrations of the samples were equalized by matching of their Soret peaks at 410 nm. The X-band EPR spectra at 8K were obtained with a Bruker 200D instrument. Cyclic and differential pulse voltammograms were recorded with an IBM EC 225 voltammetric analyzer, a BAS cell assembly, and an Ag/AgCl reference electrode. Molecular masses of the monomeric and dimeric protein from yeast were determined by size-exclusion

chromatography on a column of Sephadex G 75-50, as detailed in another report from this laboratory. [68]

Protein Labeling

Unless stated otherwise, all the incubations and chromatographic separations were done at 4° C, with phosphate buffer at pH 7.0 as a solvent and an eluent. The bakers' yeast cytochrome *c* was treated with a tenfold excess of dithiothreitol (DTT) to ensure that it is entirely in the monomeric form, [61,69] and the organic reagent was dialyzed away. Ferricytochrome *c* from horse, *C. krusei*, and yeast were incubated for 1 day with an equimolar amount of [Pt(trpy)Cl]Cl (2 mM each) in 100 mM buffer. Some reactions with the yeast protein were done at pH 8.6. The reaction was terminated by dialysis into the same buffer, the protein was oxidized with 1 equivalent of $[Co(phen)_3](ClO_4)_3$, and the excess oxidant was removed by dialysis. With all of the protein in the ferric state, cation-exchange chromatography was carried out on 15 cm x 1 cm columns of CM 52, equilibrated with 100 mM buffer. The typical sample contained 5 mg of cytochrome *c* in 0.5 mL of the same buffer. Regardless of the incubation conditions, the elution was done with the phosphate buffer at pH 7.0. The horse protein was chromatographed as in the previous study. [35] With both the *C. krusei* and yeast proteins, the singly labeled derivatives were eluted with 100 mM buffer at 5 mL per hour but the doubly labeled ones required a 0.05 M solution of NaCl in this buffer and an elution rate of 20 mL per hour.

Cysteine-102 in the yeast cytochrome *c* was carboxymethylated with iodoacetamide by known procedures. [61,70] The protein was treated with DTT in 85 mM Tris-HCl buffer at pH 8.6, as before. After dialysis, the protein was brought to a concentration of 200 μM. A solution of iodoacetamide, containing

0.3 mg of this chemical per 1.0 mg of the protein, was prepared in a volume of water equal to the volume of the buffered protein solution. The mixture of the two solutions was left at room temperature, in the dark, under a stream of nitrogen, for 6 hours. Subsequent incubation with $[Pt(trpy)Cl]^+$ was done as before.

Dimerization of the yeast cytochrome *c* was achieved by the standard procedure. [60,62] The protein first was incubated with 1 equivalent of $[Pt(trpy)Cl]^+$ as usual. After the dialysis of the unbound $[Pt(trpy)Cl]^+$, the mixture of the native protein and its $Pt(trpy)^{2+}$-tagged derivatives was incubated with $CuSO_4$ in the presence of air. The $CuSO_4$ was dialyzed away, and the molecular mass of the protein was determined by size-exclusion chromatography.

Peptide Mapping

The published procedures [71] were modified in a minor way. The bakers' yeast cytochrome *c* and its derivatives were treated with *p*-$HOHgC_6H_4SO_3H$ before hydrolysis. To 5.0 mL of a 200 μM solution of the protein in 100 mM phosphate buffer at pH 7.0 was added 1.0 mL of 1.0 mM solution of the organomercurial reagent in the same buffer. After 2 hours, the reagent was dialyzed away and the protein transferred into water by ultrafiltration. Tryptic hydrolysis was done at pH 7.0 in water. The protein samples were brought to a concentration of 20-200 μM and treated with 50 μL of the solution containing 2 mg of trypsin in 1 mL of 4 mM HCl. Another aliquot of trypsin was added after 6 hours, and the digestion was ended after 10 hours by lyophilization. The digest was dissolved in the starting buffer (49 mM KH_2PO_4 and 5.4 mM H_3PO_4) and subjected to HPLC separation with a linear gradient between the buffer and acetonitrile; the fraction of the latter component increased from 0 to 45% in 2 hours. The $Pt(trpy)^{2+}$-tagged peptides

were detected at 342 nm and identified by their retention times or by their composition, or by both properties.

Samples for amino acid analysis were hydrolyzed at 165 °C for 45 minutes under argon; they were then dried and diluted with water of HPLC grade. The derivatives were prepared and separated with an Applied Biosystem 420A instrument. The quantities of amino acids were calculated with a Model 920A module.

RESULTS AND DISCUSSION

Synthesis of Protein Complexes

Labeling of the proteins occurs easily under mild conditions--incubation with an equimolar amount of $[Pt(trpy)Cl]^+$ for 1 day. After dialysis of the unbound Pt reagent, the protein is chromatographed on CM 52 with phosphate buffer at pH 7.0 as an eluent. The greater the number of the cationic $Pt(trpy)^{2+}$ labels covalently attached to the protein, the slower the elution of the derivative from the cation exchanger. The chromatographic bands of the two proteins are designated with the initials of the corresponding organisms: C for *C krusei* and Y for (bakers') yeast. The *C. krusei* cytochrome *c* yielded, in addition to the native protein (band C1), three fractions: two singly labeled (bands C2 and C3) derivatives and one doubly labeled (band C4) derivative. The yeast protein yielded, in addition to the native protein (band Y1), six fractions: three singly labeled (bands Y2, Y3, and Y4) and three doubly labeled (bands Y5, Y6, and Y7) derivatives. The native and singly labeled fractions are eluted with the 100 mM buffer, and the doubly labeled ones required higher ionic strength, achieved by the addition of NaCl. Despite many attempts, separation of fractions Y3 and Y4 and complete separation of fractions Y3 and Y4 and complete separation of fractions C2 and C3 was not achieved. The yields are listed in Table XI.

The characteristic strong absorption bands of the $[Pt(trpy)His]^{2+}$ chromophore at 328 and 342 nm are prominent in the region in which the protein absorbance is low. The presence of one or two labels per protein molecule is clearly evident. Similar spectra are obtained with the yeast protein. The number of singly and doubly

Table XI. Products of reactions between cytochromes *c* and $[Pt(trpy)Cl]^+$, separated by cation-exchange chromatography

cytochrome *c*	fraction	binding sites	no. of tags	yield
horse heart	H4	26 and 33	2	10
	H3	26	1	5
	H2	33	1	50
	H1		0	35
C.krusei	C4	33 and 39	2	10
	C3	39	1	30
	C2	33	1	30
	C1		0	30
bakers' yeast	Y7	33 and 39	2	5
	Y6	102 and 33	2	5
	Y5	102 and 39	2	5
	Y4	39	1	20
	Y3	33	1	15
	Y2	102	1	10
	Y1		0	40

labeled derivatives indicates the presence of two binding sites in the *Candida* protein and three such sites in the yeast protein. The representative absorption spectra of the native and labeled cytochromes *c* from *C. krusei* are shown in Figure 1.

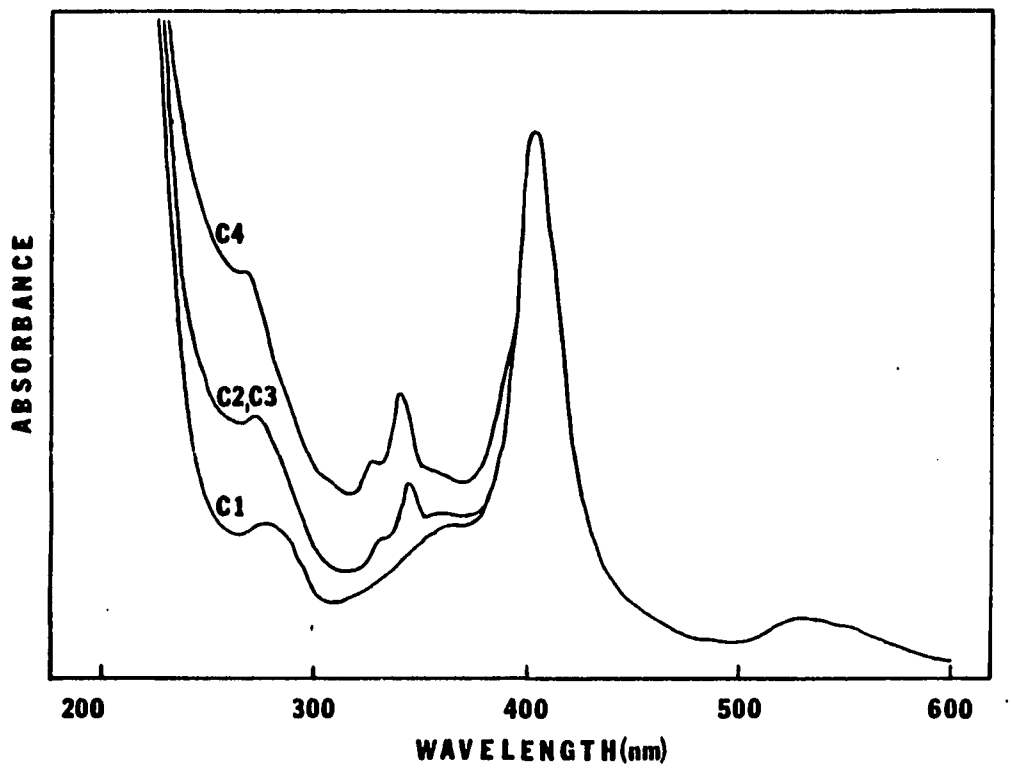


Figure 1. Absorption spectra: native cytochrome *c* from *C.krusei*(C1); mixture of its derivatives singly labeled with $Pt(trpy)^{2+}$ at His-33(C2) and at His-39(C3); derivative doubly labeled at His-33 and His-39(C4)

Structural and Redox Properties

The modified and native proteins were compared with one another by various physical methods in order to determine whether labeling with the novel inorganic reagent alters the properties of cytochrome *c*. Since the singly labeled derivatives C2 and C3 are only partially resolved, and Y3 and Y4 are unresolved, the measurements involving them are done with the mixtures. The findings are summarized in Tables XII and XIII. The absorption spectra indicate that the electronic structure of the heme is not noticeably perturbed. The reduction potentials of the native and modified proteins from the same organism, determined by cyclic and differential pulse voltammetry, are nearly identical within the margin of experimental error. [72,73] The similarities among the EPR g values rule out significant electronic or structural perturbations of the heme. [74] The evidence against perturbation is reliable even when obtained with a mixture of tagged proteins.

The ^{1}H NMR resonances of the paramagnetic ferriheme, especially those shifted by hyperfine effects, depend markedly on the interactions between the iron atom and its axial ligands and between the heme periphery and the neighboring amino acid residues. [75-84] The spectra are assigned according to previous studies. [81,85-88] Labeling evidently leaves the protein structure, at least the features manifest in the four properties measured, virtually unperturbed. Our previous study of cytochromes c from horse and tuna [35] and this study both indicate that $[Pt(trpy)Cl]^+$ is a noninvasive reagent for protein modification.

Table XII. Redox and spectroscopic properties of the *C. krusei* cytochromes c and of its $Pt(trpy)^{2+}$-tagged derivatives [a]

	C1	C2 and C3 [b]	C4
redn. potential [c]	273	275	272
EPR [d]			
g_x	1.20	1.20	1.20
g_y	2.23	2.23	2.09
g_z	3.07	3.06	3.18
^{1}HNMR [e]			
CH_3 at ring IV	33.70	33.73(0.03)	
CH_3 at ring II	31.02	31.35(0.33)	
β-CH of propionate IV	16.94	16.55(0.39)	
β-CH of propionate III	14.19	14.25(0.06)	
CH_3 at ring III	10.56	10.54(0.02)	
CH_3 in Met 80	-23.69	-23.29(0.040)	
S-CH-CH_3 at ring I	-3.19	-3.14(0.05)	
S-CH-CH_3 at ring II	-2.39	-2.37(0.02)	

[a]For the designation of the protein derivatives and for the tagged amino acids, see Table XI.

[b]Mixture of two singly labeled derivatives.

[c]Solution composition: 0.1-0.4 mM in the protein, 0.1 M in $NaClO_4$, 0.01 M in 4,4'-bipyridyl, and 0.085 M in phosphate buffer at pH 7.0 and 25° C.

[d]Measured with 0.1-1.0 mM solutions of the proteins in 85 mM phosphate buffer at pH 7.0 and 8 K.

[e]Measured with 1-3 mM solutions of the proteins in D_2O, with chemical shifts in ppm downfield from DSS; deviations from the native values are given in parentheses.

Table XIII. Redox and spectroscopic properties of the bakers' yeast cytochrome *c* and its $Pt(trpy)^{2+}$-tagged derivatives [a]

	Y1	Y2	Y3 and Y4 [b]	Y7
redn.potential [c]	282	287	280	277
EPR [d]				
g_x	1.20		1.30	1.20
g_y	2.16		2.19	2.19
g_z	3.12		3.14	3.12
1H NMR [e]				
CH_3 at ring IV	34.75	34.92(0.15)	34.25(0.50)	
CH_3 at ring II	30.85	31.12(0.27)	30.57(0.18)	
β-CH of propionate IV	15.17	15.29(0.012)	15.01(0.16)	
β-CH of propionate III	14.37	f	f	
CH_3 at ring III	11.65	11.19(0.46)	11.35(0.30)	
CH_3 in Met-80	-22.67	-23.12(0.45)	-22.39(0.28)	
S-CH-CH_3 at ring I	-3.14	-3.06(0.08)	-3.13(0.01)	
S-CH-CH_3 at ring II	-2.26	-2.31(0.05)	-2.16(0.10)	

[a]For the designations of the protein derivatives and for the tagged amino acids, see Table XI.

[b]Mixture of two singly labeled derivatives.

[c]Solution composition: 0.1 mM - 0.4 mM in the protein, 0.1 M in $NaClO_4$, 0.01 M in 4,4'-bipyridyl, and 0.085 M in phosphate buffer at pH 7.0 and 25° C.

[d]Measured with 0.1-1.0 mM solutions of the proteins in 85 mM phosphate buffer at pH 7.0 and 8 K.

[e]Measured with 1-3 mM solutions of the proteins in D_2O, with chemical shifts in ppm downfield from DSS; deviations from the native values are given in parentheses.

[f]Not detected.

Binding Sites on the Cytochromes *c*

Our qualitative study of reactivity of $[Pt(trpy)Cl]^+$ toward all the amino acids that contain heteroatoms in their side chain showed that only Cys and His are reactive under the conditions of protein labeling [35,36] Kinetic experiments confirm these conclusions quantitatively. Horse and tuna cytochromes *c*, neither of which contains free Cys residues, are modified exclusively at His residues at pH 5.0. [35] The yields of the singly labeled derivatives were proportional to the accessibility of the respective binding sites. In the horse protein the major site is His-33, a residue fully exposed on the protein surface; the minor site is His-26, which is only slightly exposed. The tuna protein lacks His-33 and has tryptophan, an amino acid inert toward $[Pt(trpy)Cl]^+$, in its place; consequently, the binding occurs solely at His-26. The binding sites in the horse and tuna proteins were determined by UV-vis spectrophotometry and peptide mapping. The latter method was augmented with control experiments involving amino acid and peptide complexes as models for the tryptic fragments of the modified proteins.

Potential Sites

Since the heme environment is not perturbed by protein modification (see section on structural properties), binding to His-18, an axial ligand to the Fe atom, can be ruled out. The potential binding sites are the following: His-26, His-33, and His-39 in both proteins; and Cys-102 only in the iso-1 protein from yeast because the protein *C. krusei* has serine, an amino acid inert toward $[Pt(trpy)Cl]^+$, in position 102.

All the cytochromes *c* under consideration have similar amino acid sequences and are folded in the same, characteristic

manner. [89-91] Since the crystal structures of the proteins from horse, [92] tuna, [93-96] and bakers' yeast [97,98] are known and since the protein from *C. krusei* is homologous with them, especially with that from yeast, characteristics of the potential binding sites in all four proteins are known. Polypeptide segments 19-26 and 27-33 retain their conformations in all of the cytochromes c of interest. [99] Both His-26 and His-33 lie on the protein surface, but in different environments. [93] The former is hydrogen bonded in a hydrophobic pocket and largely shielded from the exterior, whereas the latter belongs to a hydrophilic region and is exposed to the exterior. [93,100] Residue 39 (Lys in the proteins from horse and tuna, and His in that from yeast) is exposed on the surface. [93,98] These considerations of structure show clearly that His-33 and His-39 are likely and that His-26 is unlikely to react with $[Pt(trpy)Cl]^+$.

Since cytochromes *c* adopt very similar structures in the crystalline state and in solution, [79] exposure of residues can be inferred from labeling studies (among which is our own, mentioned above). [35] Indeed, His-33 and His-39 in the bakers' yeast cytochrome *c* are modified with diazonia-1H-tetrazole, whereas His-26 is not. [101] Since Cys-102 in the yeast protein is very reactive toward various compounds [60-66] and since the protein dimerizes easily, [61,62,65] the free thiol is generally considered to be accessible from solution. [64]

Histidine Residues

Subtraction of the UV-vis spectrum of the native protein from the spectra of its singly tagged derivatives in all cases except in the case of Y2 yields the difference spectrum characteristic of the model complexes with imidazole ligands. One such comparison is shown in Figure 2 a,b. Clearly, the binding sites in all the derivatives except Y2 are His residues; consideration of their

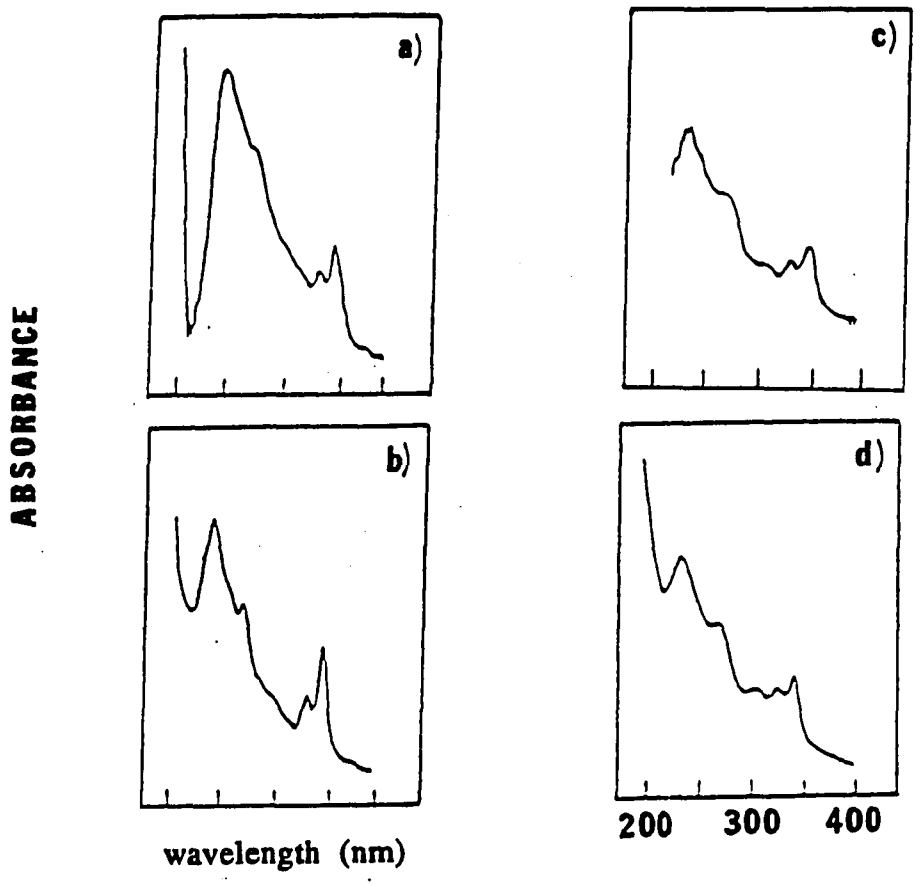


Figure 2. (a) Differences between the absorption spectra of the singly labeled (Y3 and Y4) and native (Y1) bakers' yeast cytochrome *c*. The former is a mixture of derivatives tagged with $Pt(trpy)^{2+}$ at His-33 (Y3) or at His-39 (Y4); (b) Absorption spectrum of $[Pt(trpy)(Gly\text{-}His\text{-}Gly)]Cl_2$, a complex in which the tripeptide is coordinated through the imidazole ring; (c) Difference between the absorption spectra of the singly labeled (Y2) and native (Y1) bakers' yeast cytochrome *c*. The former contains a $Pt(trpy)^{2+}$ tag at Cys-102; (d) Absorption spectrum of [Pt(trpy)(γ-Glu-Cys-Gly)], a complex in which glutathione is coordinated through the thiolate group

exposure on the protein surface (see above) points at His-33 and His-39, both of which are accessible from solution.

In a previous study of cytochromes *c* from horse and tuna, the relative intensities of the absorption bands of $[Pt(trpy)His]^{2+}$ proved sensitive to the environment of the His residue to which the chromophore is attached. The tag in the hydrophobic region (at His-26) had a $\varepsilon_{342}/\varepsilon_{328}$ quotient of 1.15, whereas the one in the hydrophilic region (at His-33) had a quotient of 1.58. [35] In this study, the chromophores attached to both His residues in both proteins had the high $\varepsilon_{342}/\varepsilon_{328}$ quotient, 1.45 in the *C. krusei* derivatives and 1.50 in the yeast derivatives. Both of the His residues involved seem to be in hydrophilic environments, *i.e.*, exposed to the solution. Indeed, both His-33 and His-39 are located in such environments. Residue His-26, a very minor site in the horse and tuna proteins, is not tagged in the *C. krusei* and yeast proteins because the latter two proteins have a greater number of potential binding sites than the former two. This minor site (His-26) could be labeled slightly in competition with one other site (His-33), but it cannot compete with two other sites (His-33 and His-39).

The minor perturbations of the ^1H NMR resonances of the methyl groups in the heme rings III and IV are evident in the labeled yeast protein but not in the labeled horse [35] and *C. krusei* proteins; see Tables XII and XIII. The binding sites His-33 and His-39 (especially the former) are near these rings in all the homologous proteins, but the tertiary structure of the first one is less stable [102] than those structures of the other two proteins. Hence the slight perturbations only in the spectrum of the yeast protein.

Cysteine residues

The singly labeled derivative Y2 of yeast cytochrome *c* yielded a UV-vis difference spectrum characteristic of $[Pt(trpy)Cys]^+$; compare parts c and d of Figure 2. The $\varepsilon_{342}/\varepsilon_{328}$ quotient is 1.14 for this model complex and 1.28 for the difference spectrum Y2-Y1. The yield of the tagging at Cys-102 is surprisingly low in view of the high reactivity of this residue toward organic reagents [60-66] and in view of the complete selectivity of $[Pt(trpy)Cl]^+$ for Cys over His in the model reactions.

Peptide Mapping

Tryptic digests of the various labeled proteins (those from the yeast cytochrome *c* were pretreated with $HOHgC_6H_4SO_3^-$) were analyzed by HPLC, and tagged peptides were detected easily owing to the high absorptivity of the $[Pt(trpy)L]^{n+}$ chromophores at 342 nm. Pretreatment with the organomercurial is necessary in order to suppress (as in equation (7)) the migration of the $Pt(trpy)^{2+}$ tag from the His (*i.e.*, imidazole) ligand to the Cys (*i.e.*,thiolate) ligand (eq 4). The identity of the tagged amino acid (His or Cys) was clear from the UV-vis spectra of the peptides. The labeled peptide from Y2 was identified on the basis of its UV-vis spectrum, and such peptides from Y3 and Y4 were identified by amino acid analysis. The remaining peptides were identified on the basis of their retention times in comparison with those of the definitely recognized peptides. The assignments were unambiguous because no tryptic peptide contains more than one amino acid reactive toward $[Pt(trpy)Cl]^+$. The protein derivatives, $Pt(trpy)^{2+}$-labeled peptides (designated as usual), and their retention times (in min in parentheses) are as follows: Y2, T20 (52); mixed Y3 and Y4, T8 (35) and T9 (48); mixed C2 and C3, T6 (36) and T7 (44); doubly labeled

C4, T6 (36) and T7 (42). Peptides T8, T9, and T20 of yeast cytochrome c contain His-33, His-39, and Cys-102, respectively. Peptides T6 and T7 of C. krusei cytochrome *c* contain His-33 and His-39, respectively. The reversed-phase HPLC experiments with protein digests allowed the determination of the yields of those singly labeled cytochromes *c* that could not be separated by cation-exchange chromatography. The Y3 and Y4 fractions in one experiment and C2 and C3 in another were digested together and the two $[Pt(trpy)His]^{2+}$ chromophores detected by monitoring at 342 nm. Since both peptides are singly labeled, the ratio of their peak areas equals the ratio of the two singly labeled cytochromes in the mixture. The findings are included in Table XI. The derivative C4 yielded equimolar amounts of two labeled peptides, whose retention times were identical with those of the peptides obtained separately from the C2 and C3 derivatives. The two binding sites in the doubly labeled *C. krusei* protein (C4) evidently are the same as the sites in the two singly labeled proteins (C2 and C3).

The lack of resolution of C2 and C3 and of Y3 and Y4 may well be a consequence of the similarity between His-33 and His-39. The overall charge of the protein is the same whichever residue is tagged. Even the charge distribution probably does not depend much on the binding site because the two residues are relatively close to each other on the protein surface.

