1986

Reduction of methemerythrin in the erythrocytes of Phascolopsis gouldii

Ron E. Utecht
Iowa State University

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REDUCTION OF METHEMERYTHRIN IN THE ERYTHROCYTES OF PHASCOLOPSIS GOULDII

Iowa State University PH.D. 1986

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Reduction of methemerythrin in the erythrocytes of *Phascolopsis gouldii*

by

Ron E. Utecht

A Dissertation Submitted to the
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GSH	Glutathione (reduced form)
DCIP	2,6-Dichloroindophenol
Hr	Hemerythrin
Hb	Hemoglobin
Mb	Myoglobin
Triton X-100	OctylPhenoxypolyethoxyethanol $(CH_3)_3CCH_2C(CH_3)_2C_6H_4O(CH_2CH_2O)_{9.5}H$
DTT	1,4-Dithiothreitol
EDTA	Disodium ethylenediamine tetraacetate
HEPES	N-2-Hydroxyethylpiperazine -N'-2-ethane-sulfonic acid
MES	2(N-Morpholino)ethanesulfonic acid
NADH	Nicotinamide adenine dinucleotide (reduced form)

DEDICATION

To my wife Jane and my son James.

I. INTRODUCTION

Aerobic organisms require oxygen for the efficient utilization of energy sources. In organisms that have a single cell to a few cells, oxygen can easily diffuse to all the cells. As the size of the organism increases, it is necessary to provide some sort of oxygen transport and storage system to supply sufficient oxygen to all the tissues. Three types of oxygen carrying proteins have evolved: hemoglobin (Hb), hemerythrin (Hr), and hemocyanin (Hc). The distribution of oxygen carriers is shown in Figure I-1 (1). The oxygen carriers vary widely in their method of oxygen binding. Some of the properties of oxygen carriers are summarized in Table I-1 (1).

A. Hemerythrin

1. General considerations

Hemerythrin is a nonheme, oxygen binding protein, found in the erythrocytes of one annelid and all sipunculids, priapulids, and brachiopods so far examined. These four phyla are closely related. Hemerythrin chemistry has recently been reviewed (1).

In the sipunculid Phascolopsis gouldii, at normal burrow conditions, the oxygen partial pressure of the coelomic fluid

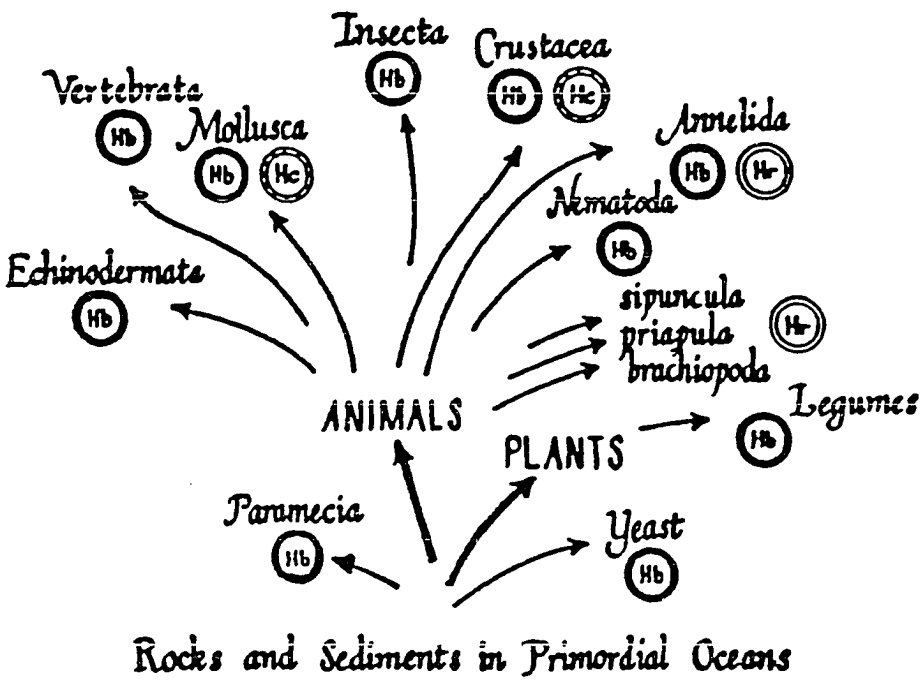


Figure I-1. Distribution of oxygen carriers (reprinted with premission from reference 1)