Low Reactivity of Cys-102

Four findings prove that the yeast protein is labeled primarily at His residues. (1) The difference spectra of the kind shown in Figure 2, obtained with the major labeled derivatives, are conclusive. Even when the incubation is carried out at pH 8.6, at which the highly nucleophilic thiolate form predominates over the thiol form, His-labeled derivatives remain the major ones. (2) The

large difference in the rates with which imidazole and thiolate ligands displace the Cl^- ion from $[Pt(trpy)Cl]^+$ (see Table II in Section I) permits a kinetic proof of the major binding sites. The rate of protein tagging is identical, within the margin of experimental error, with the average rate of the reactions involving imidazole-containing ligands. The horse protein, which does not contain free Cys and is labeled only at His residues, reacts at this rate. (3) A mixture of the labeled derivatives of yeast cytochrome *c* behaves like the native protein when it gives a dimeric protein held by a disulfide bond. Size-exclusion chromatography of the mixture oxidized with air in the presence of Cu^{2+} ions yielded a very small amount of the monomeric protein, thus proving that Cys-102 is labeled sparingly. The dimer of the native protein and the dimer of the labeled protein derivatives are eluted from the size-exclusion column with the same retention time, 1.60 times the void time. The $Pt(trpy)^{2+}$ chromophores on the protein dimer are evident in the UV-vis spectrum--the proof that the tags were not displaced from the Cys-102 residues prior to the coupling of the protein molecules. (4) Carboxymethylation of Cys-102 with iodoacetamide (under the conditions at which this reagent does not block His residues) prior to the incubation with $[Pt(trpy)Cl]^+$ prevents the formation of derivatives Y2, Y5, and Y6, but does not affect the yields of other derivatives. This finding confirms Cys-102 as the single labeled residue in Y2 and as one of two such residues in the derivatives Y5 and Y6. It also confirms His-33 and His-39 as the labeled residues in the doubly labeled derivative Y7. Peptide mapping experiments with derivatives Y3 and Y4 proved that Y2 is not admixed to them. No $Pt(trpy)2^+$-labeled peptide was eluted at 52 min, the retention time of the labeled peptide T20 from derivative Y2.

Explanation

The low reactivity of the Cys residue and reactivity of the His residues toward the platinum complex are surprising in view of several facts. Relative nucleophilicities of the two potential ligands,[17,59] manifest in the outcomes of the reactions in equations (4) and (5) and in the complete selectivity of $[Pt(trpy)Cl]^+$ toward γ-Glu-Cys-Gly over Gly-His-Gly (see Table II in Section I), predict the opposite. Residue Cys-102 is modified with various organic reagents.[60-66] The whole experience with $[Pt(trpy)Cl]^+$ indicates that the yield of its reaction with a given ligand in the protein is proportional to the exposure of that ligand on the protein surface. The low reactivity of Cys-102, therefore, means that this residue is inaccessible. If it were accessible, it would have been labeled rapidly and completely. If it were somewhat accessible, its partial labeling would have raised the overall rate of the reaction between the yeast protein and $[Pt(trpy)Cl]^+$. The low reactivity of $[Pt(trpy)Cl]^+$ toward Cys-102 indeed demonstrates the noninvasiveness of this new labeling reagent. We conclude, in spite of the general assumption to the contrary,[64] that Cys-102 must be inaccessible from solution and that the reagents that modify Cys-102 completely have to perturb the protein in a way that enhances the exposure of this residue. The commonly used iodoacetamide may well be such an invasive reagent. Indeed, the treatment of the carboxymethylated yeast protein with $[Pt(trpy)Cl]^+$ under the usual conditions results in denaturation of *ca.* 50% of the protein. The partial unfolding of the C-terminal helix, necessary for the carboxymethylation of Cys-102, destabilizes the protein structure and perhaps renders His-18, an axial ligand to the heme, accessible to the platinum reagent.

Controlled partial unfolding of the *C. krusei* protein in the phosphate buffer containing 30% of methanol,[102] followed by

incubation with $[Pt(trpy)Cl]^+$, caused an increase in the yields of labeling at His-33 and His-39. Similar preliminary experiments with the yeast protein showed that Cys-102 remains a minor binding site even in the presence of methanol.

These two conclusions--inaccessibility of Cys-102 and invasiveness of the reagents that modify it with large yields--were reached without knowledge of the protein structure. They were confirmed by the preliminary crystallographic analysis of the iso-1 cytochrome *c*, which was communicated to us afterward. Cysteine-102 indeed is buried in a hydrophobic region of the protein and inaccessible to the solvent. [98] Modifications with organic reagents and dimerization indeed are achieved by partial unfolding of the helical segment at the C terminus. [98] The new reagent, $[Pt(trpy)Cl]^+$, evidently does not perturb the protein in this way nor does it alter the structural and redox properties of the protein when it modifies the His residues on the surface.

ADVANTAGES OF NEW LABELING REAGENT

Complex $[Pt(trpy)Cl]^+$ is well suited to tagging biological macromolecules on account of its reactivity under very mild conditions and of its noninvasiveness, which permits selectivity toward exposed residues. An interplay between the steric and electronic properties of the terpyridyl ligand makes the selectivity of this complex opposite from that of the common reagent, $PtCl_4^{2-}$.[35] Stability of the $Pt(trpy)^{2+}$ tags permits storage, dialysis, and even cation-exchange chromatography of the modified proteins. The tags can be removed, however, and the native protein restored easily, by treatment with highly nucleophilic ligands.[35]

A particular advantage of the new reagent lies in the strong UV-vis absorption by the $[Pt(trpy)L]^{n+}$ chromophore (extinction coefficients of 9000-30000 $M^{-1}cm^{-1}$), which permits its easy detection and quantitation. The charge-transfer bands in the region 300-350 nm, largely unobscured by the protein absorption, are sensitive not only to the identity of the binding site (*e.g*, His vs. Cys) but also to its environment.

The new inorganic reagent, unlike many well-known organic ones, does not perturb the protein to which it binds. Because of this important property, $[Pt(trpy)Cl]^+$ may perhaps be used as a reliable probe of the exposure of various binding sites on the protein surface. This study showed that $[Pt(trpy)Cl]^+$ exhibits great preference for cysteine and its peptides over histidine and its peptides but is nevertheless capable of labeling histidine residues in high yield in a protein that contains a reactive Cys residue. The seeming reversal of selectivity between amino acids and peptides on the one side and the yeast protein on the other is actually a consequence of the noninvasiveness of the novel reagent, which does not alter the protein conformation. The contrast between the

molecules without and with tertiary structure may serve as a reminder that what is true about small models need not be true about biological macromolecules.

SECTION V.

LABELING OF SERINE PROTEASES WITH $Pt(trpy)Cl^+$

INTRODUCTION

Serine protease, and α–chymotrypsin in particular, have been much studied by various methods. [103-111] All of these enzymes contain a triad of serine, histidine, and aspartic acid at the active site. Their proteolytic action is commonly explained by the charge-relay mechanism, for which all these three residues are essential. [112] But very recent theoretical calculations of electrostatic energies in proteins [113,114] and biochemical studies have cast doubt on this mechanism. Methylation of His-57 in α-chymotrypsin [115] and even removal (by mutagenesis) of this residue and of the entire triad in subtilisin [116-118] do not completely inactivate these enzymes.

Histidine 57 of α-chymotrypsin has been modified irreversibly with various organic reagents [115,119-122], but only the methylated α-chymotrypsin has been studied for its enzymatic activity. [115, 123-125] It has recently been shown that histidine residues in proteins can be labeled selectively and noninvasively with the new inorganic reagent, chloro(2,2':6',2"-terpyridine)platinum(II) or $[Pt(trpy)Cl]^+$. [35,36] Displacement of the Cl^- ligand of the imidazole group yields the complex $[Pt(trpy)His]^{2+}$. which is easily detected and quantitated in the protein because of its strong, characteristic UV absorption spectrum. Although the complex is stable both kinetically and thermodynamically, the $Pt(trpy)^{2+}$ tag can easily be removed from the protein by treatment with ligands more nucleophilic than imidazole. [35] This combination of noninvasiveness, detectability, stability, and removability makes $[Pt(trpy)Cl]^+$ well suited to investigation of enzymes. We used the new reagent with advantage to study the esterase and amidase activity of α-chymotrypsin and α-lytic protease.

EXPERIMENTS

Chemicals

Bovine α-chymotrypsin (type II), PMSF, TPCK, BTEE, ATEE, BAPNA, AAPNA, N-succinyl-AAPF-p-nitroanilide, N-acetyl-AAPA-β-naphthylamide, proflavin, and lima bean trypsin inhibitor for affinity chromatography were obtained from Sigma Chemical Co. Pure α-lytic protease was donated by W. W. Bachovchin. Chloro(2,2',6',2"-terpyridine)platinum(II) chloride dihydrate, [Pt(trpy)Cl]Cl·2H_2O, was obtained from Strem Chemicals; it is easily synthesized and is now available from Aldrich Chemical Co. [126] Amicon YM-5 membranes for ultrafiltration had a cutoff limit at the molecular mass of 5 kDa. Tris buffer was 50 mM and was adjusted with H_2SO_4, usually to pH of 8.0. Sodium acetate buffer was 20 mM and had a pH of 5.0. Sodium phosphate buffer usually was 85 mM and usually had a pH of 7.0. Different concentrations and pH values are specified in particular procedures. The Fisher Accumet 805 pH meter was calibrated at 4.00 and 10.00.

α-Chymotrypsin

Ultrafiltration of the commercial preparation with 20 mM sodium acetate buffer of pH 5.0 and spectrophotometric measurements (with IBM 9430) at 280 nm, where the enzyme absorptivity is 50000 $M^{-1}cm^{-1}$ [127], showed that α-chymotrypsin constituted 49% of the original dry weight. Because two different commercial lots have nearly identical activities per milligram of solid, only one lot was analyzed for the protein content. One milligram of the commercial preparation was chromatographed on a 1 x 10 cm column of lima bean trypsin inhibitor [128] previously equilibrated with Tris buffer. Peptides and salts were eluted in the void volume

with this buffer, and the enzyme was eluted with 100 mM solution of Na_2SO_4 adjusted to pH 3.0 with H_2SO_4; both solvents flowed at 20 mL per hour

α-Lytic Protease

The enzyme was used without further purification. Its concentration was determined by the absorbance at 280 nm, where the absorptivity is 19400 $M^{-1}cm^{-1}$.[129]

Enzyme Labeling and Purification

The platinum reagent was always used as a 30 mM aqueous solution, containing 16.1 mg of [Pt(trpy)Cl]Cl·$2H_2O$ per mL. In order to retard autodigestion of α-chymotrypsin, its labeling was done at pH 5.0. To a solution containing 25 mg of the commercial preparation in 25 mL of acetate buffer was added 1.60 mL of the stock solution of [Pt(trpy)Cl]Cl. After 1 day at room temperature the reaction was stopped, and the small molecules were removed, by ultrafiltration into Tris buffer. The platinated enzyme was purified by affinity chromatography as explained above, except the second eluent had a pH of 3.5. The solution of the purified platinated enzyme was frozen at -5° C and thawed at 4° C immediately before use in other experiments.

Labeling of α-lytic protease was done in 50 mM Tris buffer of pH 8.0. All other incubation conditions were the same as for α-chymotrypsin. To a solution containing 1.2 mg of α-lytic protease in 1.0 mL of Tris buffer was added 100 μL of the stock solution of [Pt(trpy)Cl]Cl. The reaction was stopped after 1 day, and the platinated enzyme was frozen at -5° C.

To inactivate α-chymotrypsin, 750 μL of a 2.50 mM solution of TPCK in methanol was added to a solution containing 2.50 mg of

the commercial preparation in 25.0 mL of phosphate buffer of pH 6.1. To block the active site, 2.50 mL of a 10 mM solution of BTEE (a substrate) or 100 mL of a 50 mM solution of indole (an inhibitor) in methanol was added to 25.0 mL of a 40 μM solution of the commercial preparation in phosphate buffer of pH 7.5 for the substrate and in acetate buffer of pH 5.0 for the inhibitor. The inactivated, substrate-bound, or inhibited α-chymotrypsin was then incubated with 160 μL (for TPCK) or 1.67 mL (for BTEE and for indole) of the stock solution of [Pt(trpy)Cl]Cl. In the case of TPCK and indole, this incubation lasted for one day. In the case of BTEE, it lasted for 2 hours, and fresh 2.00 mL portions of the substrate solution were added every 30 minutes. In another attempt to block the active site, 1.25 mL of a 2 mM solution of proflavin in water was added to 20 mL of a 50 μM solution of α-chymotrypsin in 20 mM acetate buffer of pH 5.0. The inhibited enzyme was then incubated with 1.67 mL of the stock solution of [Pt(trpy)Cl]Cl for 18 hours.

Quantitation and Stability of the $[Pt(trpy)His]^{2+}$ Tags

The enzymes, TPCK, and the $[Pt(trpy)His]^{2+}$ chromophore all absorb at 280 nm, but only the last chromophore absorbs at 342 nm. Since the absorptivities of all three are known [35,36,127,129], the number of tags, n, could be calculated from the absorbances (A) at these two wavelengths; see equation (11). The

$$n=\frac{\varepsilon_{280}A_{342}}{14000A_{280}-19000A_{342}} \qquad (11)$$

absorptivities ε_{280} (in $M^{-1}cm^{-1}$) are as follows: 50000 for α-chymotrypsin, 52800 for α-chymotrypsin first modified with

TPCK, and 19400 for α-lytic protease. Incubation, ultrafiltration, and spectrophotometry showed that the $Pt(trpy)^{2+}$ tags remain attached to α-chymotrypsin in the pH range 1.5-12, in buffers containing 3% methanol or 10% of DMSO, and after purification by affinity chromatography.

The strong metal-to-ligand charge-transfer bands of the $[Pt(trpy)His]^{2+}$ chromophore are at 342 and 328 nm; the enzymes do not absorb at either wavelength. These absorption maxima permit spectrophotometric quantitation of the $Pt(trpy)^{2+}$ tag attached to the proteins. The A_{342}/A_{328} quotients for 10 μM solutions of $[Pt(trpy)Im]Cl_2$ in 20%, 40%, 60%, and 80% mixtures by volume of dioxane and water were 1.72, 1.64, 1.39, and 0.68, respectively.

Activity and Kinetics

The α-chymotrypsin activity at a single concentration of BTEE was expressed as the number of micromoles of this substrate hydrolyzed by 1.0 mg of enzyme/min at pH 8.0 at room temperature. The α-lytic protease activity was measured at a single concentration of N-acetyl-AAPA-β-naphthylamide and was expressed in the same manner as the α-chymotrypsin activity. The Lineweaver-Burk kinetic assays for α-chymotrypsin were done with BTEE, ATEE, BAPNA, and N-succinyl-AAPF-*p*-nitroanilide, whose hydrolyses in buffered solutions, at (25.0 ± 0.1)° C, were monitored at 256, 300, 410, and 410 nm, respectively. In the assays with BTEE and ATEE, the α-chymotrypsin concentration was 0.5 μM, the substrate concentrations varied between 55 and 500 μM, and the buffer contained 1.5% by volume of methanol. In the assays with BAPNA, the enzyme concentration was 1 μM, the concentration of the L-BAPNA (half of the racemic amide) varied between 180 μM and 1.25 mM, and the buffer contained 5% by

volume of DMSO. In the assays with the tetrapeptide, the enzyme concentration was 0.1 μM, the substrate concentration varied between 55 and 500 μM, and the buffer contained 2.5% by volume of DMSO. The Lineweaver-Burk kinetic assays for α-lytic protease were done with AAPNA and N-acetyl-AAPA-β-naphthylamide. In the assays with AAPNA, the enzyme concentration was 1.5 μM, the substrate concentrations varied between 278 μM and 2.5 mM, and the buffer contained 2.5% by volume of DMSO. In the assays with the tetrapeptide, the enzyme concentration was 0.2 μM, the substrate concentration varied between 55 and 500 μM, and the buffer contained 2.5% by volume of DMSO. The data were fitted to a hyperbolic curve. [130] The error bounds reported for the k_{cat} and K_m values are those from individual Lineweaver-Burk plots; the error bounds for the averages from different plots, obtained with different batches of the enzyme, were greater.

Before all assays the platinated enzymes were incubated with a 3-fold molar excess of PMSF for 3 hours at 4° C, and PMSF was present throughout the measurements. The platinated enzymes for some assays were also incubated with a 50-fold molar excess of [Pt(trpy)Cl]Cl for 18 hours at 4° C, and this compound was present (together with PMSF) throughout the measurements. The reaction mixtures for some assays were made 1 mM in EDTA.

Assays with BTEE were done in acetate buffer of pH 5.0, in phosphate buffers of pH 6.0-7.5, in Tris buffers of pH 8.0 and 8.5, and in carbonate buffer of pH 9.0. The ionic strength always was 50 mM.

Binding of proflavin to the native and to the platinated α–chymotrypsin was monitored spectrophotometrically in a solution that was 22 μM in this dye and 32 μM in the enzyme. [131] Mixtures that were 100 μM in proflavin and 10 μM in [Pt(trpy)Cl]Cl or in [Pt(trpy)His]Cl, with Tris buffer as a solvent, were monitored spectrophotometrically for 1 day. Some assays for the ATEE

hydrolysis by the platinated α-chymotrypsin were done in the absence and presence of 50 and 100 μM proflavin.

Acid-Base Titrations

The titrations were followed by measuring both pH and the UV spectra. All solutions were in water. A 10 μM solution of platinated α-chymotrypsin at pH 7.50 was taken to pH 1.90 with 50.0 mM H_2SO_4 and to pH 12.5 with 100.0 mM NaOH. A 15.0 mM solution of [Pt(trpy)Im]Cl_2, whose ionic strength was kept at 200 mM with Na_2SO_4, was taken to pH 11.32 with 100.0 mM NaOH, to pH 3.24 with 100.0 mM HCL, and to pH 1.0 with 50.0 mM H_2SO_4. The last two titrations yielded identical UV spectra. All the absorbance changes proved reversible.

Removal of the $Pt(trpy)^{2+}$ Tags

A 40 μM solution of the platinated α-chymotrypsin in Tris buffer was made 20 mM in thiourea, kept for 30 min at 4° C, ultrafiltered, and examined spectrophotometrically.

Computer Graphics

Steric interactions between the $Pt(trpy)^{2+}$ tag, whose platinum atom is placed at 2.30 Å from the Nε2 atom of His 57, and its environment in α-chymotrypsin were examined with the program FRODO 6.3 and an Evans and Sutherland PS 300 terminal. The van der Waals radii of H, C, N, O, and Pt were set at 1.2, 1.7, 1.6, 1.5, and 1.7 Å, respectively. The overlap between the van der Waals spheres was minimized visually.

RESULTS AND DISCUSSION

Binding Sites and Labeling

The new reagent, [Pt(trpy)Cl$^+$, reacts only with the cysteine and histidine side chains. [35,36,132,133] Since all the cysteine residues in both α-chymotrypsin and α-lytic protease form disulfides, [134,135] toward which [Pt(trpy)Cl]$^+$ is unreactive, the only potential binding sites are His-40 and His-57 in α-chymotrypsin and His-57 in α-lytic protease. All of these residues are on the protein surface, and His-57 belongs to the catalytic triad at the active site.

The labeling was achieved simply by incubation under mild conditions. The progress of the reaction over time was followed by occasional measurements of the enzymatic activity toward BTEE as a substrate for α-chymotrypsin and toward N-acetyl-AAPA-β-naphthylamide as a substrate for α-lytic protease. The activity of α-chymotrypsin decreased to *ca.* 2% of the native value already after 5 hours, and the activity of α-lytic protease decreased to *ca.* 40% of the native value after 1 day. The incubation was prolonged to 1 day for α-chymotrypsin and to 2.days for α-lytic protease to ensure complete labeling.

Since both enzymes are transparent at wavelengths greater than *ca.* 300 nm (Figure 1A), the Pt(trpy)$^{2+}$ chromophore is easily detected by UV spectrophotometry. Comparison between panels B and C of Figure 3 shows the presence of [Pt(trpy)His]$^{2+}$ in labeled α-chymotrypsin. The model complex in Figure 3C and several other complexes [Pt(trpy)L]$^{2+}$ in which L represents imidazole-containing ligands [35,36] have identical λ_{max} values. Comparison between panels C and D in Figure 3 shows that the absorption pattern in the range 300-350 nm depends markedly on the ligand L [35,36]. Since the ratio [Pt(trpy)His]$^{2+}$ to enzyme is 2.00 ± 0.08 for α-chymotrypsin and 1.00 ± 0.10 for α-lytic protease, both His-40

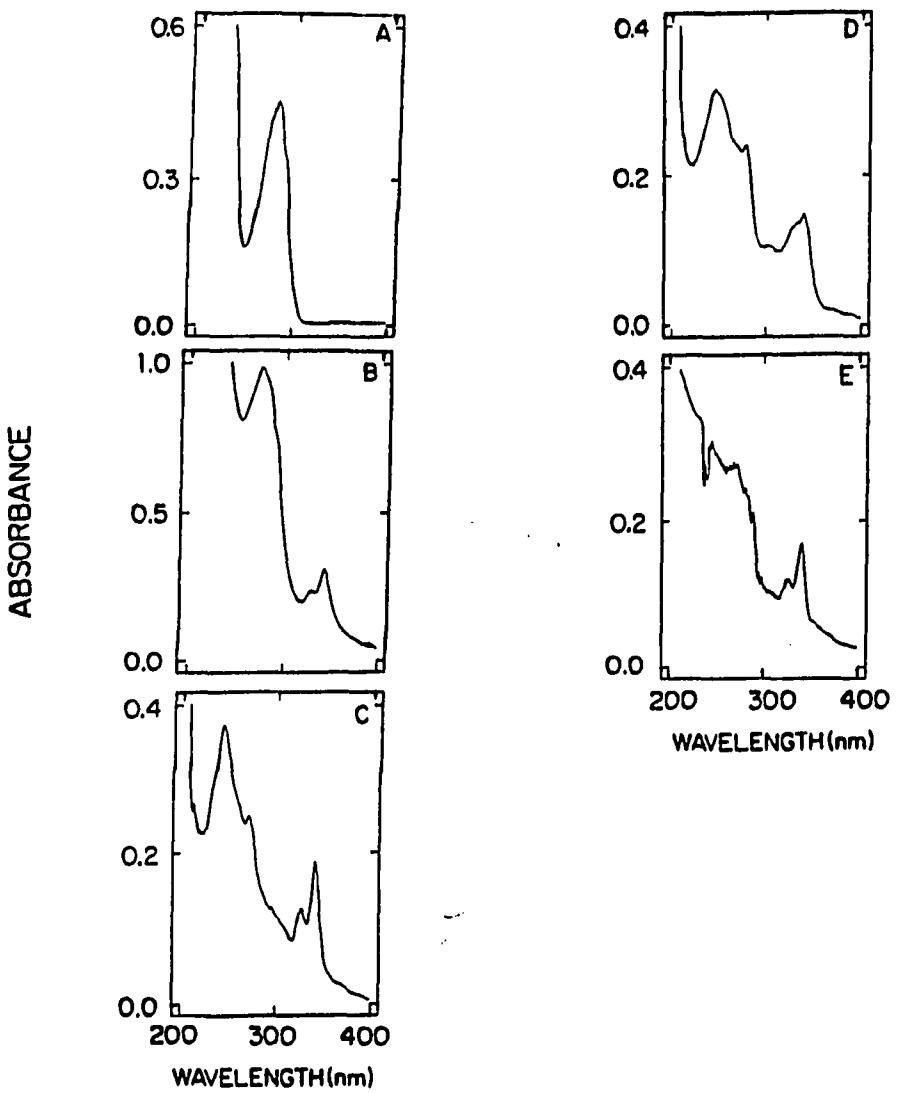


Figure 3. Absorption spectra of 10 μM solutions in 50 mM Tris buffer of pH 8.0. (A) α-Chymotrypsin; (The spectrum of α-lytic protease is similar.) (B) α-Chymotrypsin tagged with $Pt(trpy)^{2+}$ at His 40 and His 57; (C) [Pt(trpy)(Gly-His-Gly)]Cl_2, in which the tripeptide is coordinated to the Pt atom via the imidazole ring; (D) [Pt(trpy)Cl]Cl; (E) Difference between (B) and the spectrum of α-chymotrypsin modified with TPCK and tagged with $Pt(trpy)^{2+}$ only at His 40

and His-57 are modified in the former, and only His-57 is modified in the latter. Since the ratio of $[Pt(trpy)His]^{2+}$ to α-chymotrypsin is unaffected by long incubation of the platinated enzyme (in buffers at 4° C), by changes of pH in the range 1.5-12, by repeated ultrafiltrations, and by affinity chromatography, the tags are stable. Indeed, coordination complexes of Pt(II) are known for both thermodynamic and kinetic stability. [136] The $Pt(trpy)^{2+}$ tag, once attached to α-lytic protease, also is stable, but this enzyme at pH 5.0 is less reactive than α-chymotrypsin toward $[Pt(trpy)Cl]^{+}$ probably because of electrostatic and steric factors. First, α-lytic protease has a charge of +9 at pH 5.0, and an appreciable positive charge even at pH 9.0. [129] Second, α-lytic protease (a bacterial enzyme) and α-chymotrypsin (a mammalian one) have similar active sites but different tertiary structures around them. [135,137]

Labeling of the Active Site

When α-chymotrypsin is first modified at the active site with TPCK [119] and is then platinated, the diagnostic bands (Figure 3E) match those in the model complex (Figure 3C), and the ratio of $[Pt(trpy)His]^{2+}$ to the enzyme is 1.01 ± 0.15. The single tag evidently is attached to His-40. Attempts at selective platination of His 40 without the use of TPCK failed. Neither indole, a competitive inhibitor, nor BTEE, a substrate, could shield His-57 from attack by $[Pt(trpy)Cl]^{+}$, and the doubly platinated derivative was obtained in both cases. The competitive inhibitor proflavin did lower the yield of doubly platinated enzyme but did not eliminate this product. The imidazole ring of His-57 evidently remains accessible to $[Pt(trpy)Cl]^{+}$. Indeed, this residue is known to move upon substrate binding and upon enzyme modification with reagents that mimic the tetrahedral intermediate. [138,139]

Catalytic Activity

The platinated α-chymotrypsin is retained by the affinity column. Since its residual esterase activity for amino acid substrates (*ca.* 2% of the native value) is low, the platination must involve the active site; [140,141] chemical modification elsewhere would not produce such a large effect. [142,143] The platinated α-chymotrypsin hydrolyzes N-succinyl-AAPF-*p*-nitroanilide, a specific substrate for the native enzyme, at approximately 25% of the rate with which the native enzyme does it. Again, the large retardation indicates labeling at the active site. Similarly, platination of α-lytic protease substantially reduces the activity toward N-acetyl-AAPA-β-naphthylamide. Since, however, the residual activities of both enzymes are appreciable, a $Pt(trpy)^{2+}$ tag at His-57 must not completely inactivate them.

As Table XIV shows, the K_m values for the platinated α-chymotrypsin are, on the whole, only a little higher than the corresponding K_m values for the native enzyme. Similar findings have been reported for α-chymotrypsin modified at Nε2 of His-57 with small reagents [125,144,145] and for a mutant subtilisin in which His-57 was replaced with alanine. [117] It is surprising, however, that the bulky $Pt(trpy)^{2+}$ tag so little impedes substrate binding. This finding may reveal something about enzyme-substrate interaction, but its proper analysis must await a crystallographic study of our platinated enzyme.

Until then, a preliminary examination of platinated α-chymotrypsin by computer graphics will suffice. When the platinum atom sits at approximately the bonding distance from His-57, the $Pt(trpy)^{2+}$ tag seems not to crowd the other two members of the catalytic triad. The tag appears to sit in the so-called primary-specificity pocket, defined by the residues Tyr-146, Ser-190, Cys-191, Met-192, Val-213, Ser-214, Trp-215, and Tyr-228, which

interact with substrates. [112] The tag does not much affect the K_m values because it is much smaller than the overall substrate-binding region. Table XV shows the K_m values for platinated α-lytic protease.

The turnover numbers, k_{cat}, for the platinated α-chymotrypsin are the following fractions of the corresponding

Table XIV. Activity at pH 8.0 and 25° C of native α-chymotrypsin and of the derivative tagged with Pt(trpy)2+ at His 40 and His 57

	native		platinated[a]		platinated[b]	
substrate	K_m(μM)	$k_{cat}(s^{-1})$	K_m(μM)	$k_{cat}(s^{-1})$	K_m(μM)	$k_{cat}(s^{-1})$
BTEE[c]	140±10[d]	100±1.0[d]	165±65	1.66±0.12	269±11	2.21±0.04
ATEE[e]	93±10	52±1.0	426±150	0.51±0.14	886±150	0.76±0.14
BAPNA[g]	2100±920	$7x10^{-3}$	8430±400	$8x10^{-4}$	15700	$3x10^{-4}$
AAPF[h]	59±11	19±1.1	73±20	5.1±50	47±14	4.40±0.40

[a] With 3-fold excess of PMSF over total enzyme.
[b] With 3-fold excess of PMSF and 50-fold excess of [Pt(trpy)Cl]Cl over total enzyme.
[c] N-Benzoyl-L-tyrosine ethyl ester.
[d] From reference 146a.
[e] N-Acetyl-L-tryptophan ethyl ester.
[f] From reference 146b.
[g] Nα-Benzoyl-DL-arginine-*p*-nitroanilide.
[h] N-Succinyl-L-alanine-L-proline-L-phenylalanine-*p*-nitroanilide.

Table XV. Activity at pH 8.0 and 25° C of native α-lytic protease and of the derivative tagged with $Pt(trpy)^{2+}$ at His 57 toward N-Acetyl-L-Ala-L-Ala-L-Pro-L-Ala-β-naphthylamide

enzyme	$k_{cat}(s^{-1})$	$K_m(\mu M)$
native	15.6 ± 2.0	420 ± 94
platinated[a]	0.23 ± 0.02	495 ± 73

[a]With 50-fold excess of $[Pt(trpy)Cl]^+$ over total enzyme.

native values: *ca.* 1-2% for amino acid esters, as much as *ca.* 10% for amino acid amides, and as much as 25% for a peptide toward which the native enzyme is specific. These values are considerably higher than the turnover numbers of α-chymotrypsin methylated at the Nε2 atom of His-57; the methylated enzyme proved unreactive toward amides.[123,124,146] This difference in catalytic activity may result from the difference between the electrostatic or steric properties of the $Pt(trpy)^{2+}$ and CH_3^+ tags.

The electric dipoles around the so-called oxyanion hole stabilize the anionic tetrahedral intermediate (designated t^-), and the anionic Asp-102 residue stabilizes the cationic His-57 residue in the intermediate (designated His^+t^-). Although the presence of either tag lowers the turnover number, the dipositive $Pt(trpy)^{2+}$ may be more effective than the formally monopositive CH_3^+ in stabilizing t^- in the oxyanion hole. (The tagged His-57 residue probably still is stabilized by the Asp-102 residue.) Unlike mutagenesis, which removes His-57 and disrupts the electrostatic network of the active site, platination of this residue does not preclude the crucial electrostatic interactions during catalysis.

Alternatively, the bulkier Pt-(trpy)$^{2+}$ tag at the Nε2 atom may cause a greater reorientation of the His-57 side chain, so that the Nδ1 atom facilitates the proton transfer from Ser-195 to His-57. Similar rotation of the imidazole ring has already been invoked in enzymatic mechanisms.[147]

The rate of hydrolysis by α-chymotrypsin depends on the number and spatial distribution of enzyme-substrate interactions.[148] The platinated α-chymotrypsin is more efficient toward the specific substrate (a peptide) than toward the nonspecific ones (an amide, and even esters, of amino acids) probably because the scissile bond in the larger substrate is better oriented for catalysis than are these bonds in the smaller substrates. Orientation seems to be more important than the intrinsic reactivity of the scissile bond (amide vs ester).

Platination lowers the catalytic activity of α-lytic protease more than the activity of α-chymotrypsin perhaps because the substrate fit is even more important with the former enzyme than with the latter one.[149] Neither native nor platinated α-lytic protease can hydrolyze the amino acid derivative AAPNA even at a high concentration of this substrate. The platinated enzyme hydrolyzes the specific substrate N-acetyl-AAPA-β-naphthylamide with a turnover number that is only *ca.* 1% of the native value.

The activation energies were calculated with equation (12).[113]

$$k_{cat}=(k_bT/h)\exp(-\Delta G_{cat}/k_bT) \qquad (12)$$

Replacement of His-57 with Ala in subtilisin raises ΔG by *ca.* 6 kcal·mol^{-1}.[117] Platination of α-chymotrypsin, however, raises ΔG only by 1-3·kcal.mol^{-1}. This smaller effect is similar in magnitude to van der Waals interaction between a substrate and an active site.[114] The Pt(trpy)$^{2+}$ tag may also alter the effective dielectric constant at the active site.

Conventional organic reagents, such as phenylethaneboronic acid [105] or TPCK [119], inactivate α-chymotrypsin presumably because they occupy the active site and produce tetrahedral adducts that resemble the intermediates in the hydrolysis reactions. Unlike them, the inorganic tag $Pt(trpy)^{2+}$ does not completely fill the active site, is not a substrate analogue, and therefore does not completely inactivate the enzyme. The substrate apparently can still enter the hydrophobic crevice, and its functional groups can still interact in the acylamido and oxyanion-hole regions. Although these interactions may be weaker than in the case of the native enzyme, they are sufficient to permit hydrolysis of the substrate.

Several unlikely mechanisms may be hypothesized about. The hydrolysis may be catalyzed by other nucleophilic residues, such as Lys or Ser, rather than by the so-called catalytic triad. The $Pt(trpy)^{2+}$ tag perhaps disrupts the arrangement of water molecules at the active site [147] and thus lowers the enzyme activity. Finally, the Pt(II) atom may be directly involved in polarizing the substrate or in activating the residues at the active site.

Precautions about Contaminants

Various control experiments showed that the hydrolysis reactions are indeed catalyzed by the platinated enzyme and not by other agents. Chromatography of the platinated α-chymotrypsin on lima bean trypsin inhibitor removed all salts, peptides, and any other enzymes unlike trypsin. Since the treatment with TPCK completely inactivated the native enzyme toward BTEE, trypsin is absent. [151] Since addition to the assay mixture of a large excess of EDTA did not quench the hydrolysis, this reaction is not caused by some zinc-containing protease.

Because the absorptivity of the $[Pt(trpy)His]^{2+}$ chromophore

depends somewhat on its location in the protein, the error in its spectrophotometric quantitation could be as high as *ca.* 5%. Although the α-chymotrypsin after the affinity chromatography showed 2.0 chromophores, a small amount of the native enzyme could not be ruled out. To fully platinate any trace of it, a 50-fold excess of [Pt(trpy)Cl]Cl was added to the kinetic assays with both α-chymotrypsin and α-lytic protease; to inactivate any residual native enzyme, a 3-fold excess of PMSF was added to them. Since the native enzyme could, at worst, be *ca.* 5% of the total enzyme, the two reagents were in at least a 1000-fold and a 60-fold excess, respectively, over the possible contaminant. These precaution proved unnecessary because assays with just the platinated enzymes and those with added [Pt(trpy)Cl]Cl or PMSF, or both reagents, yielded kcat and Km values that differed only slightly from one another. The $Pt(trpy)^{2+}$ tags in α-chymotrypsin and in α-lytic protease indeed are stable, and the catalytic activity indeed is due to the platinated enzymes.

Additional evidence for this claim comes from the finding that proflavin, a competitive inhibitor for native α-chymotrypsin, does not inhibit the reactions of the platinated enzyme. Spectrophotometric experiments showed that proflavin does not remove the $Pt(trpy)^{2+}$ tags from the protein. In control experiments, even a 10-fold excess of proflavin failed to displace the Cl^- ligand in $[Pt(trpy)Cl]^+$ let alone the more nucleophilic His ligand in $[Pt(trpy)His]^{2+}$. The nitrogen atom of proflavin and the platinum atom in the two complexes probably are too shielded to bind to each other.[35] The proflavin absorption band at 445 nm shifts to greater wavelengths in the presence of the native[131] α-chymotrypsin, but not in the presence of its platinated derivative. Evidently, proflavin binds to the former, but not to the latter. Since the Lineweaver-Burk assays with the platinated enzyme and BTEE showed no competitive inhibition by proflavin, the catalytic activity

is not due to a native contaminant. Although this finding does not prove that catalysis occurs at the platinated active site, it rules out catalysis by the native active site of α-chymotrypsin.

Finally, platination differently affects the activity of α-chymotrypsin toward different substrates (see Table XIV). If the hydrolysis were caused by the native enzyme, the k_{cat} value of the platinated enzyme would be the same fraction of this value for the native enzyme regardless of the substrate. With the same batch of the platinated enzyme, with different substrates, this fraction varies from *ca.* 1 to *ca.* 27%. The platinated and the native enzyme clearly are different catalysts.

Effects of pH on Catalysis

Native α-chymotrypsin [152] and the derivative methylated at His-57 [115] have similar pH profiles--a bell-shaped curve with the maximum at 7.5 and two inflections on either side of it. The inflection on the basic side in both cases is attributed to the amino group at the N-terminus, Ile-16. [148] The inflection on the acidic side is attributed to the Nε2 atom of His-57 in the native enzyme, and to the Nδ1 atom of the same residue in the methyl derivative. [115,125] Data for the platinated enzyme are shown in Table XVI. The maximum occurs at 7.0, and fitting shows inflections at 6.3 and 8.6. The inflection at 8.6 may be attributed to Ile 16, but the origin of the one at 6.3 is unclear. The pK_a of the Nδ1 atom, so-called pyrrole nitrogen, in the model complex $[Pt(trpy)Im]^{2+}$ is 9.7; this value is typical of imidazole complexes with divalent transition metal. [153,154] Although the hydrophobic environment at the active site [155] and hydrogen bonding to Ser 195 can lower the basicity of this atom, it is uncertain whether such lowering actually occurs (see the next section).

This work and a previous finding that replacement of His-57

Table XVI. Catalytic activity at 25° C of α-chymotrypsin tagged with $Pt(trpy)^{2+}$ at His 40 and His 57 toward N-Benzoyl-L-tyrosine ethyl ester (BTEE) [a]

pH	$k_{cat}(s^{-1})$	$K_m(\mu M)$
5.0	0.19 ± 0.03	41 ± 36
6.0	1.16 ± 0.17	167 ± 62
7.0	2.40 ± 0.02	280 ± 5
7.5	2.15 ± 0.07	333 ± 21
8.0	2.21 ± 0.04	269 ± 11
8.5	1.11 ± 0.12	328 ± 67
9.0	0.54 ± 0.01	112 ± 2

[a]With 50-fold excess of $[Pt(trpy)Cl]^+$ over total enzyme.

with alanine in the catalytic triad of subtilisin does not abolish pH dependence of k_{cat}[117] indicate that the pH profiles of serine proteases may be affected by more factors than are now recognized. The His-57 residue, although it enhances the nucleophilicity of Ser-195 and thus increases the turnover of the substrate, is not essential for catalytic activity, as shown by site-directed mutagenesis. Even the removal of the entire catalytic triad does not completely abolish the catalytic activity.[117,118]

The $Pt(trpy)^{2+}$ Tag as a Spectroscopic Probe

The relative intensities (absorptivities), but not the positions (λ max values), of the near-UV bands (300-350 nm) in the spectrum of $[Pt(trpy)His]^{2+}$ depend on the environment of the tag in proteins.[35] Because these absorption bands arise from metal-to-ligand charge-

transitions, they are affected by the polarity of the medium and by the solvation of the chromophore. The platinum complex has absorption maxima at 342 nm and 328 nm. The quotient A_{342}/A_{328} is higher when the environment is hydrophilic than when it is hydrophobic. In this study the effect of the medium was amplified by a wide variation of the solvent hydrophobicity in studies of the $[Pt(trpy)Im]^{2+}$ complex. As the volume fraction of dioxane in water increased from 20 to 80%, the quotient A_{342}/A_{328} of the model complex decreased from 1.72 to 0.68.

For α-chymotrypsin, the composite quotient A_{342}/A_{328} for both chromophores at His-40 and His-57 is 1.38, and this quotient for the single chromophore at His-57 (from Figure 3E), is 1.41; therefore, the quotient for the chromophore at His-40 should be 1.35-1.40. Indeed, the quotient A_{342}/A_{328} for the $[Pt(trpy)His]^{2+}$ chromophore in α-lytic protease is 1.40. Both the tags at His-40 and His-57 seem to be located in hydrophilic environments. This spectroscopic evidence is consistent with the fact that platination causes only a small increase in the K_m values of the enzymes (Tables XIV and XV). The active site evidently remains accessible to substrates despite tagging of His-57. Although computer graphics indicates that the $Pt(trpy)^{2+}$ tag sits in the active site, which is hydrophobic, the tag is exposed to the solvent outside.

The titration curve in Figure 4A represents an acid-base equilibrium involving the Nδ1 atom. In addition to this process, in the diplatinated (at His-40 and His-57) and monoplatinated (at His-40) derivatives of α-chymotrypsin there is also a process in the acidic region, which we did not investigate. The similarity between the two enzyme derivatives--only one curve is shown in Figure 4B--indicates that both His-40 and His-57 in the diplatinated derivative have similar pKa values. Indeed, the corresponding $[Pt(trpy)His]^{2+}$ chromophores have similar A_{342}/A_{328} quotients. The inflection at pH 6.3 in the pH profile of the diplatinated enzyme

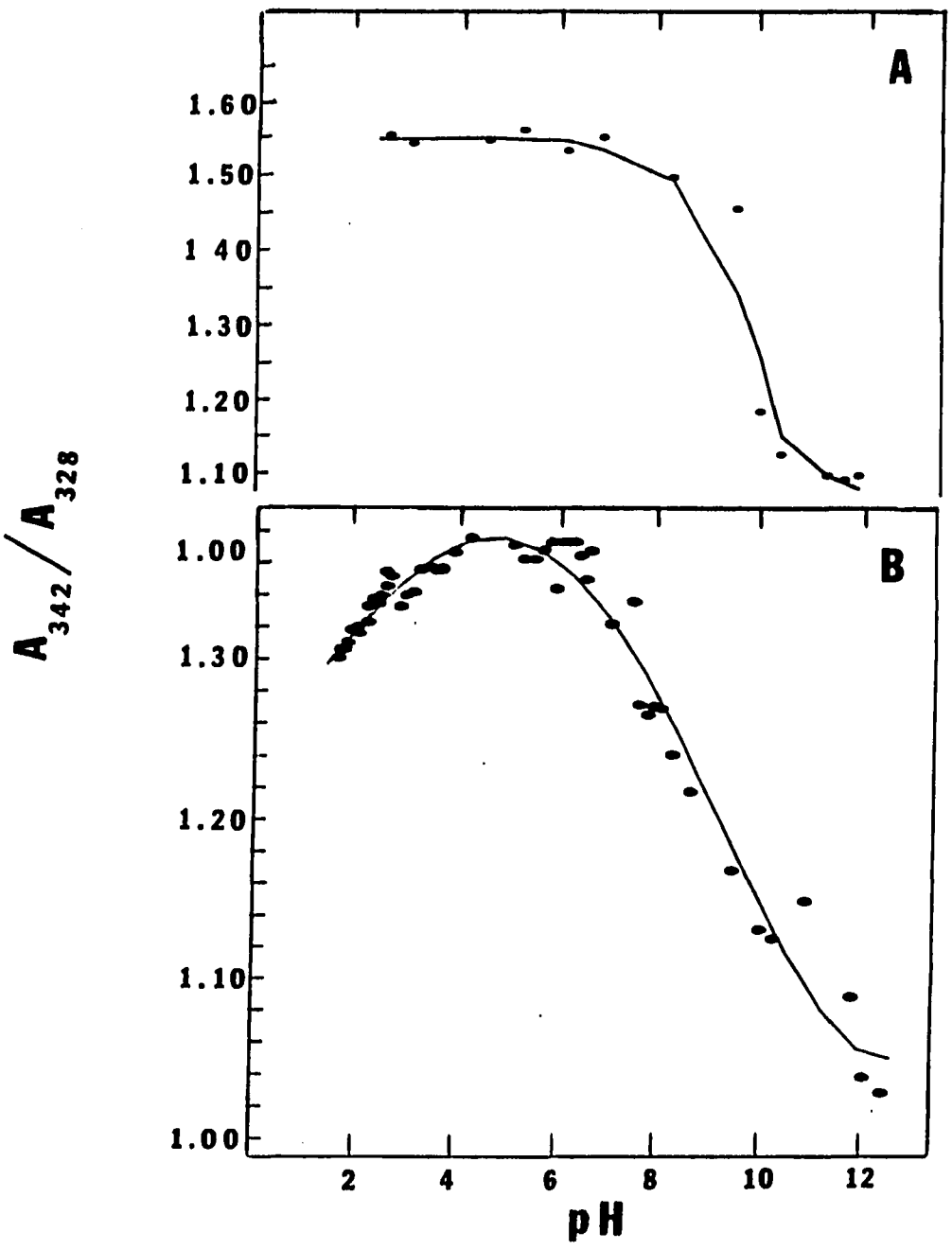


Figure 4. Spectrophotometric titrations of aqueous solutions (A) $[Pt(trpy)Im]Cl_2$ (B) α-Chymotrypsin tagged with $Pt(trpy)^{2+}$ at both His 40 and His 57

perhaps is not due to the Nδ1 of His-57.

Autodigestion of α-chymotrypsin in solution causes turbidity and precipitation over time. After 1 week at pH 5.0 and 4° C, a solution of the platinated enzyme exhibited less autolysis (no precipitation) than a comparable solution of the native enzyme (visible precipitation). The best way to keep the platinated enzyme is to freeze its solution in 100 mM Na_2SO_4 whose pH was adjusted to 3.5 with H_2SO_4. Platination of α-lytic protease similarly retards the autodigestion process. After 1 week at pH 8.0 and 25° C, the platinated enzyme showed no visible denaturation, whereas the native enzyme gave a slight precipitate. Since α-lytic protease is less susceptible to autodigestion than α-chymotrypsin,[135] the protecting effect of platination is less noticeable with it.

The $Pt(trpy)^{2+}$ tags can easily be removed, and the enzymatic activity restored, by incubation with an excess of thiourea, a sulfur nucleophile. The substitution reaction involved is shown in the simplified equation (13), in which His represents a residue in the protein.

$$[Pt(trpy)His]^{2+} + (NH_2)_2CS \longrightarrow [Pt(trpy)SC(NH_2)_2]^{2+} + His \quad (13)$$

ADVANTAGES AND PROSPECTIVE APPLICATION OF THE NEW REAGENT

The strong bands in the region 300-350 nm, where proteins usually do not absorb, permit easy detection and quantitation of the $[Pt(trpy)L]^{n+}$ chromophores. The band positions (λ max values) depend on the identity of the binding group L, and their relative intensities depend on the hydrophilicity or hydrophobicity of the environment.

Our reagent, [Pt(trpy)Cl]Cl, is particularly applicable to serine proteases, such as trypsin, chymotrypsin, subtilisin, elastase, thrombin, and lytic protease. Unlike suicide inhibitors, which recognize the entire active site and bind to it irreversibly, $[Pt(trpy)Cl]^{+}$ reacts with histidine and can be removed noninvasively. Because of its different affinity, it may prove complementary to the conventional reagents such as PMSF, TPCK, and DIFP. In particular, platination may be used as a temporary protection of proteolytic enzymes from autodigestion.

SECTION VI.

SELECTIVE LABELING OF THE SULFHYDRYL PROTEASE PAPAIN WITH $[Pt(trpy)Cl]^+$

INTRODUCTION

In this study, $[Pt(trpy)Cl]^+$ is applied to papain, a proteolytic enzyme from the papaya latex.[156,157] In previous studies this enzyme was modified[158-161] and chemically mutated with organic reagents.[162] Only three of its 212 amino-acid residues can react with $[Pt(trpy)Cl]^+$; Cys-25 and His-159 are members of the catalytic triad at the active site, while His-81 is distant from the active site.[163-165] The two kinds of residues differ from each other in the nucleophilicity of their side chains (thiolate versus imidazole), and this difference was exploited to achieve selective labeling.

EXPERIMENTS

Chemicals

Papain, L-cysteine, L-histidine, Gly-His-Gly, 2-mercaptoethanol, glutathione, and DL-dithiothreitol (DTT) were obtained from Sigma Chemical Co.; imidazole and [Pt(trpy)Cl]Cl.2H_2O, from Aldrich Chemical Co.; thiourea, from J. T. Baker Chemical Co.; and NaSCN, from Merck & Co. Distilled water was further demineralized and purified. The solvent always was 50 mM acetate buffer at pH 5.0.

Instruments and Software

The UV-vis spectra were recorded with an IBM 9430 spectrophotometer, whose monochromator has two gratings. In kinetic measurements it was attached to a circulating thermostat Fisher Scientific model 9500. A thermostated accessory, model SFA-11 by Hi-Tech Scientific, was used for rapid mixing. Kinetic results were fitted and analyzed with software produce by OLIS, Inc. and a Zenith 241 computer. An Accumet 805 MP pH-meter was used throughout. Dialyses were done by repeated ultrafiltrations in an Amicon 8050 cell, with Diaflo YM3 and YM5 membranes, under pressure of purified nitrogen. Platination of papain was simulated with FRODO 6.3 software and an Evans & Sutherland PS300 terminal.

Reactions of [Pt(trpy)Cl]Cl with Amino Acids

In one experiment, 10.00 mL of a 0.100 mM solution of [Pt(trpy)Cl]Cl was mixed separately with 1.00 mL of 1.00 mM solutions of cysteine and of histidine; in another experiment, the [Pt(trpy)Cl]Cl solution was added to a mixture of the two amino-acid

solutions. In both cases the reaction mixtures were left overnight, diluted to be 25.0 μM in platinum, and examined by UV-vis spectrophotometry.

Molar Absorptivities (Extinction Coefficients) of the Model Complexes

Equimolar amounts (3.00 mL of 3.00 mM solutions) of [Pt(trpy)Cl]Cl and of different bioligands (imidazole, histidine, Gly-His-Gly, 2-mercaptoethanol, cysteine, and glutathione) were mixed and allowed to react overnight. The reaction mixtures were diluted 60-fold (to 25 μM concentration) and examined by UV-vis spectrophotometry.

Reaction of Papain with [Pt(trpy)Cl]Cl

The commercial preparation of papain, which is twice crystallized and lyophilized, was further treated with a 10-fold excess of dithiothreitol for 15 min and dialyzed by repeated ultrafiltrations. The enzyme concentration was determined by UV-vis spectroscopy (ε_{280} = 58.4 $mM^{-1}cm^{-1}$).[166] It was incubated overnight with equimolar amounts and with 10-fold, 20-fold, and 50-fold excesses of [Pt(trpy)Cl]Cl at room temperature. The unspent platinum reagent was dialyzed away. The $Pt(trpy)^{2+}$ tags in the enzyme were identified and quantitated on the basis of the absorbance quotients A_{278}/A_{342} for the two specified wavelengths.

Kinetics of [Pt(trpy)Cl]Cl Reactions with Papain and Model Ligands

The concentration of [Pt(trpy)Cl]Cl was 20.0 μM, and the entering bioligands were always in excess. Pseudo first-order rate constants

were determined by the Guggenheim method (for papain and glutathione, which react faster) or on the basis of an estimated absorbance at infinite time (for histidine, which reacts slower). Bimolecular rate constants were determined in experiments with the following concentrations of the bioligands: 270, 240, 180, and 150 μM for papain; 2.00 and 1.00 mM and 500, 250, and 150 μM for glutathione; and 2.00 and 1.00 mM and 750, 500, 250, and 150 μM for histidine. The papain solutions were prepared fresh before each run. The glutathione solutions were prepared and stored under nitrogen. The histidine solutions were stored in closed flasks. The [Pt(trpy)Cl]Cl and bioligand solutions, 1.00 mL of each, were mixed in the standard spectrophotometric cuvette with a pipette or, more correctly, with a stopped-flow accessory attached to the spectrophotometer. The reaction with the enzyme was followed at 344 nm, and the reactions with the two model ligands were followed at 275 nm. The absorbances were recorded every 30.0 s in the reactions with papain and with glutathione and every 5.00 min in the reaction with histidine. Each run consisted of one hundred points. Each observed rate constant was an average value from one to three separate runs. All the reactions were done at (25 ± 0.5)°C.

Removal of $Pt(trpy)^{2+}$ Tags from the Bioligands

The following stock solutions were prepared: 3.00 mM each of [Pt(trpy)Cl]Cl, cysteine, histidine, NaBr, NaSCN, and thiourea (designated tu); 1.5 mM each of [Pt(trpy)His]Cl_2 and [Pt(trpy)Cys]Cl; and 150 mM of NaBr. The complexes [Pt(trpy)Br]Cl, [Pt(trpy)SCN]Cl, and [Pt(trpy)tu]Cl_2 were prepared by mixing 2.00 mL each of the corresponding stock solutions. The stock solutions of [Pt(trpy)His]Cl_2 and of [Pt(trpy)Cys]Cl were treated with equimolar amounts (2.00 vs. 1.00 mL) or with 10-fold molar excesses (0.50 vs.

2.5 mL) of NaBr, NaSCN, and thiourea, and with a 100-fold molar excess (1.00 vs. 1.00 mL) of NaBr. Solutions that were 1.50 mM in cysteine and NaSCN, in cysteine and thiourea, in histidine and NaSCN, and in histidine and thiourea were prepared by mixing 1.00 mL each of the corresponding stock solutions. To each mixture was added 1.00 mL of the [Pt(trpy)Cl]Cl solution. The various reaction mixtures were left overnight at room temperature. Any precipitate was filtered off. The clear solutions were diluted to be 25 μM in the platinum complex and examined by UV-vis spectrophotometry.

Papain was treated with one equivalent or with 10-fold, 20-fold, and 50-fold molar excesses of [Pt(trpy)Cl]Cl and dialyzed. It was then incubated with equimolar amounts and with 10-fold molar excesses of NaBr, NaSCN, and thiourea and also with a 100-fold molar excess of NaBr.

Computer Graphics

The van der Waals radii of hydrogen, carbon, nitrogen, oxygen, and platinum were set at 120, 170, 160, 150, and 170 pm, respectively. The platinum atom in the $Pt(trpy)^{2+}$ group was positioned 200 pm away from the sulfur atom in Cys-25 and from the δ (the so-called pyrrole-type) nitrogen atom in His-159 at the active site of papain.

RESULTS AND DISCUSSION

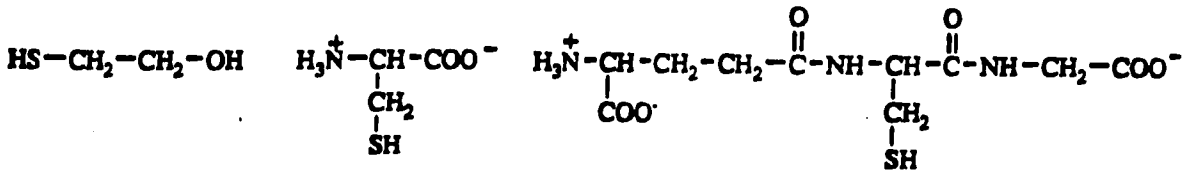
Potential Binding Sites in Papain

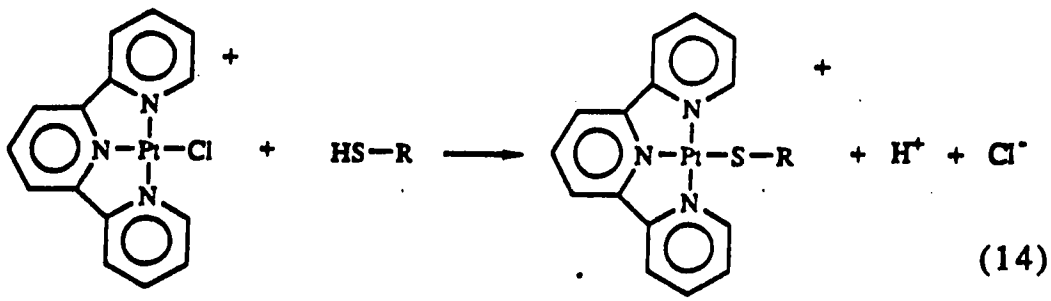
The active site of this enzyme contains the catalytic triad composed of Cys-25, Asp-158, and His-159. There is also His-81, remote from the active site. The crystal structure of this well-characterized enzyme shows that all these three potential binding sites for $[Pt(trpy)Cl]^+$ are accessible from the exterior.[163-165] But the thiolate group is a stronger nucleophile than the imidazole ring.

The Model Complexes

We examined the reactions of $[Pt(trpy)Cl]^+$ with three sulfhydryl-containing ligands (2-mercaptoethanol, cysteine, and the tripeptide glutathione, γ-Glu-Cys-Gly) and with three imidazole-containing ligands (imidazole, histidine, and the tripeptide Gly-His-Gly). The formulas (at pH 5.0) of the ligands are shown below, and their displacements of the chloride ligand in $[Pt(trpy)Cl]^+$ are shown schematically in equations (14) and (15). The two nitrogen atoms are equivalent in the imidazolium cation but not in the (unprotonated) imidazole. The atom in the ε position (the so called pyridine-type nitrogen) has the pK_a of *ca.* 6 and readily binds to metal ions; the atom in the δ position (the so-called pyrrole-type nitrogen) has the pK_a of *ca.* 14 and does not readily bind to metal ions. [167]

The UV-vis absorption properties of the model complexes are summarized in Tables XVII and XVIII. The bands below 300 nm are due to electronic transitions within the aromatic terpyridine ligand, and the bands





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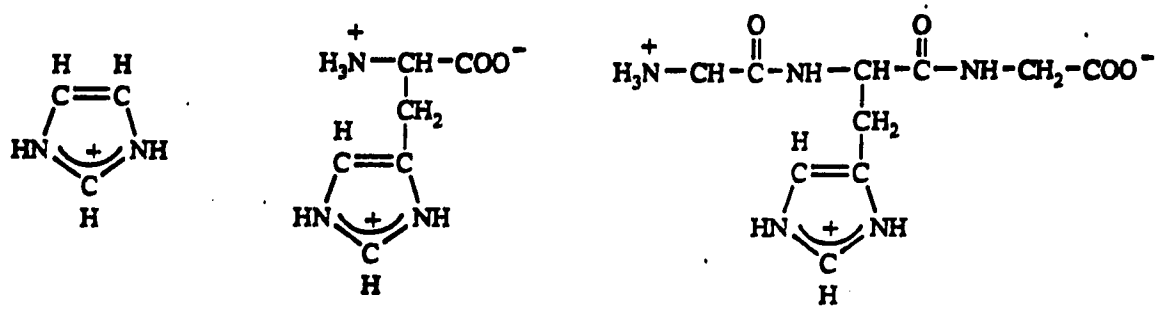
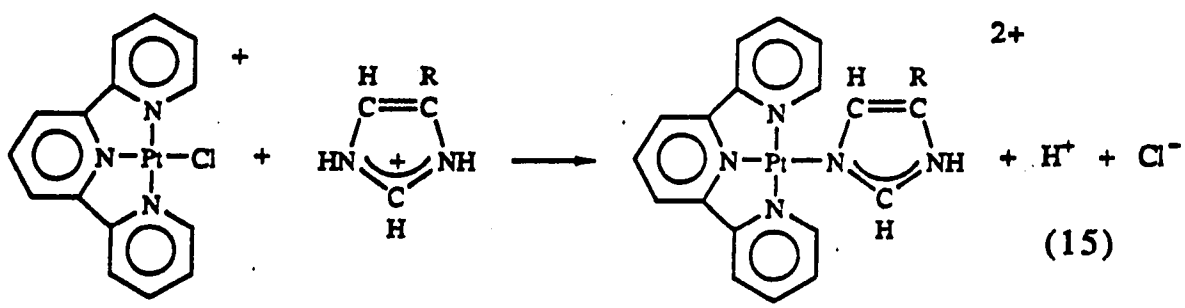




(15)

in the region 300-350 nm probably are due to metal-to-ligand charge-transfer (MLCT) transitions. The absorptivities (extinction coefficients) in Table XVII are useful for quantitation of the $Pt(trpy)^{2+}$ tags in the enzyme, while the absorption maxima and relative intensities of the MLCT bands in Table XVIII are diagnostic of the unidentate (fourth) ligand in the coordination sphere of platinum(II). The absorptivities depend on the concentration of the complex and on other species, such as the buffer and other salts, present in solution.[35,36,59]

Table XVII. Absorptivities at two wavelengths for 25 μM solutions of the model complexes $[Pt(trpy)L]^{n+}$ in 50 mM acetate buffer at pH 5.0

L	n at pH 5.0	Absorptivity/$mM^{-1}cm^{-1}$	
		278 nm	342nm
Im	2	22.2	13.8
His	2	21.2	14.5
Gly-His-Gly	2	17.0	13.7
Average for the imidazole-containing ligands		20.1	14.0
$-SCH_2CH_2OH$	1	18.4	11.3
Cys	1	17.1	11.0
γ-Glu-Cys-Gly	0	16.2	10.1
Average for the thiolate-containing ligands		17.2	10.8

Table XVIII. Absorption maxima[a] and relative absorbancies at two wavelengths for the 25 μM solutions of $[Pt(trpy)L]^{n+}$ complexes in 50 mM acetate buffer at pH 5.0

L	n at pH 5.0	λ_1/nm	λ_2/nm	A_1/A_2
Cl^-	1	344.8	328.8	1.27
His	2	343.6	328.8	1.30
Cys	1	342.0	327.6	1.18

[a] ±0.4 nm.

The kinetics of the displacement reactions (14) and (15) was studied with glutathione and histidine. The respective bimolecular rate constants, 30.2 and 0.274 $M^{-1}s^{-1}$, confirm that the sulfhydryl group is much more nucleophilic than imidazole toward platinum(II).

The competition experiments with model complexes confirmed this conclusion. When equimolar amounts of cysteine and histidine compete for an equimolar amount of $[Pt(trpy)Cl]^+$, the product shows MLCT absorption bands at (341.6 ± 0.4) and (327.2 ± 0.4) nm, and their absorbance quotient is 1.17. According to the values in Table XVIII, this product is $[Pt(trpy)Cys]^+$.

Papain Labeling

The labeling procedure was simple--incubation of the enzyme with $[Pt(trpy)Cl]^+$ overnight and removal of the unspent metal reagent by ultrafiltration. These experiments were done at pH 5.0 in order to minimize papain autodigestion. For the sake of consistency, all the other experiments also were done in this same buffer. When

the reagent and the enzyme are in equimolar amounts, only one $Pt(trpy)^{2+}$ tag can be incorporated. The kinetic and competition experiments with the model complexes showed this tag to be attach to Cys-25 at the active site. Indeed, the bimolecular rate constant for the $[Pt(trpy)Cl]^+$ reaction with papain (21.3 M^{-1} s^{-1}) is much larger than that with histidine (0.274 M^{-1} s^{-1}) and similar to that with cysteine (30.2 M^{-1} s^{-1}). Although the catalytic triad has evolved in a way that enhances the nucleophilicity of Cys-25, this amino acid in the enzyme reacts a little slower than free cysteine with $[Pt(trpy)Cl]^+$. This retardation may have two causes. First, the natural substrates for the enzyme are polypeptides, not a platinum(II) complex. Second, and probably more important, a protein-bound amino acid is shielded by other residues from the approaching metal complex. When a residue is exposed on the protein surface--for example, His-33 in cytochromes *c* from horse heart and from bakers' yeast--it reacts with $[Pt(trpy)Cl]^+$ as fast as a free amino acid.[5] The kinetics of binding of our inorganic reagent evidently reveals details of the topography of protein surfaces.

When the reagent is present in excess over the enzyme. UV-vis spectra show two $Pt(trpy)^{2+}$ tags in the enzyme. Even with a 50-fold molar excess of $[Pt(trpy)Cl]^+$, no more than two tags are found in papain. This inorganic reagent evidently is selective in its reaction with the enzyme.

The number, identity, and locations of these two $Pt(trpy)^{2+}$ tags were determined by UV-vis spectrophotometry and computer graphics. On the basis of the known absorptivities of papain and of the model complexes $[Pt(trpy)His]^{2+}$ and $[Pt(trpy)Cys]^+$, the theoretical values in Table XIX are calculated. The A_{278}/A_{342} quotient for the doubly-labeled papain was 4.0 ± 0.1, in reasonable agreement for the value expected if Cys-25 and one of the histidine residues were labeled. Computer graphics showed the orientation of the His-159 side chain to be unfavorable for

Table XIX. Expected absorbance quotients for papain variously labeled with $Pt(trpy)^{2+}$ chromophores.

Labeled residues	A_{278}/A_{342}
Cys	7.00
His	5.63
Cys and His	3.88
2 His	3.57
Cys and 2 His	3.03

platination because the trigonal (so-called pyridine-type) nitrogen atom in the ε position in imidazole points toward the interior of the protein.[163-165] In the case of the enzyme α-chymotrypsin, whose His-57 at the active site readily accepts a $Pt(trpy)^{2+}$ tag, this nitrogen atom points toward the exterior.[132] Moreover, the first $Pt(trpy)^{2+}$ tag on Cys-25 probably obstructs the approach of the second such tag to the proximate His-159.[168] The second $Pt(trpy)^{2+}$ tag likely is attached to His-81. See Figure 5.

Selective Removal of $Pt(trpy)^{2+}$ from Papain

Selective labeling of a biological macromolecule with smaller reagents usually is achieved by selective attachment. To our knowledge, this study is the first attempt at selective labeling by means of selective removal of some but not the other metal labels from the multiply-labeled biomolecule.

We take advantage of the difference in nucleophilicities between the two binding residues, Cys-25 and His-81. Relative nucleophilicity of a ligand N toward platinum(II), designated n_{Pt}, is

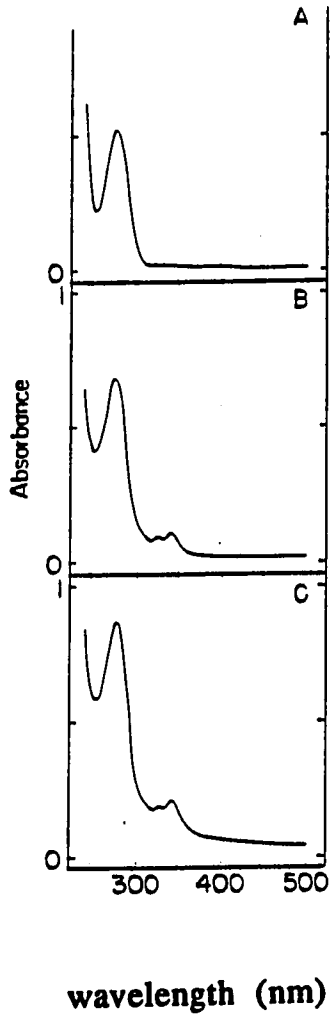


Figure 5. Ultraviolet-visible absorption spectra of 10 μM solutions in 50 mM acetate buffer at pH 5.0 (A) Papain (B) Papain labeled with $Pt(trpy)^{2+}$ at Cys 25 (C) Papain labeled with $Pt(trpy)^{2+}$ at both Cys 25 and His 81

defined in equation (16), in which k_N and k_S are

$$n_{Pt} = \log \frac{k_n}{k_s} \qquad (16)$$

rate constants for the displacement, by the nucleophile and by the solvent, of a given ligand in a reference complex of platinum(II). The reference solvent usually is methanol, and the reference complex usually is trans-$[Pt(py)_2Cl_2]$. [17,59] We chose three nucleophiles intermediate between imidazole (as a model for His-81) and thiophenolate (as a model for Cys-25). Their n_{Pt} values are as follows: Im, 3.44; Br^-, 4.18; SCN^-, 5.75; thiourea ($(NH_2)_2CS$), 7.17; and $C_6H_5S^-$, 7.17.

The histidine ligand in the model complex is displaced by equimolar amounts of the two nucleophiles according to equations (17) and (18). It is not displaced by

$$[Pt(trpy)His]^{2+} + SC(NH_2)_2 \longrightarrow [Pt(trpy)\{SC(NH_2)^2\}]^{2+} + His \qquad (17)$$

$$[Pt(trpy)His]^{2+} + SCN^- \longrightarrow [Pt(trpy)SCN]^+ + His \qquad (18)$$

either an equimolar amount or a 10-fold molar excess of bromide, but apparently is displaced by a 100-fold molar excess of bromide. The cysteine ligand in $[Pt(trpy)Cys]^+$ is not displaced by any of the three nucleophiles in the equimolar amounts, but is partially displaced by 10-fold excesses of thiourea and thiocyanate. All of these findings are consistent with the relative nucleophilicities of the ligands.

Contrary to the expectations on the basis of these model studies, thiourea and thiocyanate that were equimolar with the doubly-labeled papain proved unsuitable for the selective removal

of $Pt(trpy)^{2+}$ from His-81. This goal was achieved by successive treatments of the diplatinated enzyme with 100-fold molar excesses of bromide, followed by dialysis. At the end of this procedure, the UV-vis spectrum showed only one $Pt(trpy)^{2+}$ tag per papain molecule. It probably is bonded to Cys-25.

CONCLUSION

This study showed some of the advantages of transition-metal complexes as new reagents for selective modification of enzymes, including their active sites. Because the rate of the chloride displacement in $[Pt(trpy)Cl]^+$ depends on the nucleophilicity and accessibility of the amino-acid side chains, kinetic studies can reveal important properties of the active site. Selectivity in labeling can be achieved by selective removal of labels from the protein as well as by their selective attachment to the protein.

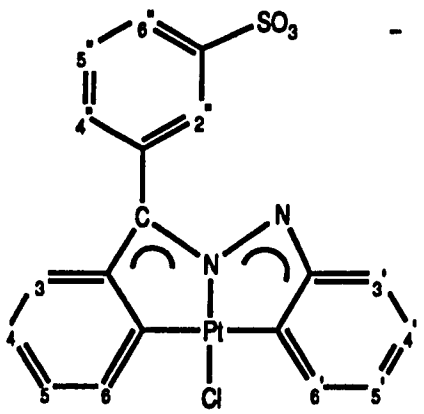
SECTION VII.

COVALENT MODIFICATION OF CYTOCHROME *c* WITH A HYDRAZONE COMPLEX OF PLATINUM(II)

INTRODUCTION

Tridentate ligand pyridine-2-aldehyde 2'-pyridylhydrazone, designated paphy, forms stable complexes with numerous transition metals;[169-172]

platinum(II) is one of them. [172] These complexes are useful in extraction of metal ions. [173] The conjugated π-electron system in the paphy complexes causes high absorptivity and facilitates colorimetric determination of metals. The present study concerns a derivative designated sbpaphy, in which the conjugation is extended over a 3-sulfobenzoyl substituent. Ionization of the sulfonic acid renders this compound anionic in aqueous solution. The complex Pt(sbpaphy)Cl⁻ is shown below with its protons numbered for the assignment of ^{1}H NMR signals (*vide infra*).



The new complex retains the advantages of Pt(trpy)Cl⁻. The high absorptivity of Pt(sbpaphy)Cl⁻ allows for its easy detection and quantitation. Since in both complexes only the unidentate Cl⁻ ligand is substituted easily. The new reagent, too, should bind to the protein in a simple one-step reaction--displacement of the Cl⁻ ligand by an amino-acid side chain. But the terpy and sbpaphy ligands, and thus their homologous Pt(II) complexes, differ in

charge. The imino N atom of the coordinate chelate is deprotonated already in the neutral and weakly basic solutions so that it is the sbpaphy form which is ligated to platinum.[169] It is interesting to compare the reactivity of the oppositely charged $Pt(trpy)Cl^+$ and $Pt(sbpaphy)Cl^-$, complexes toward horse-heart cytochrome *c* , a protein with positive overall charge of *ca.* +7 at pH 7.0. [35] Studies such as this may contribute to the understanding of electrostatic effects in protein-protein and enzyme-substrate interactions.

EXPERIMENTS

Chemicals and Apparatus

Ligand sbpaphy was obtained from Aldrich Chemical Co. under the name 2-(3-sulfobenzoyl)-pyridine 2-pyridylhydrazone. Amino acids and horse-heart cytochrome *c* (of type VI) were obtained from Sigma Chemical Co. The protein, although nearly homogeneous as received, was purified further by chromatography on CM 52 cellulose [174] and only the major fraction was used for the labeling experiments. Ultrafiltration was performed with Amicon YM 5 membranes under the pressure of purified nitrogen at 4°C. The 1H NMR spectra were recorded in D_2O solutions with a Nicolet NT 300 spectrometer. Absorption spectra were measured with an IBM 9430 UV-vis spectrophotometer, equipped with a two-grating monochromator. Cyclic and differential-pulse voltammograms were obtained with an IBM EC225 analyzer and a BAS cell assembly, according to published procedures. [175] All solutions were made in 85 mM phosphate buffer at the pH indicated. This buffer was also used as the eluent for cation-exchange chromatography.

Pt(sbpaphy)Cl$^-$ complex

An 8 mM solution of sbpaphy was prepared by dissolving 29.9 mg of it in 10 mL of the buffer at pH 7.0; the dissolution required heating at 70°C for 2 hours. An 8 mM solution of K_2PtCl_4 was prepared by dissolving 33.2 mg of it in 10 mL of the same buffer at room temperature. A solution of the ligand was added dropwise to a stirred orange-red solution of K_2PtCl_4 and the mixture, 4 mM in each reactant, was kept in the dark at room temperature. The color changed from orange to purple in one day and the title complex in solution remained stable. The reaction was also carried out in the

buffer at pH 5.5. For monitoring by ^1H NMR spectroscopy, the reaction was carried out in D_2O according to the same procedure except with one-tenth of the stated volumes and weights.

Reactions with Amino Acids

Glycine and amino acids containing heteroatoms in the side chain were incubated separately with an equimolar amount of Pt(sbpaphy)Cl$^-$ for several days at room temperature, that is, under the conditions used for the incubation of cytochrome *c*. A 4 mM solution of the amino acid or of its N-acetyl derivative in the buffer at pH 7.0 was added dropwise to an equal volume of a stirred 4 mM solution of Pt(sbpaphy)Cl$^-$ in the same buffer. The following amino acids caused no significant change in the UV-vis spectrum of the chelate complex; Gly, Met, Lys, Arg, Trp, Glu, Gln, Thr, Asp, and Asn. Only two amino acids, Cys and His, produced a color change. Imidazole (Im) behaved as His did. The reactions of Pt(sbpaphy)Cl$^-$ with His and with Im were faster at pH 7 than at pH 5.5, as evidenced by rate of color change.

Protein Modification

Ferricytochrome *c* from horse heart (typically 25 mg or 2 μmol) was incubated with two equivalents of Pt(sbpaphy)Cl$^-$ in the buffer at pH 5.5 or 7.0, in the dark, at room temperature. The reaction mixture was made by adding dropwise 500 uL of the 4 mM solution of the labeling reagent to 250 μL of the 4 mM solution of the protein; both solutions were made in the buffer of the same pH value. After two days, any particles were removed by centrifugation. R%regardless of the pH value of the reaction mixture (5.5 or 7.0), the reaction was ended by ultrafiltration into the buffer at pH 7.0, the only used as chromatographic eluent. The

components of the mixture were separated at 4°C on a CM 52 column that was equilibrated with the buffer at pH 7.0. About 1 mg of cytochrome was applied per 1 mL of the resin bed and the chromatographic procedure was identical to that by which the native protein was purified.[174] Three bands were eluted from the column. The first one, containing the unreacted Pt(sbpaphy)Cl- that was not dialyzed away, moved rapidly because of its negative charge. The second one, containing the modified protein, was diffuse. The third one, containing the native cytochrome c, was distinct. The relative chromatographic yields of the second and third bands were as follows: 13 and 87% at pH 7.0; 6 and 94% at pH 5.5. These values are averages from several experiments.

Treatment with $Pt(trpy)Cl^+$

The cytochrome *c* derivative contained in the second band was incubated separately with an equimolar amount (as determine by UV-vis spectrophotometry) of $[Pt(trpy)Cl]Cl{\cdot}2H_2O$ at pH 7.0 for one day, as described in detail in a previous report. [35] The incubation was ended by exhaustive ultrafiltration, in which the components of low molecular weight were dialyzed away. The dialyzed proteins were chromatographed as usual on CM 52 columns with the buffer at pH 7.0. The original modified protein (the second band) yielded two band upon this repeated chromatography. The first one was identical to the modified protein prior to the treatment with $Pt(trpy)Cl^+$. The second band contained native protein, resulting from the partial loss of the $Pt(sbpaphy)Cl^-$ label from the modified protein. This loss seems to be caused by binding of $Pt(trpy)^{2+}$ to the deprotonated amide N atom in the Pt(sbpaphy) label. A Pt(trpy) derivative of cytochrome *c* was not detected.

RESULTS AND DISCUSSION

The Pt(sbpaphy)Cl$^-$ Complex

This complex was the product of a simple substitution of a tridentate sbpaphy ligand for three Cl$^-$ ligands in $PtCl_4^{2-}$.

The ^1H NMR spectrum changed during the substitution reaction from that of the free tridentate ligand [176,177] to that of the single chelate complex. Coordination to the positively charged Pt(II) atom causes movement to lower field of the ^1H signals for most of the protons, as Table XX shows. The difference between the tabulated chemical shifts for the free paphy and sbpaphy are due, in part, to the different solvents that had to be used for the neutral (paphy) and for the charged (sbpaphy$^-$ and Pt(sbpaphy)Cl$^-$) solutes.

Since tridentate coordination is possible only if the imine N atom is deprotonated and sbpaphy^{2-} formed, [172] the stability of the Pt(sbpaphy)Cl$^-$ complex depends on the pH of the solution; the same is true about the complex containing the unsubstituted paphy ligand. [172] In particular, the new complex is stable at pH 7.0, at which most of the reactions for cytochrome modification were carried out. The formation of the Pt(sbpaphy)Cl$^-$ complex at pH 7.0 is accompanied by the growth of a weak absorption band at 540 nm. Over time, this band shifts to lower wavelengths as the solution turns from a purple to a red color. After three weeks, the maximum is at 505 nm. The cause of this probably is aquation of the Cl$^-$ ligand, a common substitution that does not alter the reactivity of the complex toward cytochrome *c*. As a precaution, however, all labeling reactions were done with fresh Pt(sbpaphy)Cl$^-$

Table XX. ^1H NMR chemical shifts, in ppm versus DDS, of paphy, sbpaphy and Pt(sbpaphy)Cl$^-$

	paphy[a]	sbpaphy[b]	Pt(sbpaphy)Cl[b]
H3	8.08	8.17	8.47
H3'	7.42	7.90	7.96
H4	7.91	8.07	8.12
H4'	7.78	8.05	8.04
H5	7.41	7.60	7.72
H5'	6.93	7.26	7.38
H6	8.65	8.81	8.88
H6'	8.26	8.62	8.47
H5"		7.83	7.87
H4"		7.67	7.74
H2",H5"		8.11	8.21

[a]From reference 176, for 120 mM solution in DMSO-d_6.
[b]For 2 mM solutions in D_2O at 25° C, with respect to the residual water as an internal reference.

solutions. At pH 5.5, the stability of the complex was less, as evidenced by slight precipitation within a few days, but still sufficient for the protein modification.

Reactions with Amino Acids

Selectivity of Pt(sbpaphy)Cl$^-$ toward potential ligands in a protein is the same as that of the similar complex, Pt(trpy)Cl$^+$, which has already been studied in this laboratory.[35,36] As the control reactions with glycine (and with other amino acid) show, amino and carboxylate groups of the amino-acid main chain are not reactive

toward the new complex. Among the heteroatom-containing side chains, only the thiolate (of Cys) and imidazole (of His) displace the Cl^- ligand. The reaction of Pt(sbpaphy)Cl^- with Cys produces a marked color change and is accompanied by a shift of the absorption band from 540 nm to 480 nm. Since horse-heart cytochrome *c* does not contain a free Cys residue, only the reaction with His is of interest. Since the pyrrole-type nitrogen atom is highly basic (pK_a = 14.5 in imidazole [20]) it remains protonated in these experiments. The ligating atom evidently is the pyridine-type nitrogen, whose pK_a value falls in the range 6.0-6.5.[20] The increase in the substitution rate as the pH is changed from 5.5 to 7.0 supports this conclusion. The reaction of Pt(sbpaphy)Cl- with His causes a small color change and the resulting Pt(sbpaphy)His complex has the following absorbance (ε): 14800 and 3500 $M^{-1}cm^{-1}$ at 280 and 524 nm, respectively. The color does not change further upon standing because His ligand is less amenable to aquation than Cl^- ligand.

Methionine, a soft and nucleophilic thioether ligand, is unreactive toward Pt(sbpaphy)Cl^- although the complex contains a soft and electrophilic Pt(II) atom. This unreactivity, surprising in view of the well-known affinity of such ligands and metals for each other,[17,30] has already been observed and explained in our previous study of Pt(trpy)Cl^+.[35] Molecular orbital calculations and consideration of molecular models both revealed prohibitively short contacts between the methyl H atoms in the thioether ligand and the ortho H atoms in the pyridine rings of the trpy ligand. These steric repulsions occur regardless of the Pt-S torsion angle in the thioether complex, but are virtually absent in the imidazole complex. Consideration of the molecular models of Pt(sbpaphy)L complexes with thioether and imidazole ligands as L reveals similar crowding with the former and lack of it with the latter ligand and thus explains the observed selectivity toward His over it with the

latter ligand and thus explains the observed selectivity toward His over Met. Similarity in the chemical reactivity of the trpy and sbpaphy complexes is understandable in view of their structural homology.

Protein Modification and Binding Sites

Complex Pt(sbpaphy)Cl$^-$ reacts with cytochrome *c* under mild conditions. The label on the protein is easily detectable owing to its strong UV and visible absorption bands, as Figure 6 shows. The elution order from the cation-exchange column, namely the modified protein before the native one, probably is consequence of the negative charge of the sulfonate group in the sbpaphy ligand. This order is opposite from the one found in cytochromes *c* labeled with the cationic Pt(trpy)$^{2+}$ tags, [35,36] where the derivatives eluted after the native protein under the same chromatographic conditions. Unreacted Pt(sbpaphy)Cl$^-$ is efficiently removed during the chromatographic separation. The Pt(sbpaphy) tag on the modified protein is stable at pH 7.0.

Of all the amino acids that contain potentially ligating side chain, only Cys and His react with Pt(sbpaphy)Cl$^-$ under the conditions of protein labeling. Since free Cys residues are absent from the horse-heart cytochrome *c*, the likely binding sites are imidazole side chains. Indeed, the spectrum of the labeled protein can be reproduced by adding the spectrum of Pt(sbpaphy)His to that of the native protein. The data in Figure 7 corroborate this conclusion. The difference spectrum, due to the chromophoric tag, corresponds to the spectrum of one molecule of Pt(sbpaphy)His per molecule of cytochrome *c*.

There are three possible sites, namely histidine residues in

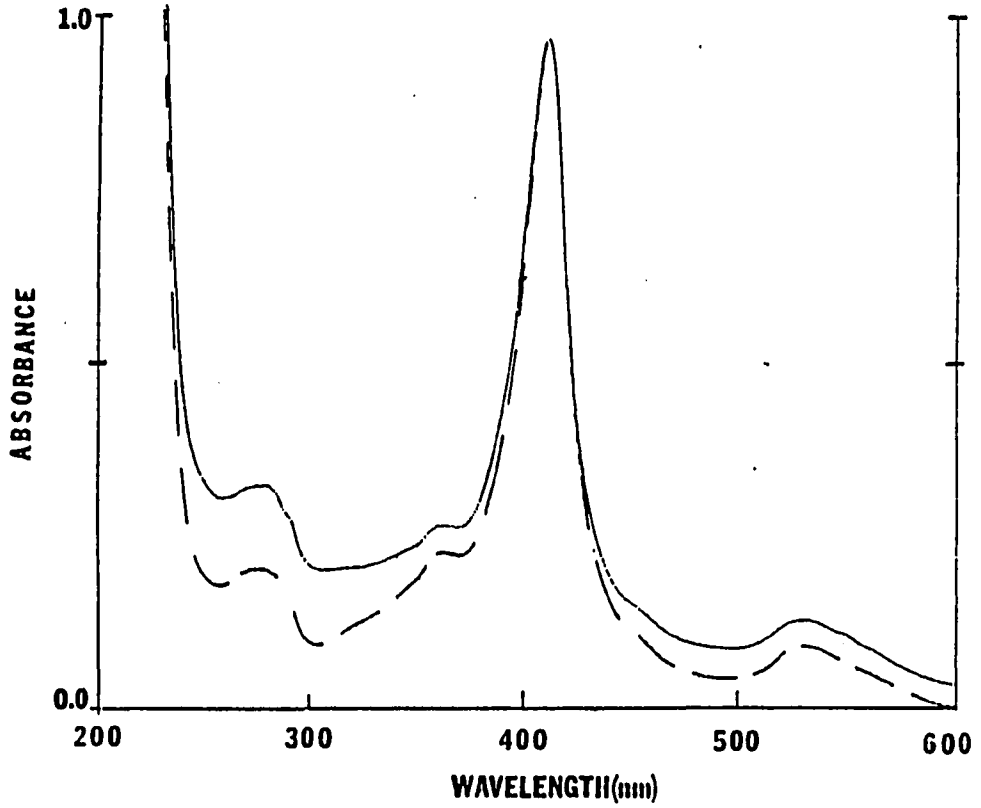


Figure 6. Absorption spectra of the native horse-heart cytochrome *c* (---) and of its derivative tagged with Pt(sbpaphy) at His 33 (——); both solutions are 10 μM, made in 85 mM phosphate buffer at pH 7.0

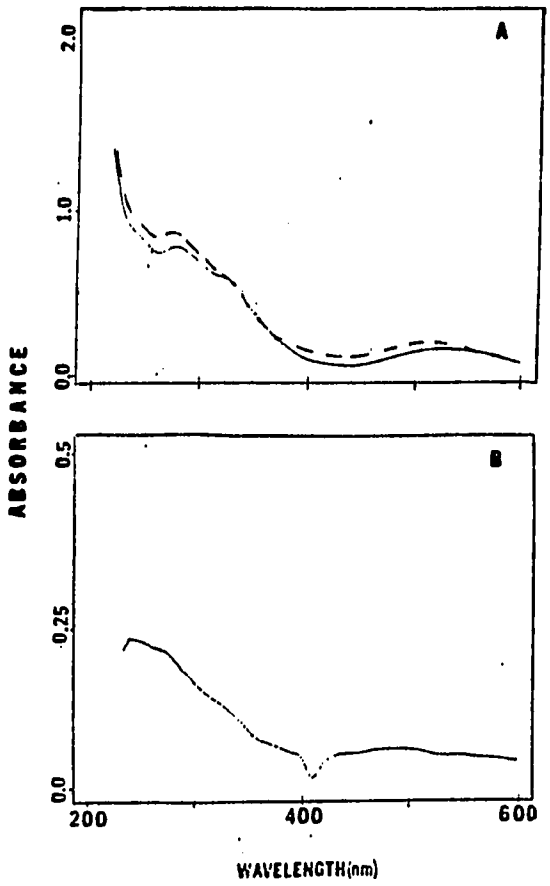


Figure 7. (A) Absorption spectra of 55 μM solutions of K[Pt(sbpaphy)Cl] (---) and of Pt(sbpaphy)His (——) in 85 mM phosphate buffer at pH 7.0 (B) Difference between the absorption spectra shown in Figure 1, namely that of horse-heart cytochrome *c* tagged with Pt(sbpaphy) at His 33 minus that of the native cytochrome *c*

positions 18, 26, and 33. Binding to His-18, an axial ligand to the Fe atom, can be ruled out. The strong ferriheme absorption bands at 410 and 530 nm [71,178] and the weak one at 695 nm, which originates in the interactions between the Fe atom and its axial ligands (Met-80 and His-18), [179,180] seem to be unperturbed by the protein modification. The reduction potential of the protein, measured by both cyclic and differential-pulse voltammetry, is unaltered by labeling; the values for the native and modified horse-heart cytochrome *c*, respectively, are 0.259 and 0.265 V versus the standard hydrogen electrode at 25°C and at pH 7.0.[181]

The remaining two residues, His-26 and His-33, lie on the protein surface, but in different surroundings. The former is hydrogen-bonded in a hydrophobic pocket and largely shielded from the exterior, whereas the latter is located in the hydrophilic region and is exposed to the exterior.[92,100] The reactivity of these two sites towards $Pt(trpy)Cl^+$ corresponds to their accessibility from solution: His-26 is barely reactive, whereas His-33 is very reactive toward this hydrophobic reagent.[35] The lower overall reactivity, and lesser hydrophobicity, of $Pt(sbpaphy)Cl^-$ in comparison with $Pt(trpy)Cl^+$ both militate against the binding of the former reagent to His-26. This reagent most likely binds to His-33. This conclusion is corroborated by the fact that the yield of the labeled protein increases as the pH increases from 5.5 to 7.9. This pH range includes the pK_a of His-33 (the normal value of 6.2 [100]), but is far removed from the pKa of His-26 (the anomalous value of 3.2, caused by hydrogen bonding [100]). The assignment of the binding site is further corroborated by the treatment of the modified protein with $Pt(trpy)Cl^+$, a reagent virtually specific toward His-33. No binding of $Pt(trpy)^{2+}$ is detected because His-33 is blocked by the Pt(sbpaphy) label.

CONCLUSION

Complex Pt(sbpaphy)Cl^- is suitable for tagging of biological macromolecules. As with Pt(trpy)Cl^+, its reactivity toward imidazole (side chain of His) and unreactivity toward thio ether (side chain of Met) are caused mainly by the steric properties of the tridentate sbpaphy ligand. This work provides yet another example[35,36,132,182,183] how selectivity of a preformed transition-metal complex toward a biological macromolecule can be controlled by ancillary ligands, which do not participate directly in the substitution reaction. An important advantage of the new inorganic reagent lies in the strong UV-vis absorption by the Pt(sbpaphy)His chromophore (ε_{280}=14800 $M^{-1}cm^{-1}$), which permits easy detection and quantitation of the tags bonded to the protein.

This study sheds some light on the importance of electrostatic factors for the interaction between small molecules and proteins. Although Pt(trpy)Cl^+ and Pt(sbpaphy)Cl^- complexes have opposite charges, they exhibit the same selectivity toward horse-heart cytochrome *c*, a protein with a substantial overall charge of *ca.* +7 at pH 7.0.[35] Moreover, the anionic complex gives lower yield than does the cationic one. The overall charge of the reactant evidently is not decisive for the outcome of the covalent modification of a protein. More important seem to be the intrinsic substitution reactivity of the inorganic complex and the distribution of (local) charges on the protein.

SECTION VIII.

ELECTRON-TRANSFER REACTIONS OF CROSS-LINKED CYT *C* AND Pc

INTRODUCTION

The thermodynamically favorable transfer of an electron from native cytochrome *c* (cyt *c*) to plastocyanin (pc) is a reaction which has been widely studied.[184-193] Since cyt *c* and pc arguably are the two best characterized metalloproteins,[90,194] the electron transfer between the two offers an excellent model for similar reactions in physiological systems.

Pc is a Cu protein essential for electron transfer in photosynthetic systems of all higher plants and some algae. The Cu atom is located in a hydrophobic pocket near the "northern" end of the molecule. It is ligated in a distorted tetrahedral environment by His 37, Cys 84, His 87 and Met 92. The imidazole ring of His 87 is accessible to solvent. It also has a concentrated patch of negatively charged acidic residues composed of two clusters (residues 42-45 and 59-61). These two patches flank a highly conserved residue, Tyr 83, which is also accessible to solvent. The physiological reductant of pc is cytochrome *f*, a 33 kDa heme protein. The physiological oxidant of pc is photosystem $P700^+$. The pc acts as an electron shuttle between cyt *f* and $P700^+$. Many studies of interactions of pc with inorganic and biological reagents have been done to understand electron-transfer processes involving pc.[195] These studies have shown that pc has two electron-transfer sites. Each electron transfer site may be specific for one of the physiological partners.

In laboratory studies, the natural reductant, cyt *f*, has frequently been replaced by a smaller heme protein, cyt *c*. Cyt *c* is an important mitochondrial protein involved in respiration and is intricately involved in the electron transfer processes accompanying respiration. Crystallographically determined coordinates are available for cyt *c*,[96] but not for cyt *f*. In cyt *c*, the heme group is attached to the protein backbone via thioether

linkages at Cys 14 and Cys 17. The Fe is axially coordinated by Met 80 and His 18. One edge of the heme, extending between the methyl groups on porphyrin rings C and D, is exposed to solvent and is surrounded by a patch of positively charged Lys residues. Although cyt *c* and pc are not biological partners, the rate constant for electron transfer from cyt *c* to pc is approximately 1 x 10^6 M^{-1} s^{-1}.[192] The rate constant for electron transfer form cyt *f* to pc is 3.6 x 10^7 $M^{-1}s^{-1}$.[186] The cyt *c* provides a good model for cyt f. In this study, electron transfer is studied between free cyt *c* or pc and a covalently cross-linked heterodimer of cyt *c* and pc. Such kinetics studies are useful in (1) identifying the site of electron transfer from cyt *c* and to pc , (2) identifying the general orientation of the two proteins in the cross-linked complex, (3) identifying secondary electron transfer sites, and (4) providing a more elaborate model for multiple-protein electron-transfer reactions.

In addition, spectroscopic studies, namely CD, MCD, and UV-vis spectroscopy are used to diagnose differences in protein structure whether the protein is in its native state, electrostatically complexed to another protein, or covalently attached to another protein. These spectroscopic analyses indicate if reorganization of protein structure plays a role in the electron-transfer systems discussed below.

EXPERIMENTS

Chemicals

Riboflavin, TNBS(trinitrobenzene sulfonic acid), and cyt c (type VI) from horse heart were obtained from Sigma Chemical Co. Plastocyanin was prepared as described below. 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDC), disuccinimidyl tartarate (DST), and ethylene glycolbis(succinimdylsuccinate) (EGS) were obtained from Pierce Chemical Co. The laser dye LD 425 was obtained from Exciton.

Preparation of Plastocyanin

Approximately 23 lbs of Burpee Tenderpod French (or snap) beans (*Phaseolus vulgaris*) were delivered to Curtiss Farm of University Farm Services. The seeds were purchased at Brekke's Town and Country seed store in Ames at $32/10 lb. Approximately 10 lbs. of this seed were planted in a 1/2 acre plot. The larger amount of seeds delivered is necessary for charging of the planter. The seeds were planted on June 8. This is a very late planting date. Planting in late April or early May is recommended.

The acreage was not irrigated and was treated with only a preemergence herbicide by Farm Services. No post emergence cultivation was done. About six weeks after planting, the bean plants were manually weeded. There was a thick growth of velvetleaf weeds which, if not removed, would have made harvesting difficult. In addition, these weeds were removed to enable the bean plants to thrive.

Harvesting of the plants started in the last week of July. By this time, the plants were fully mature and bean pods were fully formed. Plants should be harvested at 4 to 6 weeks, long before

the beans mature. This also allows for a longer harvesting period. Approximately two weeks after reaching maturity, the leaves of the plants start to rust.

The bean plants were harvested by hand. The whole plants were uprooted and placed in 30 gallon garbage bags. These were then immediately transported back to a cold room of 4° C. The leaves were stripped from the stems, and the stems and beans were discarded, along with yellow leaves. The green leaves were juiced as soon as possible. The harvest yielded 86 garbage bags of bean plants. Each bag weighed approximately 20 lbs which gives a total of 1720 lbs (0.86 tons!) of bean plants for processing. We harvested approximately 1/3 of the 1/2 acre (*i.e.*, 1/6 of an acre). The storage of the harvested plants was limited to a maximum of 2 weeks, after which, the leaves start to rust. The protein isolation procedure followed a previous method[196] with modifications as described below.

The leaves were rinsed with cold tap water 3 times. The water was drained, and the leaves were placed in dishpans of 50 mM sodium acetate buffer of pH 6.0. The leaves were dipped, not soaked, in the buffer. The bean leaves were juiced with 2 Champion juicers. It takes 8 intense person-hours on one juicer to produce 20 liters of juice, if the juicer is in prime operating condition. The juicer blades wore down quickly and replacement blades should be ordered prior to undertaking this task. After hours of operation, the juicers become clogged repeatedly. An estimate of juice production per person per day at the end of the season is close to 4 liters per day. The by-product of pulp was collected in garbage bags and discarded. Approximately 5 bags of bean plants (100 lbs.) could fill one 20 L carboy with juice. Our total yield of raw juice was approximately 300 L.

Juice was collected in 5 gallon buckets. After a bucket was full, sediment settled and the foam was removed by skimming the

surface with a small strainer. The foam was discarded. The juice was then stirred and poured into 20 L carboys and frozen at -40° C in an ultrafreezer or it was stored in a meat locker (courtesy of the Meat Sciences Laboratory) at -18° C. The juice can be indefinitely stored at this stage.

After thawing, the bean juice was shaken while in the carboy to disperse settled sediment. The juice was poured into 500 mL centrifuge bottles and centrifuged at 3000 rpm for 30 minutes. This speed corresponds to about 10000 g. A heavy green pellet formed. The juice was decanted and filtered through 2 layers of cheesecloth and stored at 4° C. When 10 L of clarified juice was collected, the juice was brought to 40% saturation with $(NH_4)_2SO_4$ (240 g/L). The solid ammonium sulfate was usually added over the course of one or two hours and was stirred 6-18 hours at 4° C. This juice was centrifuged for 15 minutes at 6000 rpm in a JA-10 rotor in a Beckman J-21B or J2-21 M/E centrifuge at 4° C. The pellets were removed and the juice was treated with 320 g/L of $(NH_4)_2SO_4$ to bring to 80% saturation. The ammonium sulfate was again added over 1-2 hours and stirred overnight. This juice was centrifuged at 6000-8000 rpm for 15 minutes, and the pellets were isolated and were resuspended in 50 mM acetate buffer of pH 6.0. The volume of juice had decreased from 10 liters to 500 mL. This juice was centrifuged for 15 minutes at 6000-8000 rpm, and the pellets were removed. The remaining juice was adjusted to pH 4.0 with cool 50% glacial acetic acid and was centrifuged as soon as possible. The juice was centrifuged 15 minutes at 6000-8000 rpm, and the pellets were removed. The juice was brought to pH 6.0 with 6 M NaOH at 4° C.

The juice from the final step above was concentrated in 400 mL Amicon cells with YM-5 membranes to approximately 1/5 of its original volume. Aliquots of the juice were then loaded on a 2.5 x 75 cm G-25 column equilibrated with 50 mM acetate of pH 6.0.

Two unresolved bands were seen. A dark black-brown band eluted first, followed by a fainter red-brown band. The black-brown band was collected and was concentrated to 1/5-1/10 of its volume in an Amicon cell.

An aliquot of this juice (30-50 mL) was treated with approximately 100 mg of K_3FeCN_6 and was applied to a 2.5 x 50 cm column of DEAE preequilibrated with 50 mM acetate buffer of pH 6.0. One liter of the acetate buffer was passed through the column. The same buffer with 0.1 M NaCl was then started. A blue band emerged from the black band at the top of the column. After the blue band separated from the black band, buffer which was 0.2 M in NaCl was started. The pc was eluted from the DEAE column with this buffer. The DEAE resin became brown and was impossible to restore to its original condition. It is best to discard the top layers of the resin and to reuse the DEAE resin. The resin was reused after washing with 50 mM acetate buffer which was 0.5 M in NaCl and reequilibrating with the 50 mM acetate buffer of pH 6.0.

The blue band was concentrated with a YM-5 membrane in an Amicon cell and was placed on a 2.5 x 75 cm G-75 column and eluted with 50 mM acetate buffer of pH 6.0. A light brown band eluted ahead of the blue protein. The collected blue band was treated with a few grains of $K_3Fe(CN)_6$ and was applied to a 2.5 x 30 cm column of A-25 DEAE Sephadex equilibrated with 50 mM acetate buffer of pH 6.0. After sample application, the buffer was stepped up to 0.1 M NaCl and eventually to 0.2 M NaCl. The isolated protein was frozen at -40° C. The pc should be frozen at any stage between chromatographic procedures if the process is not to be continued immediately. If left at 4° C, the impure pc will denature quickly.

The purity of pc was measured by the absorbance quotient A_{280}/A_{595}. Values less than 1.3 of the fully oxidized proteins were considered satisfactorily pure.

Preparation of cytochrome *f*

Since cyt *f* is the physiological partner of pc, its isolation was attempted. The following is an account of the procedure used in the attempt to isolate cyt *f* from red rape (*Brassica napus*).

One-half acre of red rape seed was planted on Curtiss farm on June 9, 1989. Fifty pounds of the seed were needed to charge the planter, but only 10 pounds of seed were used. The seeds were purchased for a price of $18/50 lbs. The acreage was not irrigated and was only treated with a preemergence herbicide. No post emergence cultivation was done.

Approximately 6 weeks after planting, the rape plants reached full maturity as evidenced by the emergence of yellow flowers. The crop should have been harvested at this time, but was neglected because all efforts were focused on the concurrent bean crop. After reaching maturity, the crop rapidly deteriorated and was completely lost within 2 weeks. It is recommended that harvesting begin long before the onset of maturation. A new crop was planted in mid August in an attempt to get another harvest before winter. A moderate harvest was obtained in late September and throughout October. The immature plants were hearty in cold weather. Optimal planting time, however, is in the spring. The rape was harvested and placed into 30 gallon garbage bags. A total of 18 bags of plants at 20 lbs each were collected. This collection did not even decimate the 1/2 acre crop. The plants were stored at 4° C until processed. Leaves were processed as soon as possible.

The isolation procedure was the same as that reported [197] with minor modification. The rape leaves were juiced with Champion juicers in order to separate juice and pulp. Before juicing, the leaves were washed with cold tap water and were dipped in a solution of 0.2 M K_2HPO_4 and 10 mM EDTA. The juice was collected into 5 gallon buckets. The top layer of foam was removed from the

juice with a strainer. The juice was frozen at -40° C until further use.

Approximately 750 mL of liquid rape juice was placed in a 4 L beaker with 1500 mL of methyl ethyl ketone. The mixture was stirred vigorously at 4° C with a magnetic stir bar for 5-10 minutes. The buffer and ketone were refrigerated at 4° C before use. After stirring, the layers settled. The upper green phase (the ketone layer) was poured off and the lower phase (the buffer layer) was passed through 4 layers of cheesecloth. The two layers were recombined, and the emulsion was centrifuged at 12000 g (8500 rpm JA-10 rotor) for 15 minutes. The bottom buffer layer was removed by aspiration. Chilled acetone (4° C) was added to the yellowish buffer layer (1.2 volumes). The acetone mixture was centrifuged at 8500 rpm in a JA-10 rotor for 15 minutes. The pellets recovered were reddish-brown and were suspended in 50 mL of 40 mM K_2HPO_4 and 2 mM EDTA. This suspension was centrifuged at 8500 rpm for 15 minutes. The recovered supernatant was faint reddish brown and should contain the cyt *f*.

The reddish-brown liquid was concentrated in an Amicon cell with a YM-10 membrane. The concentrated solution was passed through a 27 x 3.5 cm column of Sephadex G-25 equilibrated with 1 mM sodium phosphate buffer at pH 7.5. The collected eluate was stored at -40° C until sufficient extracts were accumulated for further purification. The thawed extracts were centrifuged to remove turbidity. The extracts were concentrated to 1/5 of their volume. The extracts were applied to a 7.5 x 2.0 cm DEAE cellulose (Whatman DE52) column equilibrated with 1 mM sodium phosphate of pH 7.5. A brown band was retained at the top of the column. Ideally, a pink band should be eluted with 10 mM sodium phosphate at pH 7.5 and 100 mM sodium phosphate should remove polyphenol-modified cyt *f*. Even washing with 5 M NaCl in 100 mM phosphate did not remove anything from the top of the column. No

pink band was observed.

A possible explanation for the problems experienced with this procedure is that the cyt *f* may be extremely modified with polyphenols. Mature plants are likely to be highly modified with polyphenols. The whole procedure was repeated with juvenile plants. As of this writing, the outcome of the procedure with the juvenile plants is unknown.

Preparation of Cyt *c*-Pc Dimers

Cross-linking of cyt *c* and pc with EDC proved optimal if both proteins were 80 μM and the EDC cross-linking reagent was 1 mM. The buffer was 5 mM MOPS of pH 6.5. The incubation was done at room temperature for 1 day. Yields of dimeric protein ranged from 70-90%.

Cyt *c* and pc were cross-linked with DST in 5 mM MOPS buffer at pH 7.0. The proteins were 50 μM each and the DST was at a concentration of 400 μM. The incubation lasted 6 hrs. at room temperature.

The analogous reaction with EGS was done under a variety of conditions. In addition to the conditions above, the following reaction conditions were tried: 500 μM in each protein, 30-fold excess of EGS, 39 hours reaction time; 500 μM in each protein, 30-fold excess of EGS, 39 hours reaction time, 0.2 M NaCl; 30 μM in each protein, 33-fold excess of EGS, 39 hours reaction time, pH of 6.5; 30 μM in each protein, 33-fold excess of EGS, 39 hours reaction time, pH of 6.5, 0.2 M NaCl.

All reaction mixtures were dialyzed in an Amicon ultrafiltration cell to remove excess cross-linking reagent and to concentrate the reaction mixtures for sample application in chromatographic experiments. The reaction mixtures were chromatographed on a 1 x 75 cm G-75 Sephadex gel filtration

column, equilibrated with 85 mM phosphate buffer of pH 7.0. The dimeric protein obtained from this column was concentrated and oxidized with $K_3Fe(CN)_6$ and placed on a 1.5 x 20 cm CM52 column equilibrated with either 5 or 10 mM phosphate buffer of pH 7.0. These chromatographic conditions proved optimal for the EDC-cross-linked proteins.

Other chromatographic conditions were tried for the EGS- and DST-cross-linked proteins. In addition to conditions described above, chromatography was tried on a DEAE column with a gradient from 10 mM to 44 mM phosphate buffer or to 85 mM phosphate buffer at a ramp of 2 mM buffer per hour. An isocratic elution was attempted on DEAE with 10 mM phosphate buffer or 85 mM phosphate buffer, too. In addition, chromatography was tried on both CM52 and DEAE resins with 10 mM cacodylate buffer of pH 6.4 and on CM52 with 1 mM phosphate buffer of pH 7.0.

Modification of EGS Cross-linked Dimers

Lysine, native cyt *c*, and EGS cross-linked dimer were labeled with TNBS (trinitrobenzenesulfonic acid)[198] under the following reaction conditions. Lys solutions were prepared such that final concentrations were 10 μM, 50 μM, 70 μM, and 100 μM. In each Lys solution were 500 μL of 0.1 M borate buffer of pH 9.0 and 2 mL of a 1.5 mM sulfite/0.1 M NaH_2PO_4 solutions. The total volume of each Lys solution was 3.00 mL. To each solution, 100 μL of 5% TNBS (commercial preparation, 0.171 M) was added. Absorbances were measured at 420 nm to quantitate extinction coefficients of Lys modified with TNBS. Blanks were prepared sans Lys and used in the reference compartment of the spectrophotometer. Since the Lys was not acetylated, 2 TNBS labels per Lys residue were incorporated. The calculated ε for the attached chromophore on the ε N of Lys is 13500 $M^{-1}s^{-1}$. The reaction was repeated with cyt *c*

and with EGS-cross-linked cyt *c* and pc. Cyt *c* concentrations used were 0.4, 1.4, and 8.6 μM. The EGS heterodimer concentration was 1.1 μM. Incubation times varied from 5 minutes to 3 hours.

Reduction Potentials of the EGS Dimer

$Co(III)(trpy)](ClO_4)_3$ and $[Co(II)(trpy)](ClO_4)_2$ were prepared according to published procedures[199]. Cobaltous chloride (0.24 g) and trpy (0.50 g) were refluxed in 40 mL H_2O with 22 mL Br_2 water. On addition of 70% $HCLO_4$, yellow crystals precipitated. The crystals were filtered, washed with ice-water, and dried over $CaCl_2$. Cobaltous chloride (0.16 g) and trpy (0.4 g) were dissolved in 5 mL of boiled water. $NaClO_4$ (7 mL of a 2-3 g/10 mL solution) was added yielding red crystals. After cooling in ice, the crystals were filtered, washed with 75% ethanol and then with absolute ethanol. The solid was dried in air.

Redox titrations were used to determine the reduction potentials of the proteins in the EGS-cross-linked complex. Oxidized protein was treated with varying amount of $[Co(III)trpy](ClO_4)_3$ and $[Co(II)(trpy)](ClO_4)_2$ to determine the Cu reduction potential of Cu in pc and varying amounts of K_3FeCN_6 and K_4FeCN_6 were used to determine the reduction potential of Fe in cyt *c*. A plot is made of log $([titrant]_{red})/([titrant]_{ox})$ versus log $[protein]_{red}/[protein]_{ox}$. The reduction potential of cyt in the EGS dimer was estimated by monitoring the absorbance at 550 nm at different concentration ratios of the two iron complexes. The iron reduction potential used in calculations was 420 mV. Absorbance changes were monitored at 557 nm, an isosbestic point for cyt *c*, to quantitate the ratio of cupric to cuprous metal in pc in the Co titration. The cobalt complex reduction potential is 280 mV vs. NHE.

Flash photolysis

A PRA-1100 dye laser with a pulse width of 0.4 μsec was used. The dye used was LD 425 in methanol with a λ_{max} emission at 431 nm. All samples were bubbled with argon prior to kinetic experiments and were blanketed with argon during experimentation. Most reactions of the diprotein complexes were followed by monitoring a large change in the heme absorbance at 550 nm. Some reactions were also followed by monitoring the smaller change in the copper absorbance at 557 nm, an isosbestic point for ferrocytochrome *c* and ferricytochrome *c*. Riboflavin was dissolved in 3 mM phosphate buffer such that the flavin concentration was 70 μM. An extinction coefficient of 14000 $M^{-1}cm^{-1}$ at 442 nm was used to determine the exact concentration. The final buffer was 0.5 mM in EDTA which was added in the dark immediately prior to experiments. The ionic strength of the buffer was either 10, 50, 150, 250, or 500 mM. Ionic strength, μ, is calculated from solution molarities as follows:

$$\mu_{(KH2PO4)}=(0.5)(C_{Pi}))(3.53)$$
$$\mu_{(EDTA)}=(0.5)(C_{EDTA})(11.0)$$
$$\mu_{(NaCl)}=(C_{NaCl})$$

All kinetic samples were 3 mM in phosphate buffer, 0.5 mM in EDTA and were adjusted to the desired ionic strength with NaCl.

MCD and CD Spectra

All measurements were run on a Jasco Model ORD/UV-5 with a CD attachment. The spectrophotometer was equipped with a permanent magnet of field-strength 600 Gauss. This value was

determined using $K_3Fe(CN)_6$ as a standard where $\Delta\varepsilon_{422}/H = 3.0$ $(M.cm.T)^{-1}$. [200] The concentration of the $Fe(CN)_6^{3-}$ was measured based on $\varepsilon_{420} = 1040\ (Mcm)^{-1}$. $\Delta\varepsilon_m$ values were calculated using equation (19), where b, c,

$$\Delta\varepsilon_m = \frac{[\Theta]}{33bcB_0}$$

and B_0 are in units of cm, M, and T respectively. $[\Theta]$ values for MCD measurements were corrected by taking measurements of the sample with the field on and the field off (i.e., field off is removal of the magnet from the sample chamber). All scans, MCD and CD, were run from 600 nm to 230 nm at a scan rate of 100 nm h^{-1}. At least two passes were made to ensure reproducibility. The path length for all MCD measurements was 0.1 cm (limited by magnet geometry) and the path length was either 1 cm or 0.1 cm for CD measurements. MCD sample concentrations were adjusted such that A_{410} was equal to 1.06 in a 1 mm cell. For CD, the concentration was adjusted such that A_{410} was equal to 1.6 in a 1 cm cell. All spectra were run at ambient temperature.

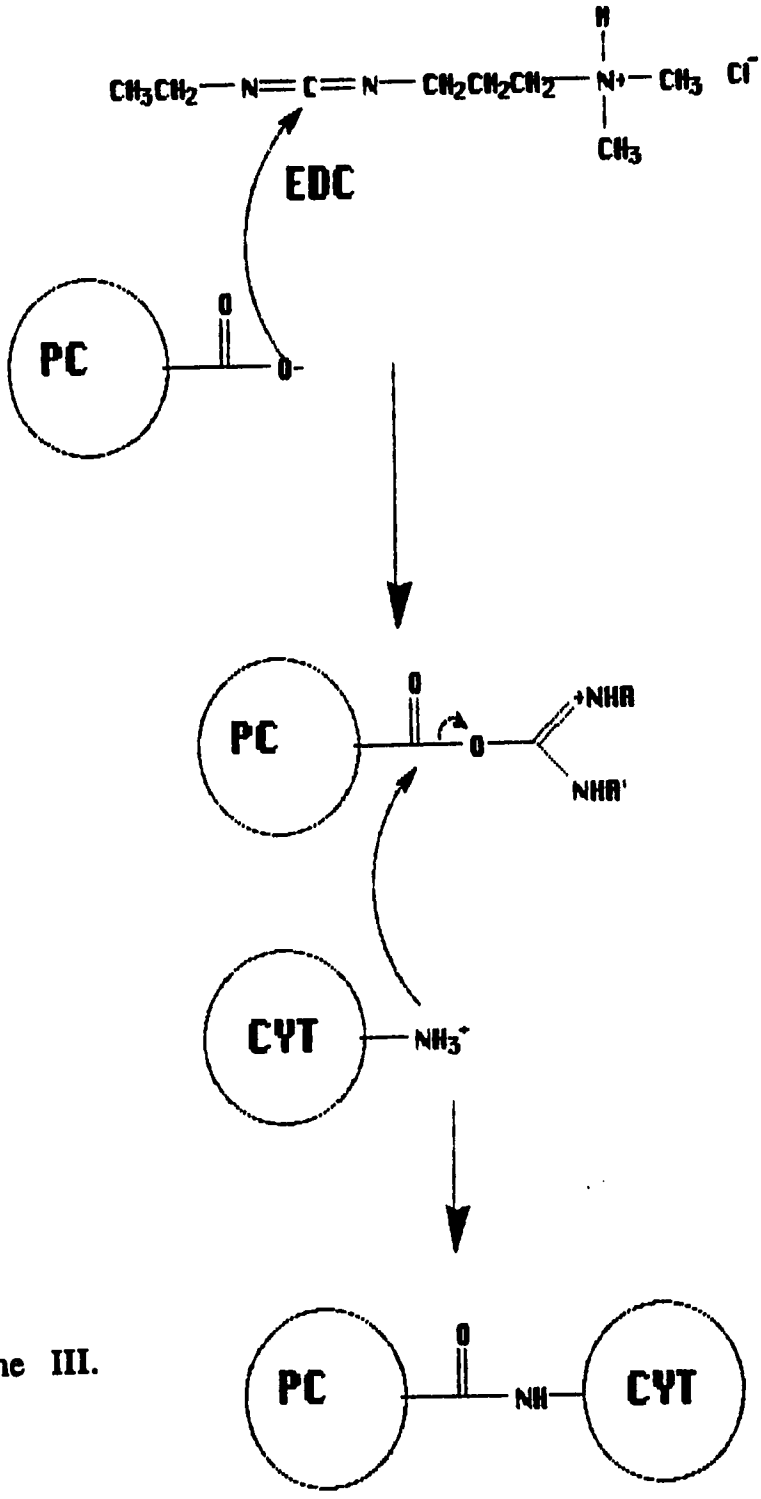
RESULTS AND DISCUSSION

Reaction Products

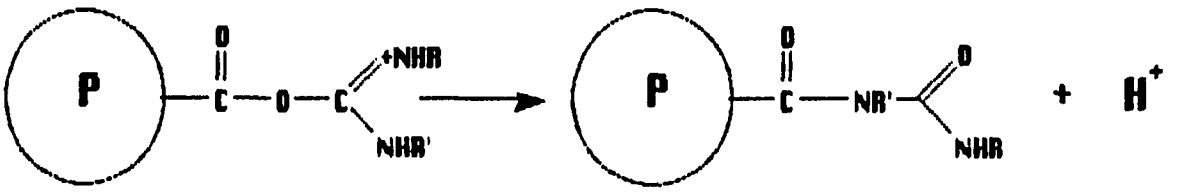
Previously in this laboratory, cyt c and pc were cross-linked with EDC, a reagent which activates direct covalent attachment between 2 reactive groups on the proteins.[184] This reaction produces heterodimeric protein in high yield 70-90%. A simplified mechanism of this reaction is shown in Scheme III.

In the cross-linking reaction, a carboxylate on pc attacks the imide carbon on EDC which, in turn, activates the carboxylate carbon on pc toward nucleophilic attack by a cyt c amine (Lys).[52b] If an amine is not present, the carboxylate carbon is attacked by water in which the labeled carboxylic group is hydrolyzed back to its native state with concomitant release of the urea group.[52b] If a carboxylate protected from solvent is modified, then a stable N-acylurea group may remain attached to the protein.[201] Studies with cyt c show that the propionic side chains of the heme can be irreversibly modified with EDC.[202] Also, Glu 62 has been shown to be labeled with EDC.[202] Since there are 19 Lys on cyt c and 5 on pc, it is not surprising to find 12 different derivatives of the EDC-cross-linked proteins. Irreversible modification may also occur at Tyr residues. An example of this type of modification is shown in Scheme IV. Characterization of these derivatives is currently underway.[203] Some initial spectroscopic characterization of the EDC-cross-linked cyt *c* and pc is discussed below.

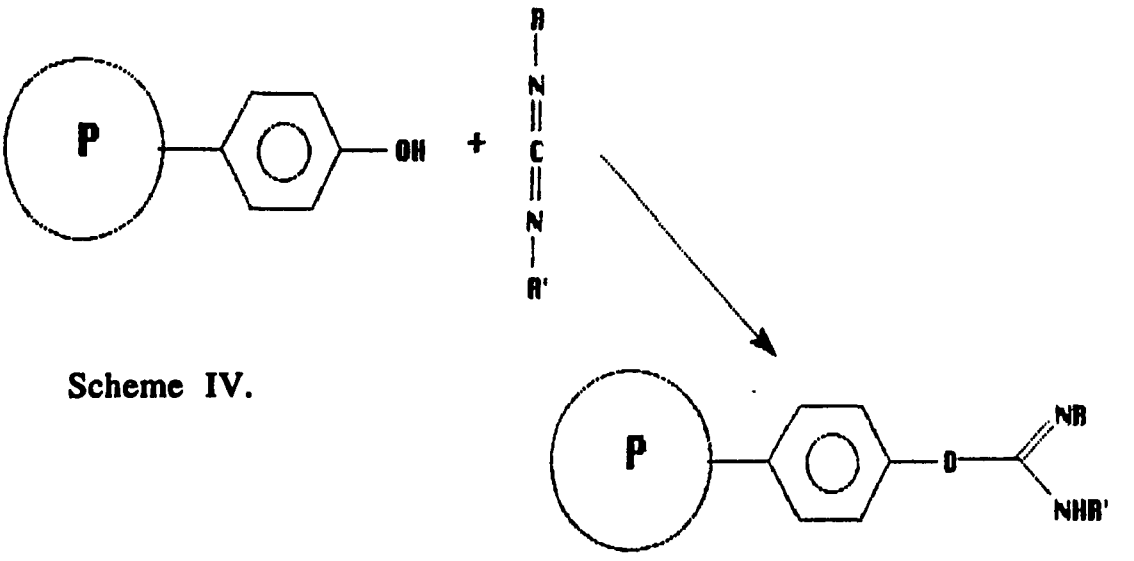
Ion-exchange chromatography on CM52 with 5 mM phosphate buffer of pH 7.0 yields a minimum of 12 bands. Four of these bands make up the majority of the cross-linked complex. These bands correspond to the first, second, fourth, and seventh bands eluted from the column. These major bands are labeled B1, B2, B3, and B4, in order of elution. It is not entirely understood how these



Scheme III.



MIGRATION

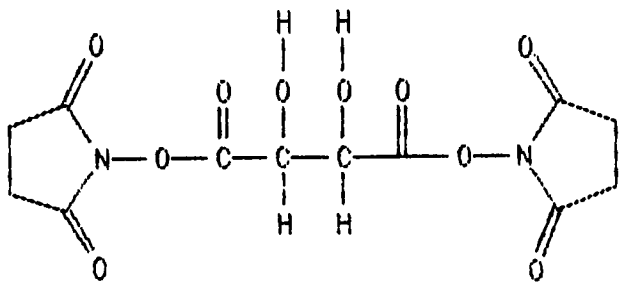


Scheme IV.

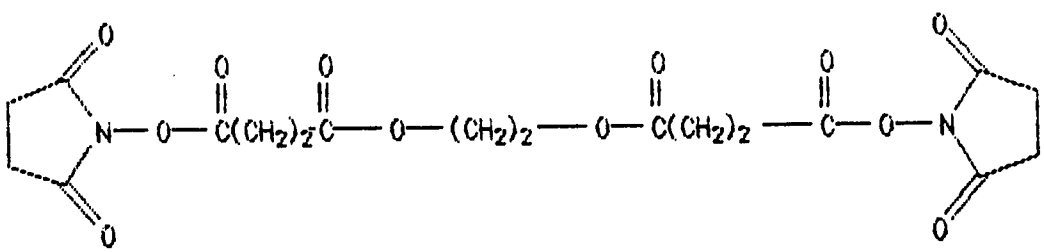
STABLE AT NEUTRAL PH

bands differ. We believe the different bands are from additional external modification of the proteins.

Since the EDC cross-linked complexes are rigid, cross-linking was done with more flexible tethers. DST, a 6 Å spacer and EGS, a 16 Å spacer were used to cross-link cyt *c* and pc. The structures of these cross-linkers are shown below. This provides a continuum of lengths for the cross-links between the proteins. The differences in properties of these "variable-spaced" proteins may provide some insight into how covalent attachment of proteins affects their structure and function. Although these tethers have the advantage of having long



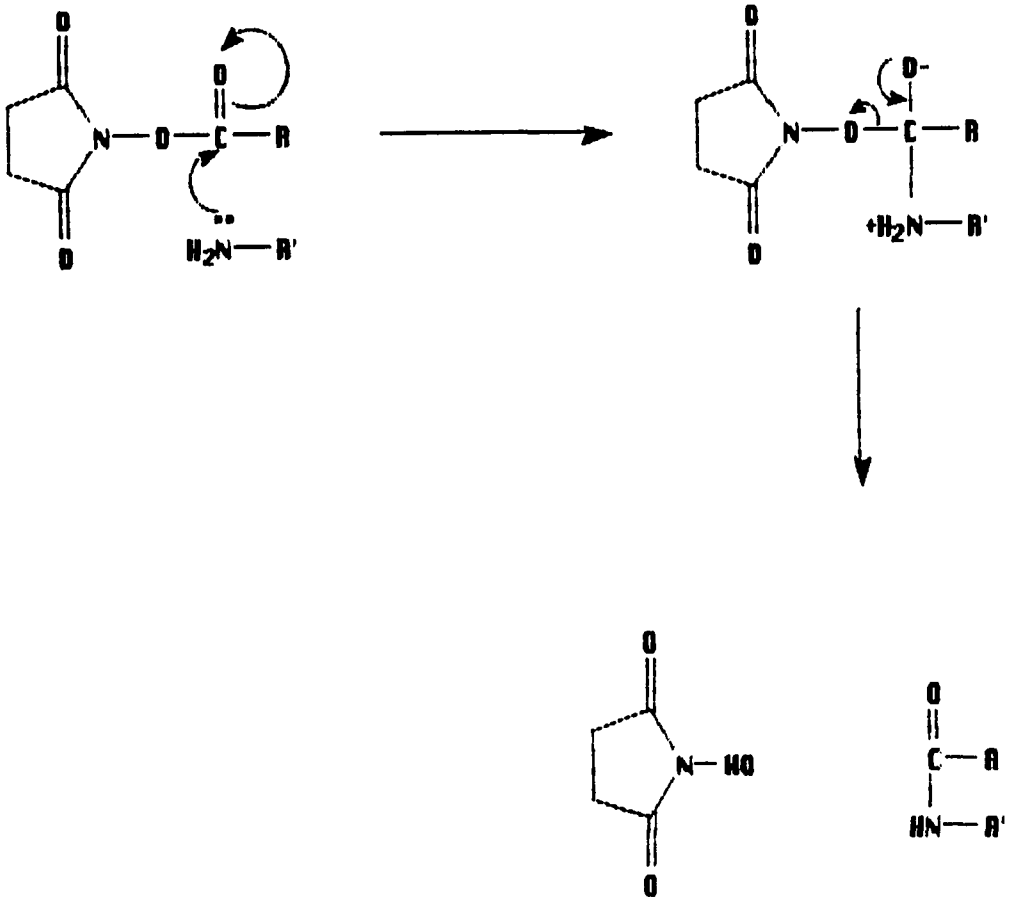
DST, 6 Å spacer



EGS. 16 Å spacer

spacers between the functional groups, they have the disadvantage of being homobifunctional. The reagent reacts with primary amines (*i.e.*, Lys groups) in the pH range of 7-9. The Lys on a protein attacks the carbonyl carbon to form a tetrahedral intermediate with subsequent loss of hydroxysuccinimide. The mechanism is shown below in Scheme V. Since the reagent will attach to Lys on both cyt *c* and pc, it is unlikely that a formed, cross-linked complex will have the general orientation of the electrostatic complex where carboxylates on pc interact with Lys residues on cyt c. Low yields of cross-linked heterodimers are obtained because of this. In low ionic strength media, positively charged residues (*i.e.*, Lys) are generally not going to be in orientation favorable for cross-linking. The highest yields of heterodimer are *ca.* 15%. Increasing ionic strength, by the addition of 0.2 M NaCl, slightly lowered the yield of heterodimer. At low protein concentrations, increasing ionic strength would make cross-linking a more random event, which appears to be less efficient than attempts of cross-linking the prearranged electrostatic complex at low ionic strength. Increasing protein concentration results in an increase of cyt *c* homodimer. Increasing ionic strength at high protein concentration seems to have no effect on dimer formation. At 500 μM protein, the ionic strength from the proteins themselves may cause random orientation of the proteins in solution. The addition of 0.2 M NaCl would not change how the proteins see each other. Increases in incubation time or larger excesses of cross-linking reagent had no effect on the yield of heterodimer formed. Cross-linking with DST gives lower yields than cross-linking with EGS. The shorter length of DST may be detrimental since two like charged residues on two different proteins must approach each other. The longer EGS, once

MECHANISM:



ACYLATION

Scheme V.

Table XXI. Yields of heterodimer produced in cross-linking reactions of cyt *c* and pc

reagent	protein[a] conc.	cross-link[a] conc.	pH	t(h)	[NaCl](M)	%cyt (in dimer)	%pc
DST	50	400	7.0	6	0	5	5
EGS	50	400	7.0	6	0	14	12
EGS	500	15000	7.0	39	0	31	16
EGS	500	15000	7.0	39	0.2	24	18
EGS	30	990	6.5	39	0	5	5
EGS	30	990	6.5	39	0.2	0	0

[a]Concentrations are reported in μM.

attached to a protein, can attach its other end to a protein 18 Å away. Reaction conditions and yields are given in Table XXI.

Another explanation for the low yield may be the competing reaction of hydrolysis of the cross-linking reagent. Both DST and EGS readily hydrolyze to form hydroxysuccinimide and a carboxylic acid.

The DST and EGS cross-linked dimers are heterogeneous because of multiple binding sites on the proteins. To purify derivatives becomes a difficult task, especially since the yields of heterodimeric protein are low. If it is assumed there are 2 Lys on cyt *c* and 2 Lys on pc that will form a cross-link, then there are 4 possible isomers. This heterogeneity is complicated by the probable formation of single-end hits, where only one end of the tether attaches to the protein and the other end is hydrolyzed to a carboxylic acid. The carboxylate end of the tether dangles from the protein. There are a total of 24 Lys residues on both proteins. For

one of the 4 cross-linked isomers, there are 22 possible isomers of a dimer with a single-end hit. This implies a total of 88 possible isomers with a single-end hit for all 4 cross-linked isomers. The number of isomers increases when multiple cross-links are considered and when multiple single-end hits are considered.

Ion-exchange chromatography of the DST and EGS cross-linked isomers should single out the isomer with a single cross-link and no single-end hits. Since the charge of native cyt *c* is +7 and that of pc is -8 at neutral pH, the modification of one Lys on each protein would give an overall charge of -3 on the dimer. No other isomer carries this net charge. Chromatography did produce at least 3 bands from the EGS heterodimer. In spite of the overall negative charge on the heterodimers, chromatography on CM52 with 1 mM phosphate seemed to work the best. Apparently the Lys residues on the cyt *c* are dominant in the interaction of the dimer with the ion-exchange resin. The dimeric mixtures were strongly retained on DEAE resin as expected. The dimers do have an overall negative charge. Cacodylate buffer was tried since cacodylate does not bind to cyt c. Phosphate does bind to cyt c and may interfere with chromatographic properties. The use of cacodylate buffer did not improve the chromatographic separation of derivatives.

There is a group of derivatives with an overall net charge of -5, -7, -9 and so on. If overall charge could be considered to be the dominant factor of elution order, which is not always true, then the uppermost band on the CM52 column would correspond to the dimer with a net charge of -3. The first and second bands could be assigned as having charges of -7, and -5, respectively. Further characterization of these bands proved difficult because of low yield.

Modification of EGS Heterodimer

Lys residues are quantitated spectrophotometrically with TNBS (trinitrobenzene sulfonate).[198] As a control, free Lys was modified with TNBS. The reagent is capable of modifying both the α-amino and ε-amino groups on the amino acid. A plot of A_{420} versus Lys concentration gave a linear plot. The overall extinction coefficient for both amino groups was 31,400 $M^{-1}cm^{-1}$. The contribution from the ε-amino group is 19200 $M^{-1}cm^{-1}$ according to the literature.[198] Incubation of cyt *c* with TNBS gave sporadic results. The number of Lys residues labeled was not reproducible. If the unmodified Lys residues in a heterodimer could be quantitated with TNBS, then the number of cross-links and single-end hits on a given derivative could be determined. This, in conjunction with chromatographic behavior, would give some indication of how the separated dimers differ. The number of Lys residues labeled on a heterodimer fraction from a DEAE column was 30. This is clearly erroneous, since the number of potential residues is 24. The reason for the problems experienced with TNBS are unclear. The reagent behaved well for the free amino acid, Lys, but not so well for cyt *c* . Perhaps under extremely rigorous control of reaction conditions, quantitation could be done. Perhaps the commercial preparation is impure. The author recommends purification of commercial lots of TNBS prior to use. The technique proved unsatisfactory for analysis of the EGS dimer. Since it is a destructive technique (*i.e.*, the TNBS can not be removed once attached), this analytical approach was terminated. Future studies with TNBS and EDC-cross-linked heterodimer, which is produced in comparatively high yield, may prove fruitful.

Reduction Potentials of the EGS Dimer

The reduction potential of both cyt *c* and pc in the EGS dimer were not significantly perturbed from the reduction potentials of the native proteins. The measurement, made on the heterogeneous dimer mixture, is only an estimate. Not enough protein could be isolated for measurement of individual derivatives of the cross-linked heterodimer. The reduction potential for the cross-linked cyt was 260 mV vs. NHE. The native value is generally reported as 256 mV vs. NHE. The Cu(II)/Cu(I) potential was 360 mV, the same as reported for the native protein. The reduction potential for cyt *c* is virtually unperturbed. The length of the EGS tether evidently prevents the negatively charged pc from approaching close enough to cyt *c* to perturb its reduction potential. The loss of positive charge on the Lys residues(s) from EGS itself also does not seem to perturb the reduction potential. In the EDC-cross-linked complex, a significant drop is seen in the reduction potential of cyt *c* .[184] This was attributed to neutralization of the Lys residue by EDC, but it may be from the negative electrostatic field of pc. which, in the EDC complex, is proximate to cyt *c* . The Cu in pc is not perturbed since the Lys modified is likely far from the Cu site. The lack of perturbation also indicates that the positively charged cyt is effectively not seen by the Cu in pc. The DST or EGS likely binds to pc at a Lys on the west side of the protein. Three of the five Lys (26, 30, and 54) residues are located here. If attachment were to Lys 95 on the east side of pc, a perturbation would be expected in the reduction potential of pc. Attachment to Lys 77 on the south side of the protein probably would not perturb the reduction potential of pc.

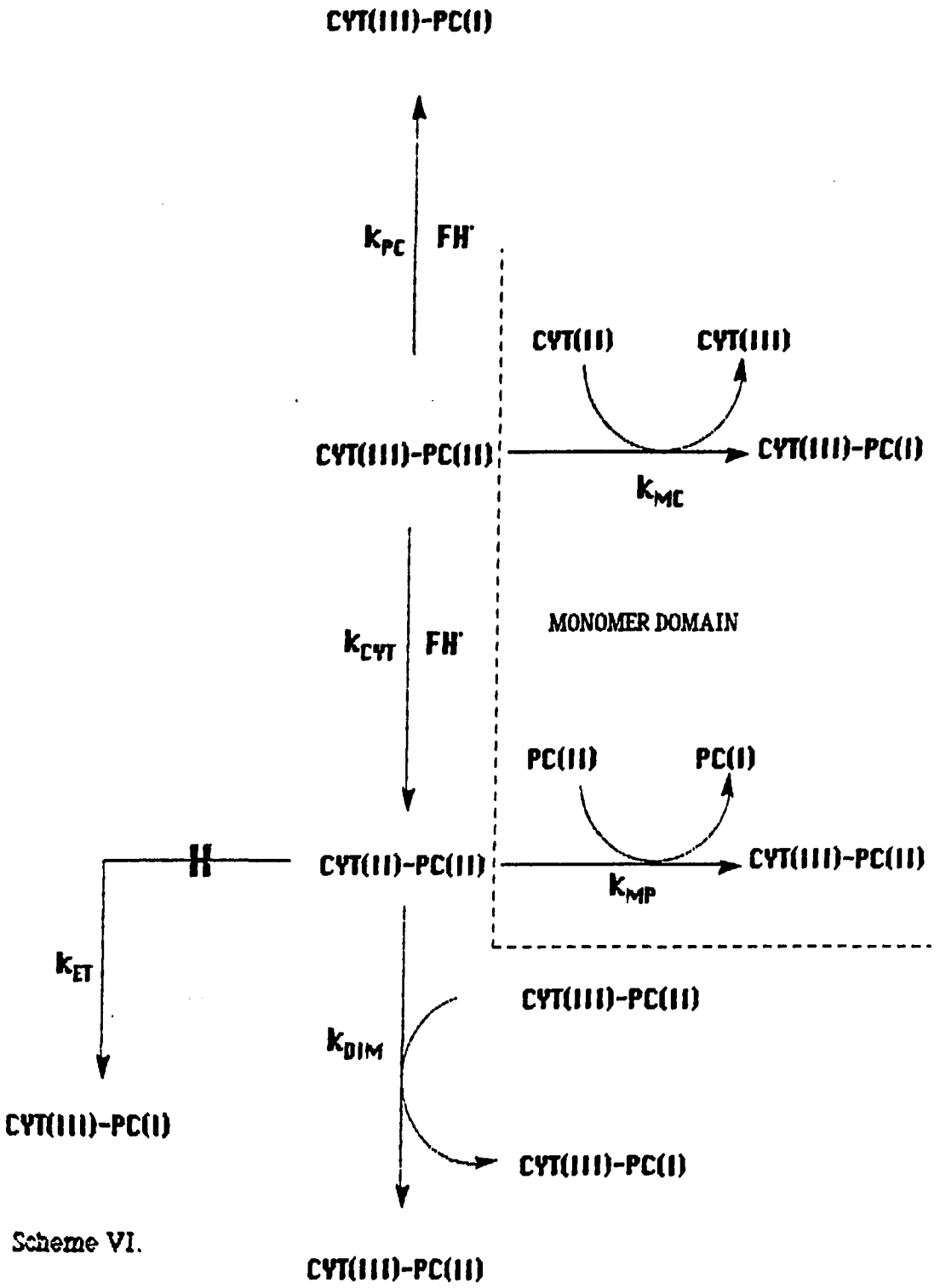
Electron-transfer Kinetics of EGS and DST dimers

Some of the reactions that can occur in kinetic experiments with the cross-linked protein complexes and monomeric proteins are shown in Scheme VI. The riboflavin semiquinone, generally designated RFH. was generated by EDTA reduction of riboflavin, designated RF, in the triplet state. Riboflavin has a ribose side chain at N10 in the isoalloxazine ring. Riboflavin has a net charge of 0 at neutral pH. The estimated reduction potential is -230 mV vs NHE for the RF/RFH. couple.

The cyt(III)-pc(II) in the kinetic scheme represents either EDC, DST, or EGS cross-linked cyt *c* and pc. The section within the dotted lines, designated the monomer domain, represents reactions of the protein dimers with monomeric proteins. These reactions will be discussed in detail below. The k_{dim} reaction represents reaction of the dimeric cyt(II)-pc(II) with the cyt(III)-pc(II) in the bulk of the solution. The amount of cyt(II)-pc(II) produced is limited by the concentration of RFH. produced, which is generally 0.5 μM. Cyt(III)-pc(II) is in pseudo-first-order excess over cyt(II)-pc(II). Similarly, the pc in the monomeric domain is in pseudo-first-order excess over cyt(II)-pc(II). Also, the cyt(III)-pc(II) is in pseudo-first-order excess over the cyt(II) in the monomer domain.

First consider the kinetic scheme sans the monomeric proteins. As shown in Scheme VI, the RFH. can either reduce the pc (k_{pc}) which leads to a thermodynamic end product or it can reduce the cyt (k_{cyt}) in the dimer. In the latter case, the cyt(II)/pc(II) complex is in a state such that electron transfer is thermodynamically favorable. Cyt *c* has a reduction potential of 256 mV and pc has a reduction potential of 360 mV.

Theoretically, the reaction designated k_{et} should be possible. It has been shown previously in this laboratory, that intramolecular


CYT(III)-PC(I)
k_{PC} FH·
CYT(II)
CYT(III)
CYT(III)-PC(II)
CYT(III)-PC(I)
k_{MC}
MONOMER DOMAIN
k_{CYT} FH·
PC(II)
PC(I)
CYT(II)-PC(II)
CYT(III)-PC(II)
k_{MP}
k_{ET}
CYT(III)-PC(II)
k_{DIM}
CYT(III)-PC(I)
CYT(III)-PC(I)
CYT(III)-PC(II)

Scheme VI.

ground-state electron transfer does not occur when the proteins are cross-linked with EDC.[184] The lack of electron-transfer in the ground-state reaction may be from cross-linking the proteins in a nonproductive orientation. Modelling has shown that there are three energetically favorable families of precollision orientations all directing the positive region surrounding the heme edge of cyt *c* toward the acidic patch of pc.[204] These favored orientations should converge to productive complexes promoting an electron transfer pathway from the cyt c heme edge to Tyr 83 of pc.[204] It is possible that EDC cross-linking locks the two proteins in one of the precollision positions which is yet not a productive electron-transfer complex. The general orientation of the two proteins is probably close to the orientation found in the electrostatic complex. The proteins were cross-linked at low ionic strength such that the orientation from electrostatic forces would not be perturbed.

When the cyt is replaced with Zncyt, then the thermodynamic driving force is much higher and electron transfer does occur at approximately 10% of the rate of electron transfer in the electrostatic complex of Zncyt and pc. In this excited-state reaction, the driving force is near 1 volt. Also, different isolated derivatives of the Zncyt/pc cross-linked complexes showed identical electron-transfer rates, which indicates that all derivatives are linked in the same general orientation. The reduction in efficiency in the excited-state reaction is probably also due to a slight increase in the metal-to-metal distance. If the proteins are locked in a nonproductive complex, the high driving force may allow electron transfer to occur through a different, less efficient pathway.

If EDC locks the cyt *c* and pc in a nonproductive complex, then the DST and EGS tethers might allow enough freedom of movement of the proteins to rearrange into a productive, electron-transfer complex. There is no internal electron-transfer observed from cyt *c* to pc in either the DST or the EGS complex. See Figure 8. Once cyt

is reduced by RFH·, there is no evidence of reoxidation, at low concentrations of the heterodimers, even after 10 seconds. At higher concentrations, a concentration-dependent reoxidation is observed which is the k_{dim} reaction in Scheme VI.

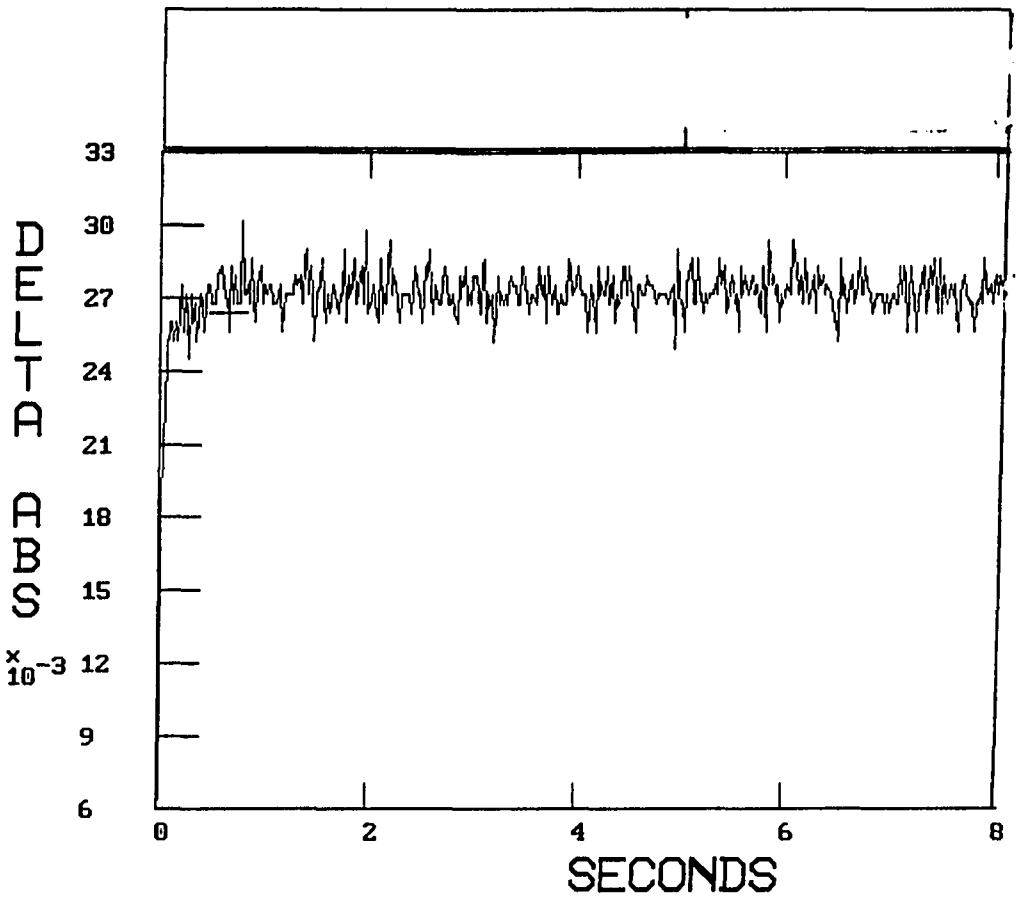


Figure 8. Absorbance (550nm) versus time trace of 10 μM EGS cyt *c*/pc dimer

The absence of intracomplex electron transfer is not surprising. The two proteins are cross-linked with a homobifunctional reagent which docks the two proteins in a "back-to-back" orientation. The positive Lys patch on cyt c is probably facing the west side of the pc. In the electrostatic complex, this positive patch faces the acidic east side of pc. Even though the EGS tether is 16 Å, it is not long enough for the protein to loop around to the east patch of pc.

If the proteins are docked such that the positive patch of cyt *c* faces the acidic patch of pc, then the length of the tether becomes detrimental since it pushes the two metal centers farther from each other, perhaps making electron transfer immeasurably slow.

The absence of electron transfer is probably a result of the first explanation. If the proteins were docked in the proper general orientation, the tethers should be flexible enough to migrate to a productive complex. Even if there are two cross-links per heterodimer, there is a degree of freedom for the two proteins to rearrange. Only 3 cross-links would freeze the proteins into a rigid, nonflexible orientation.

There are no heterobifunctional cross-linking reagents commercially available which are specific for Lys and carboxylates and which have spacers between the reactive groups. The design of such a tether would be useful in the determination of why electron transfer is absent in the EDC, DST, and EGS cross-linked complexes.

Although there is no intracomplex electron transfer between cyt *c* and pc when cross-linked with DST or EGS, both proteins are capable of electron transfer with external redox partners. Riboflavin semiquinone can reduce both cyt and pc in the heterodimers. These reactions are designated k_{cyt} and k_{pc}, respectively in Scheme VI. The bimolecular rate constant for reduction of cyt *c* by RFH· in the EGS dimer is 1.1×10^7 and 3.1×10^6 $M^{-1}s^{-1}$ at ionic strengths of 10 and 100 mM, respectively. The RFH· sees the local positive charge on cyt rather than a global

negative charge on the dimer. The RFH· species can be thought of as a zwitterion with an electron localized on the dimethylbenzene ring and the central ring such that the flavin semiquinone is seen as a dipole. The rate of reduction of cyt *c* in the heterodimer by RFH· is almost as fast as the reduction of native cyt *c* by RFH· The flexibility of the tether allows RFH· easy access to the positive patch of cyt *c* . The presence of pc attached to cyt *c* does not inhibit the reactivity of cyt c toward RFH·. Even in the presence of monomeric pc, the bimolecular rate constant for reduction of the cyt c in the heterodimer by RFH· is 1.1 x 10^7 $M^{-1}s^{-1}$ at an ionic strength of 10 mM. The added pc apparently does not block the site where the cross-linked cyt c interacts with RFH·. Apparently cyt *c* has more than one electron transfer site or the site is large enough to accommodate both RFH· and free pc concurrently, or there is no specific electron transfer site in these systems.

The cross-linked cyt *c* can also transfer an electron to the free pc where the bimolecular rate constant is 5.1 x 10^4 $M^{-1}s^{-1}$ (designated k_{mp} in Scheme VI). The covalently attached pc does not prevent access of either free pc or RFH· to the cross-linked cyt c. The reaction of the attached cyt c with free pc is inhibited compared to that of the reaction of cyt *c* with native pc. The rate constant of electron transfer between the two native proteins is 1 x 10^6 M^{-1} s^{-1} at an ionic strength of 10 mM. The tether evidently is not long enough for uninhibited access of pc to the cyt c electron transfer site. The lower rate of the k_{mp} reaction is not only from blocking of the electron-transfer site, but from the larger size of the reductant as well.

The covalently attached pc readily reacts with monomeric cyt *c* . This reaction is designated k_{mc} in Scheme VI. Either the pc is in an orientation such that its interaction site with cyt *c* is exposed to solvent or cyt *c* interacts efficiently with a second electron-transfer site. Both possibilities are viable. It is likely that the east acidic

patch of pc faces away from cyt *c* since the majority of Lys residues are on the west side of pc. If cyt *c* interacts with this site, then there should be no steric hindrance when cyt *c* approaches the cross-linked pc. If the acidic patch is inaccessible, the cyt *c* may interact with the northern hydrophobic site near the Cu atom. The bimolecular rate constant for reduction of the cross-linked pc by free cyt *c* is 7.9×10^6 $M^{-1}s^{-1}$, almost the same rate as the rate for the free proteins. Electron transfer must occur through a second, unhindered electron-transfer site. This site may be the acidic patch or the northern hydrophobic patch.

One EGS dimer is capable of transferring an electron to another dimer with a bimolecular rate constant of 1.63×10^4 $M^{-1}s^{-1}$. The cross-linked pc in one dimer probably sees a hindered cyt *c* electron transfer site, thus explaining the low reaction rate. In addition, the reaction is between two large dimeric molecules, thereby increasing the distance of closest approach between the reactants.

Kinetics of EDC Dimers with Monomeric Proteins

Since EDC heterodimer of cyt *c* and pc is formed in high yield, it was chosen to study reactions of electron transfer to monomeric proteins in further detail. The four major derivatives of the EDC-cross-linked proteins were used for kinetic studies. Kinetic studies of EDC-cross-linked Zncyt *c* and pc indicate that all derivatives are cross-linked in the same general orientation, as reflected by similar rates of intracomplex transfer in each derivative. The derivatives differ by modification of carboxylate residues by EDC, as discussed above.

The rate constants for reaction of the four major EDC derivatives with monomeric cyt *c* and pc are listed in Table XXII. It should be noted that overall, the observed trends are slight and

the reactivities of the derivatives toward monomeric proteins are similar. The general orientation of the cyt *c* and pc with respect to each other are similar and that is why their reactivities toward monomeric cyt *c* and pc are not widely different.

Table XXII. Second-order rate constants of reaction of EDC derivatives with cytochrome *c* and plastocyanin

EDC derivative	$k_2 \times 10^{-5}$ ($M^{-1}s^{-1}$) μ (mM)				
	with cyt *c*				
	10	50	150	250	500
1	1.53	1.02	0.89	0.71	0.64
2	3.08	1.85	1.62	1.46	1.45
3	3.65	1.28	1.17	1.27	1.03
4	3.06	2.06	1.53	1.36	0.81
	with pc				
1	4.62	4.26	3.00	2.03	1.61
2	3.01	3.2	2.26	1.28	0.62
3	2.55	2.53	2.89	2.23	1.64
4	1.51	0.77	0.74	0.59	0.63

The rates of reaction with cyt *c* show little difference among the four derivatives at all the ionic strengths. The isomer labeled B1 reacts slightly slower with cyt *c* . This is not easily explained. In B1, the pc is more negatively charged than the other derivatives and the global charge of the B1 is less positive than the other derivatives or it may even be slightly negative. Simple electrostatics predicts that B1 should react more favorably with cyt *c* than the other three isomers. The observed data show that treatment of the proteins as point charges is not valid. The Bronsted-Debye-Huckel relationship can not be applied accurately to large molecules such as proteins.[205] The apparent charge product from graphical analysis is artifactual and cannot be considered to reflect local charges at the interface or the whole charge of the protein. The latter of these points is demonstrated in the little or no change in reaction rates when a positive charge in cyt *c* is lost due to specific Lys modification.[205] A protein is not a spherical charge distribution. A change in rate would be expected if simple monopole-monopole interactions were major contributors to productive collision.

Consideration must also be given to dipole-dipole interactions. Preorientation of cyt *c* and its reaction partner, in this case the EDC-cross-linked complex, has been suggested to allow formation of an optimally reactive complex. The dipole moment of ferric cyt *c* (where His 33 is unprotonated) is 325 Debye. The positive end of the dipole lies close to the center of the interaction domain on the front face of the molecule. Pc has a dipole moment of 360 Debye. This dipole lies such that the negative end is directed out of the acid patch on the east side of pc. The dipole vector is perpendicular to a vector from the center of mass to the Cu atom. The vector from the center of mass to the acid patch is 30° to the dipole vector. The vector from the center of mass to the site where cyt *c* interacts is 75° to the dipole vector.[192] Basically, the cyt *c* is not attracted to

the localized negative patch but rather to a site 75° from the dipole vector. If the proteins are in this general orientation when they are cross-linked, then it is likely that both the negative patch on pc and the positive patch on cyt *c* are still partially exposed to solvent. Since the proteins are not physiological partners, there is not a "lock and key" inhibition to a third approaching protein.

The reason why B1 is less reactive toward cyt than the other bands is evidently because modification of an external residue in the other derivatives causes a change in how a third protein approaches or sees the dimer complex. The derivative, B1, is likely the least modified of all the derivatives, as evidenced by its elution order from the cationic exchange column. The residue modified in B2, B3, and B4 likely occurs on a carboxylate on cyt *c* .[202] Glu 62 is labeled in horse cyt *c* since Asp 62 is labeled in tuna cyt *c* .[202] Glu 62 lies close to the heme crevice and neutralization of this residue would alter the dipole moment of the cross-linked cyt c. Since there are a proposed 2 cross-links per heterodimer, then the cross-linked complex must still have a degree of freedom. This would allow some readjustment of the 2 cross-linked proteins relative to each other and perhaps allow more accessible entrance of cyt *c* to the acid patch of pc, or alter the dipole-dipole interaction between cyt *c* and the heterodimer.

There is a weak ionic strength dependence of the reaction of cyt *c* with the heterodimer. The cyt *c* still sees a negatively charged entity for all bands, although the latter bands have an overall positive charge. Modification of carboxylates by EDC increases the overall positive charge of the complex. As cyt *c* approaches the dimer, it does not see a global charge but rather a local negative charge. The acidic east patch off pc may still be visible to cyt *c* . The effective negative charge the cyt sees is less than the two native proteins see when approaching each other since the cross-linked cyt *c* partially masks the negative charge of pc.

The rate constants for reaction of native cyt *c* with native pc are 1.1 x 10^7 and 8.3 x 10^5 $M^{-1}s^{-1}$ at 45 and 400 mM ionic strengths, a 13-fold decrease. The decrease in the rate of reaction of heterodimer with monomeric cyt *c* is only 2-fold at similar ionic strengths.

It is also possible that the monomeric cyt *c* is seeing the negative end of a resultant dipole from the two cross-linked proteins rather than a local monopole-monopole or monopole-dipole interaction.

It is not surprising to see an ionic strength dependence if the dipole interactions of the two cross-linked proteins are considered. Both proteins have dipole moments of similar magnitude. The most energetically favorable orientation for two such dipoles is for the dipoles to be aligned antiparallel rather than colinear. The two dipoles from the individual cross-linked proteins do not cancel mainly because they do not align exactly parallel to each other. The resultant dipole is small, however, since the magnitudes of the individual dipoles are similar, and the dipoles are probably nearly parallel. The small resultant dipole explains why such a small ionic strength effect is observed. Koppenol's proposal of an interaction site of pc with cyt is in agreement with such an arrangement. The positive end of the cyt c dipole is not directly pointing toward the negative end of the pc dipole but is rather to the north of it such that the opposite ends of the dipoles may also interact. This arrangement leaves a portion of the acidic patch accessible to the solvent. The free monomeric cyt *c* is in a position between the two proposed electron transfer sites in pc.

Consideration must be given to the possibility of the interaction of the monomeric cyt *c* at other sites on the covalently bound pc. Pc is known to have at least 2 electron-transfer sites. If the acidic patch is blocked by the covalently attached cyt, the northern hydrophobic site, near the Cu may be accessible to the cyt *c*. Computer calculations show the exposed imidazole ring edge of Cu

ligand His 87 and the surrounding surface to be electrostatically close to neutral.[204] At the northern end of the molecule, there is a weakly positive field pointing away from the molecular surface.[204] If monomeric cyt c were interacting at this site, a slight trend in the reverse direction would be expected.

Monomeric pc also can accept an electron from the bound cyt *c* . The reactivity of pc toward the EDC dimers is crudely C1>C2=C3>C4. See Table XXII. This trend is opposite to that expected if overall net charge is considered. The approaching pc evidently sees a more negative field in the more highly modified dimer which indicates that neutralization of individual carboxylic residues is of no importance to the approaching monomeric pc. It is likely that increased modification causes the orientation of the proteins to change slightly, as a result of dipole-dipole interactions, and alters the electric field pc experiences on its approach to the dimer.

There is a slight ionic strength effect seen in the reaction of pc with the EDC heterodimer. As with cyt, the trend is small since the cross-linked proteins are nearly electroneutral. The charge seen by pc is not global as evidenced by the decrease seen in the first two bands which are globally negative and neutral, respectively. The approaching pc still sees a slight positive charge on the cyt *c* which is cross-linked to pc. The previous explanation of dipole-dipole alignment in the heterodimer explains why a partial positive patch of the cyt *c* is still exposed to the solvent. The eleven Lys residues surrounding the exposed heme edge account for 13% of the cyt *c* molecular surface (total area 4746 $Å^2$). It is reasonable that the covalently attached pc does not mask all of this positive patch. In pc, 24% of the exposed molecular surface (total area 4095 $Å^2$)[204] has a significantly negative electrostatic potential, and covalently attached cyt c does not mask all of this negative patch.

It is clear from the ionic strength dependences that monomeric

cyt *c* and pc have different sites of interaction for electron transfer with the heterodimer. It is of interest to note that in going from derivative B1 to derivative B4, both monomeric proteins apparently see either a more negative electrostatic field or a less positive electrostatic field. Sterics evidently do not cause these differences since there is no definitive trend at high ionic strengths, especially in the case of the pc reactions. It is possible that the difference in reactivity of cyt c may be steric. Electrostatics seem to be the influential factor. Cyt *c* sees a more negatively charged interaction site with pc in the dimer in B4 than B1 and pc sees a less positively charged interaction site with cyt c in B4 than B1. These changes are probably from the way the proteins in the dimers are oriented relative to each other and how effective they are in the cancellation of charges on the other protein.

Although the discussion here focuses on dipole-dipole interactions, alternative possibilities must be considered. The transfer of an electron between the monomeric and dimeric proteins may simply be controlled by random collision and no enhancement is gained by orientation prior to collision.

Also, surface diffusion may or may not be important in the reactions of the dimers with the monomers.[204] The monomeric cyt *c* is free to diffuse around the surface of the dimeric protein complex before a productive electron transfer occurs. This is in contrast to intracomplex electron transfer within the dimers, where surface diffusion is extremely limited. The excited-state, intracomplex reaction with Zncyt *c* evidently precludes this hypothesis--diffusion in the excited-state reaction is restricted, but electron transfer still occurs.

It is still an extreme oversimplification to consider protein molecules as simple dipoles. There are several charges distributed over the surface of a protein such that several smaller dipole vectors are important in the interaction of how proteins interact

with each other. The dipole-dipole approach does provide a better model than a simpler point charge model, however.

Spectroscopic Characterization

MCD, CD, and UV-vis are good techniques for monitoring changes in protein structure when modified.[206-209] MCD, magnetic circular dichroism, is optical activity induced by the application of a magnetic field. Although MCD is largely insensitive to molecular conformation, it is sensitive to the total concentration of MCD-active chromophores and their local environment. For heme proteins, the most useful aspect of MCD is for use in analysis of redox state, spin state, and changes of ligation of the iron center.

At the concentration the samples were measured, there was no evident perturbation of the MCD spectra of the four, fully-oxidized, EDC-cross-linked cyt *c* /pc bands when compared to the MCD spectrum of native cyt *c* (III). Data are shown in Table XXIII. The MCD spectra of all four bands show them to be low-spin ferric hemes which indicates no gross perturbation of the heme environment upon modification with EDC. Timkovich reports that upon modification of tuna cyt c with EDC, there is possible loss of Met 80 as a ligand to iron. If Met is lost as a ligand, then a strong field one must replace it in order for the metal to remain low spin. Timkovich supports this claim by loss of the 695 nm band in the visible spectrum of cyt c.[202] The modification is proposed to occur at a propionate chain on the heme. UV-vis spectra of all four of the EDC-cross-linked cyt *c* /pc derivatives show the 695 nm band to be present in all samples, in agreement with the MCD findings. Evidently, the presence of pc during the cross-linking reaction prevents modification of the propionate chain from occurring under our reaction conditions. It should be noted that our reaction conditions are much less severe than those of Timkovich. We only

Table XXIII. MCD data for native and EDC-cross-linked cyt *c* (III)

sample	$\Delta\varepsilon\lambda_{max}$ (nm)	$\Delta\varepsilon_M/H$ $(M.cm.T)^{-1}$
native	400 ± 2	71 ± 10
	414	-77
B1 EDC	401	75
	414	-87
B2 EDC	403	75
	417	-81
B3 EDC	399	61
	414	-77
B4 EDC	400	73
	414	-81

use a 12.5:1 ratio of EDC to protein at pH 6.5 where they use a 156:1 ratio of EDC to cyt *c* at pH 3.7. External modification by EDC in our cross-linked dimers probably occurs at Glu 62 on cyt c and other Asp and Glu residues on both proteins.

There appears to be no change in the visible and aromatic regions of the MCD spectra of the cross-linked dimers from that of the native cyt *c*. At these protein concentrations and with the limitations of the instrument, valid comparisons warrant either measurements of these regions at higher protein concentrations or use of a better instrument.

There is no change in the entire MCD spectrum of cyt *c* when

electrostatically associated with pc. This indicates that the heme is not perturbed when associated with pc. Such perturbations are sometimes observed when physiological partners, such as cyt *c* and cytochrome *c* oxidase, are associated.[10,211] Such perturbations lower the reorganization energy for electron transfer and aid in efficient electron transfer.

One point of caution needs to be emphasized. Our magnetic field was calculated to be 600 Gauss in the MCD experiments. Most MCD spectra today are done in a field of 1.5 T (15000 Gauss!) with electromagnets or even up to 5 T with superconducting magnets.

CD (circular dichroism) is sensitive to conformational changes in a protein. If a chromophore couples with protein backbone transitions or with aromatic side chains, it is likely to be CD active. The geometry and nature of ligating groups may also affect CD active chromophores. Protoporphyrin IX has no optical activity since its symmetry is C_{2v}. In a protein environment, the chromophore is optically active. In cyt *c*, there is a positive Cotton effect from 402-405 nm, a negative Cotton effect from 416-418 nm, and a crossover point at 407-409 nm.

There are no changes in the CD spectrum of cyt *c*(III) when covalently or electrostatically bound to pc. A 1:1 cyt *c* pc (20 μM each) mixture in 3 mM phosphate buffer shows no change from the sum of the separate components. Even upon addition of NaCl such that [NaCl]=300 mM, when the electrostatic complex should be largely dissociated, there is no change. Evidently, the cyt *c* does not change conformationally when complexed to pc. To ensure that the proteins were largely associated in solution, a sample of 1:1 cyt:pc at 160 μM each was measured and showed no perturbation from the CD spectrum of free cyt *c* .

CONCLUSIONS AND PROSPECTS

The EDC-cross-linked dimers need better characterization for an accurate analysis to be made. Unfortunately, such complex systems are not easily analyzed. Even peptide mapping has proven difficult on almost identical systems.[212] Ultimately, it appears that a crystal structure needs to be done on an EDC cross-linked dimer. The use of chromophoric site-specific reagents as cross-linking reagents provides a more viable approach in the analysis of this problem. Such work is currently under way in this laboratory.[213]

A similar study with physiological partners (e.g., cyt *f* and pc) would provide an interesting comparison. Differences in both spectroscopic and kinetic properties may help uncover the reasons why cyt *c* does not transfer an electron to pc when cross-linked even when the proteins themselves are apparently not perturbed.

SECTION IX.

BLUE DYES AS PHOTOREDUCTANTS FOR PROTEINS

INTRODUCTION

Flavins and their derivatives have been used a photoreductants for biological studies for many systems.[214-216] The variety of flavin derivatives commercially available and the comprehensive literature on flavin synthesis have made these reagents useful in the study of how sterics, reduction potentials, and even electron delocalization affect the rates of electron transfer to biological systems. In addition, the occurrence of naturally existing flavoproteins allows the use of these proteins in kinetic experiments. Because of this, the flavins provide a good model system for understanding electron transfer especially in flavoprotein systems.

In this section, we introduce not a new class of photoreductants but a class whose reducing capabilities have not been quantitated. Photochemical reactions of methylene blue and other dyes have been known for several decades.[217] Studies of the participation of buffers, amines, and chelating agents in photobiochemical reactions are known, but the rates of reduction of proteins by semireduced methylene blue and related dyes have not been measured. Methylene blue and similar dyes provide a system analogous with the flavin system. Many of the properties which are of advantage in the flavins are also applicable to methylene blue. Methylene blue also provides access to spectroscopic windows in the visible region which are not attainable with the yellow flavins.

EXPERIMENTS

Chemicals

Methylene blue, methylene green, toluidine blue O, methylene violet, Nile blue, Meldola's blue, and neutral red were obtained from Sigma Chemical Co. Crystal violet, bromcresol green, and bromcresol purple were taken from a stock chemical shelf. Horse-heart cyt *c* was obtained from Sigma Chemical Co. Disodium EDTA was obtained from Fisher Scientific.

Methods

Flash photolysis was done as in Section VIII with minor changes. Rhodamine 590 in methanol was the dye used. Methylene blue and its derivatives were used as the photosensitizers. The concentration of the blue dyes in the kinetic samples were 70-100 μM. Steady-state irradiation was done with a 100 watt tungsten lamp for 5 minutes with argon bubbling.

RESULTS AND DISCUSSION

When a 100 μM riboflavin and 0.5 mM EDTA solution is pulsed with 590 nm light, there is no oxidation of the EDTA to form a flavin semiquinone. Riboflavin has an absorption maximum at 442 nm and the band extends to 510 nm, out of the range of 590 nm light. Yellow dyes, such as flavins, can not be excited with 590 nm light. This is disadvantageous since rhodamine 590 is an easy dye to work with in dye-laser systems. Dyes used with flavins, such as LD 425, tend to breakdown rapidly and generally have a lifetime of only 100 shots. Furthermore, it is difficult to reproduce the energy output of pulsed laser light when LD 425 and other dyes in this class are used. Rhodamine 590 can be used for 1000 shots and energy output is highly reproducible. Methylene blue has an absorption maximum at 610 nm and the band extends down to 500 nm. The methylene blue has a strong absorbance at 590 nm and is a good candidate for use with 590 nm excitation. In the presence of 0.5 mM EDTA and in the absence of oxygen, the 610 nm band of methylene blue bleaches under steady-state irradiation with a tungsten lamp. If cyt *c* is added and the above experiment is repeated, there is quantitative reduction of the protein as evidenced by the appearance of the visible cyt *c* bands at 550 nm and 528 nm.

This is not the first report of methylene blue and phenothiazine derivatives as reductants, or more specifically, as reductants for proteins.[218,219] The mechanism by which the methylene blue is reduced is well known.[220-223] In the presence of a reductant (EDTA), the methylene blue is excited to a triplet state which then extracts an electron from EDTA to form a semireduced state. The lifetime of the excited singlet state of the dye is 0.4 ns, too short of a time to be the reactive excited state of the dye. Oxygen must be removed from the solution since oxygen quenches the triplet of

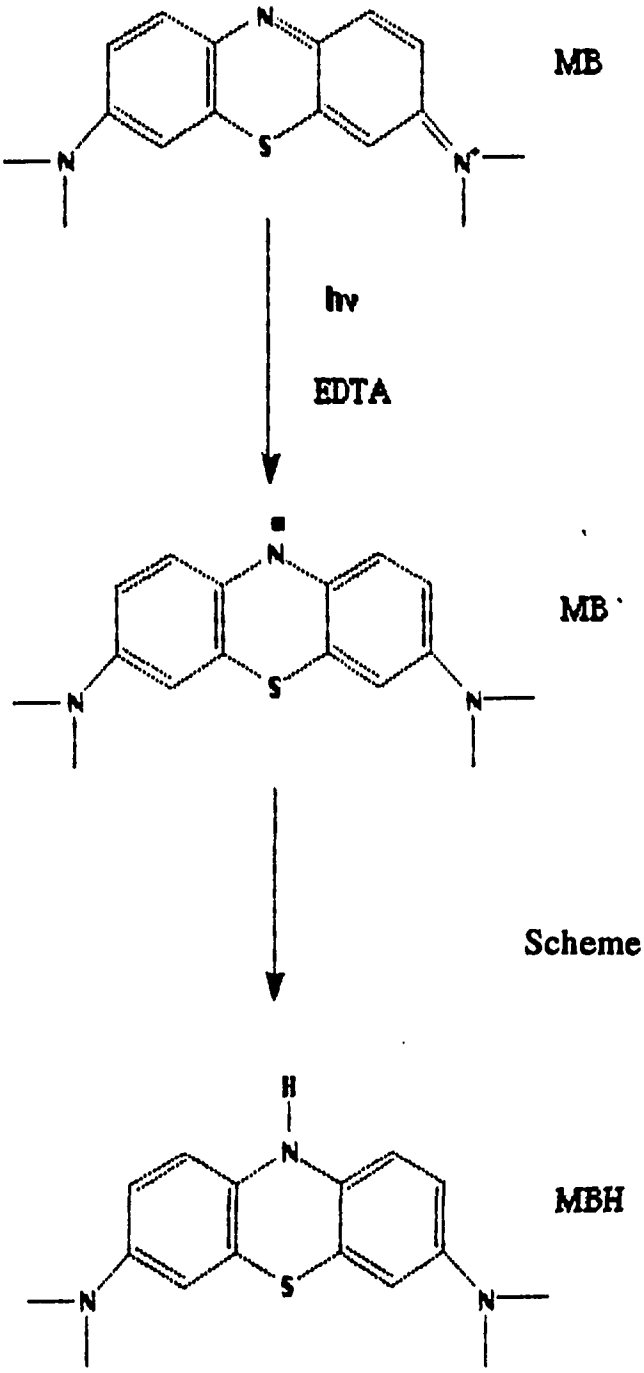
methylene blue at a rate of $k_o[O_2] = 4.4 \times 10^6\ s^{-1}$.[220] EDTA is a good choice as a reductant for the triplet state of methylene blue since amines show a general trend of tertiary > secondary > primary in the rate of reaction with the triplet state. A simplified scheme is shown below in Scheme VII. The semireduced methylene blue undergoes a disproportionation reaction which produces the fully oxidized starting material and fully reduced leucomethylene blue (MBH). It is the semireduced state which transfers an electron to the protein. The reduction of proteins by semireduced methylene blue are not well understood.

Prior studies of phenothiazine derivatives as protein reductants are qualitative and the initial reductant in such systems, usually containing membranes, is not known. Excited-state phenothiazine has been shown to reduce membrane-bound cyt *c*.[218] In that particular study, the triplet state is thought to act as a direct reductant, but it is likely that part of the phospholipid membrane present is reducing the phenothiazine to a semireduced state. The cyt *c* which is reduced in the aforementioned study is adsorbed to a phospholipid artificial membrane. In the absence of the phospholipid, the authors claim the rate of reduction is greatly reduced. This reduction is probably from the low solubility of phenothiazine in an aqueous medium. Although the mechanism is not understood, the authors show by modelling that the cyt *c* is reduced by some reactive species of methylene blue over a distance of 20 Å.

Other studies include methylene blue as an oxidant. In the presence of oxygen, methylene blue will act as an oxidant and oxidize Met residues in cyt *c* to sulfoxides.[224] This is not applicable under our conditions.

Methlyene blue is often used as a mediator between two proteins in electron transfer systems but, again, the reaction of methylene blue with an individual protein has not been studied in

METHYLENE BLUE AS A PHOTOSENSITIZER



Scheme VII.

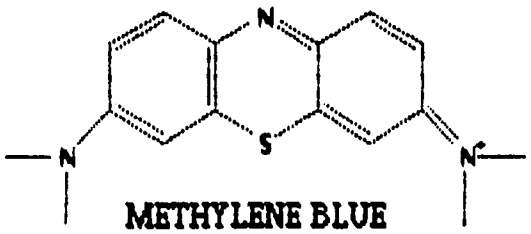
detail.[225] The methylene blue appears to transport an electron from one protein to another in such systems but rates of reaction with methylene blue have not been quantitated.

Several studies of methylene blue have been done on particulate systems containing cyt *b*.[226-228] Again, the kinetics of these studies are qualitative. Methylene blue is used as a general reductant or as a mediator to reduce cytochromes in homogenates and particulates from corn and cauliflower.

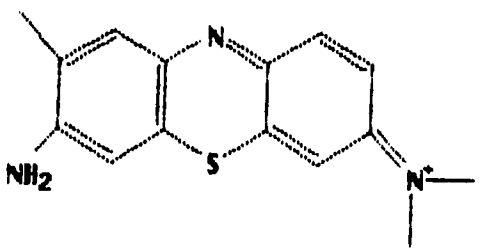
In the present study, we have studied differences in reactivity of methylene blue derivatives toward cyt *c* to better understand how these dyes interact with proteins and to establish a quantitative data base, much like the one that exists for flavins. Three dyes which successfully reduce cyt *c* are methylene blue, methylene green, and toluidine blue O. The structures are shown below. The reduction potential of the semireduced/fully oxidized methylene blue reaction couple is 5 mV vs NHE. Toluidine blue O and methylene green have reduction potentials in this vicinity. Although exact reduction potentials for methylene green and toluidine blue O are not known, the reduction potential for methylene green is probably slightly higher than methylene blue since there is an electron-withdrawing nitro group on methylene green. The electronic properties of methylene blue and toluidine blue O are similar and probably have similar reduction potentials.

The rate constants for reduction of horse-heart cyt *c* are 3.5 x 10^6, 3.0 x 10^6, and 1.4 x 10^6 M^{-1} s^{-1} for methylene blue, toluidine blue O, and methylene green, respectively. These rates are reflective of the relative reduction potentials of the dyes. Methylene green, having the highest reduction potential, is expected to be the worst reductant. The rates of reduction of cyt *c* by methylene blue and toluidine blue O are similar as expected from their similar reduction potentials.

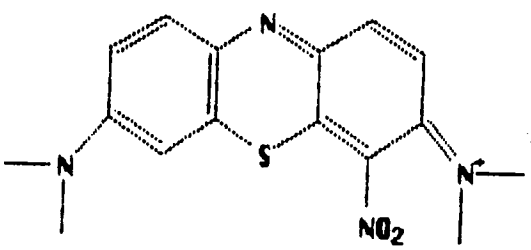
In line with the above argument, areas of electron localization



METHYLENE BLUE



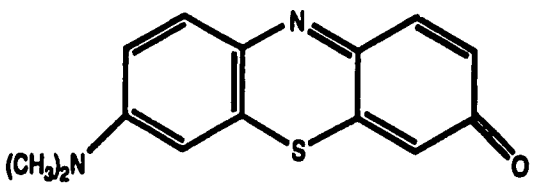
TOLUIDINE BLUE O



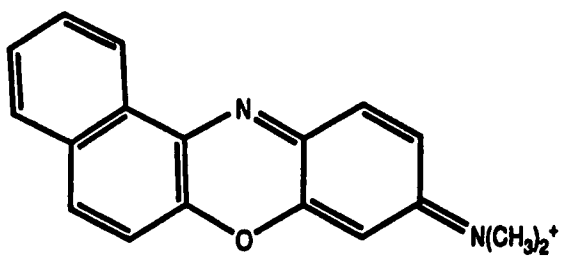
METHYLENE GREEN

can be estimated. The difference in rate of reduction of cyt *c* by methylene blue and methylene green indicates that the nitro group has an effect, albeit a very small one. This effect may either be steric in origin or may be because the electron in semireduced methylene blue is localized on the right ring. The electronic properties of methylene blue and toluidine blue O are similar but steric properties on the left ring of the structures are different. The rates of reduction of cyt *c* are similar. This, too, supports the delocalization of the electron on the right ring of the structures. A greater difference in rates probably would be observed if the electron was localized on the left ring. From these data and consideration of the dye structures, it appears that the electron may be localized on the central ring and the right side ring. A broader data base needs to be established, however.

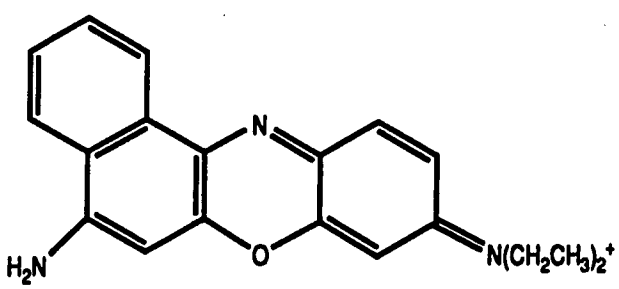
Three closely related dyes--methylene violet, Meldola's blue, and Nile blue--are all incapable of reducing cyt *c*. The structures are shown below. The presence of an oxygen atom in these systems perhaps increases their reduction potential high enough to be ineffective reductants. Control experiments do show that these three dyes do bleach in the presence of EDTA and in the absence of O_2. This bleaching process is notably slower than the other dyes. It may be that the triplet state decays too rapidly for efficient electron transfer or that it is rapidly quenched by the ground state dye. Or, it may be that the reaction of the EDTA with the triplet state of these dyes is slower and therefore the semireduced state is formed more slowly. Further characterization studies need to be done on these dyes.



Methylene Violet



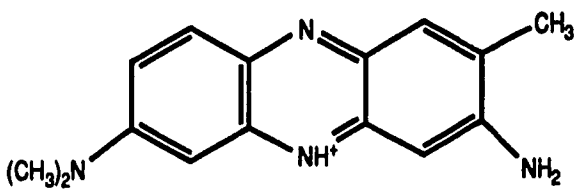
Meldola's Blue



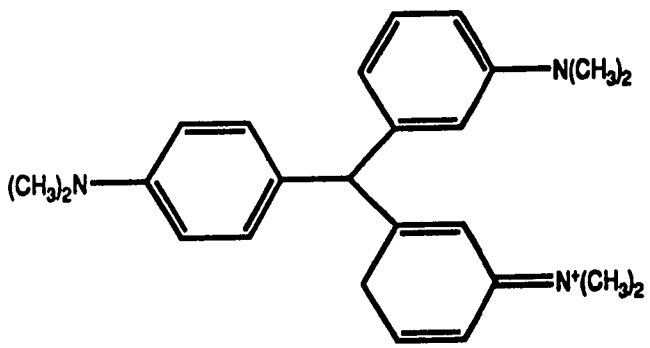
Nile Blue

Several other dyes which do absorb in the region of 590 nm but are not phenothiazine-type structures were tested as photoreductants. Crystal violet, bromcresol green, and neutral red did not show signs of oxidizing EDTA or reducing cyt *c* . Their structures are shown below.

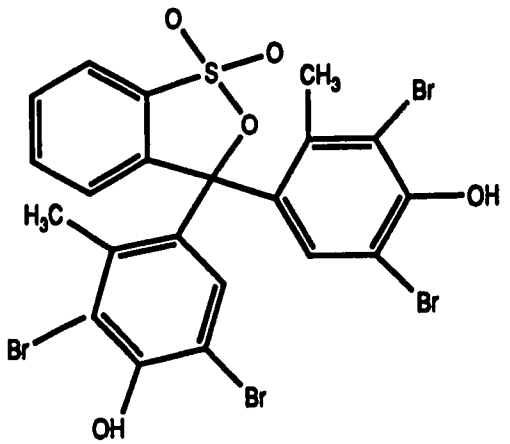
Neutral red has a structure analogous to toluidine blue O, but apparently, the triplet state of neutral red cannot oxidize EDTA. Either the triplet lifetime is too short or the reduction potentials make an electron transfer thermodynamically unfavorable. Crystal violet and bromcresol green are probably incapable of producing a stable odd-electron radical since these dyes are not as highly conjugated as methylene blue and its derivatives.



Neutral red



Crystal violet



Bromcresol green

CONCLUSIONS AND PROSPECTS

Methylene blue derivatives as photoreductants open a new class of dyes on which quantitative studies can be done. More work needs to be done on this particular study such as measurement of reduction potentials and triplet lifetimes. The family of dyes can be expanded to form a broader database of information on which factors control electron transfer from small molecules to proteins. Of particular interest would be the development of semisynthetic enzymes with methylene blue as a redox active center, much in the same way semisynthetic flavoenzymes have been developed.[229-233] This new class of blue enzymes would yield proteins with a new range of reduction potentials and a new visible band in which they can be photoexcited.

GENERAL SUMMARY

The complex $[Pt(trpy)Cl]^+$ reacts with ligands, biological and nonbiological, to form structurally and spectroscopically interesting complexes. The platinum complex exhibits very high selectivity toward the amino acids cysteine and histidine. A similar complex, $[Au(trpy)Cl]^{2+}$, is not as selective as its platinum counterpart. In addition, its oxidative properties make it an invasive reagent toward biomolecules. The spectroscopic properties and noninvasiveness of the platinum complex make it a very good bioconjugation reagent. This advantage of $[Pt(trpy)Cl]^+$ was tested rigorously on cytochromes *c* and on proteolytic enzymes. The tagging of the proteolytic enzymes α-chymotrypsin and α-lytic protease provides a new approach to study enzymatic structure and function. The tagging of the enzyme papain which has reactive Cys and His residues led to a new approach of selective labeling of biomolecules--that is, selective removal of tags by use of appropriate nucleophiles. The range of platinum complexes was expanded to $[Pt(sbpaphy)Cl]^-$, a structurally similar labeling reagent to $[Pt(trpy)Cl]^+$. Its reactivity toward cyt *c* was studied and compared to that of $[Pt(trpy)Cl]^+$.

In unrelated work, electron-transfer reactions of covalently tethered cyt *c* and pc were studied, and the reactivity of external reagents toward these proteins was investigated. No intracomplex electron transfer was observed.

Finally, the electron-transfer reactions of methylene blue derivatives toward cyt *c* were studied. This study provides a start in the quantitation of reaction rates of methylene blue derivatives toward proteins and provides potential for a new class of semisynthetic enzymes.

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