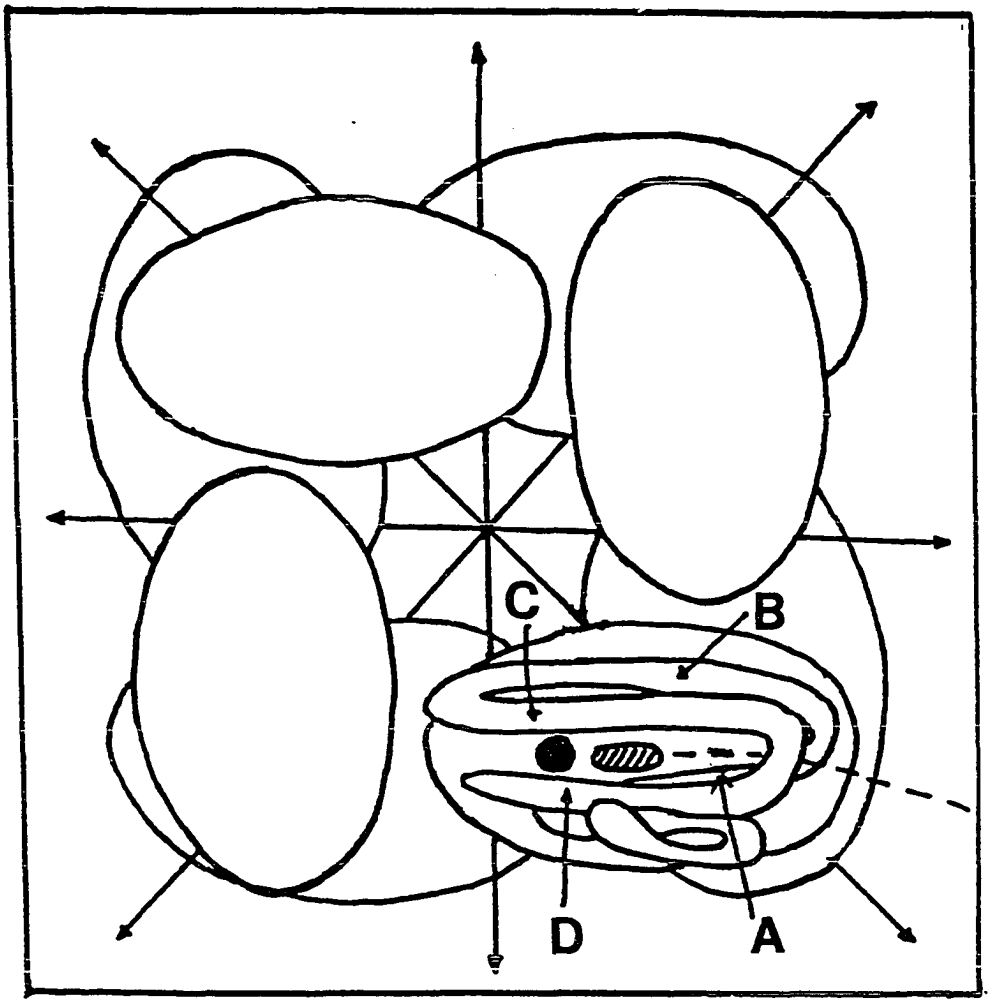
Table I-1. Properties of the oxygen carriers

	Hb	Hr	Hc
Metal atom(s)	1-Fe	2-Fe	2-Cu
Metal ligands	protoporphyrin	Amino acid side chains	
Oxidation state of bound oxygen	O_2^-	O_2^{2-}	O_2^{2-}
Oxygenated color	red	violet	blue
Deoxygenated color	violet	Colorless	

is about the same as the O_2 half-saturation pressure of coelomic Hr (P_{50} ~ 1.88 mm Hg, 0 °C). Therefore, any change in the oxygen tension will greatly affect the oxygenation state of the Hr. Hemerythrin stores oxygen for up to several hours when the environment of the worms becomes anaerobic (2).

Hemerythrins are a very homologous set of proteins. Between the sipunculids T. dyscritum, P. gouldii, and T. pyroides, 42% of the amino acid residues are invariant. The folding motif of Hr from these three different species is the same (2). The Hr subunit is composed of four nearly parallel helices. This basic motif is also present in cytochrome c', cytochrome b_{562}, the apoferritin subunit or monomer, and a portion of T4 lysozyme (3). The oxygen binding site of Hr is an iron dimer located between four nearly parallel α-helices as shown in Figure I-2. The active site of metHr is shown in Figure I-3 (4). The oxygen molecule is bound end on to the "inner" iron atom of the iron dimer. The "inner" iron atom is labelled Fe(2) in Figure I-3 and is farther from the surface in the octamer. Eight of the Hr subunits are stacked in two layers of four subunits each to form the octamer as shown in Figure I-2 (2). The octamer has often been described as a square doughnut.

Four oxidation states of Hr are known; oxy [Fe(III),Fe(III)O_2^{2-}], deoxy [Fe(II),Fe(II)], met [Fe(III),Fe(III)], and semi-met [Fe(III),Fe(II)]. The oxyHr



The ovals represent the subunits of the hemerythrin octamer. A, B, C and D are the protein helices. The dark circle represents the iron dimer. The dashed line is a proposed channel for entry of oxygen. Two-fold symmetry axes of the octamer are shown.

Figure I-2. Structure of hemerythrin

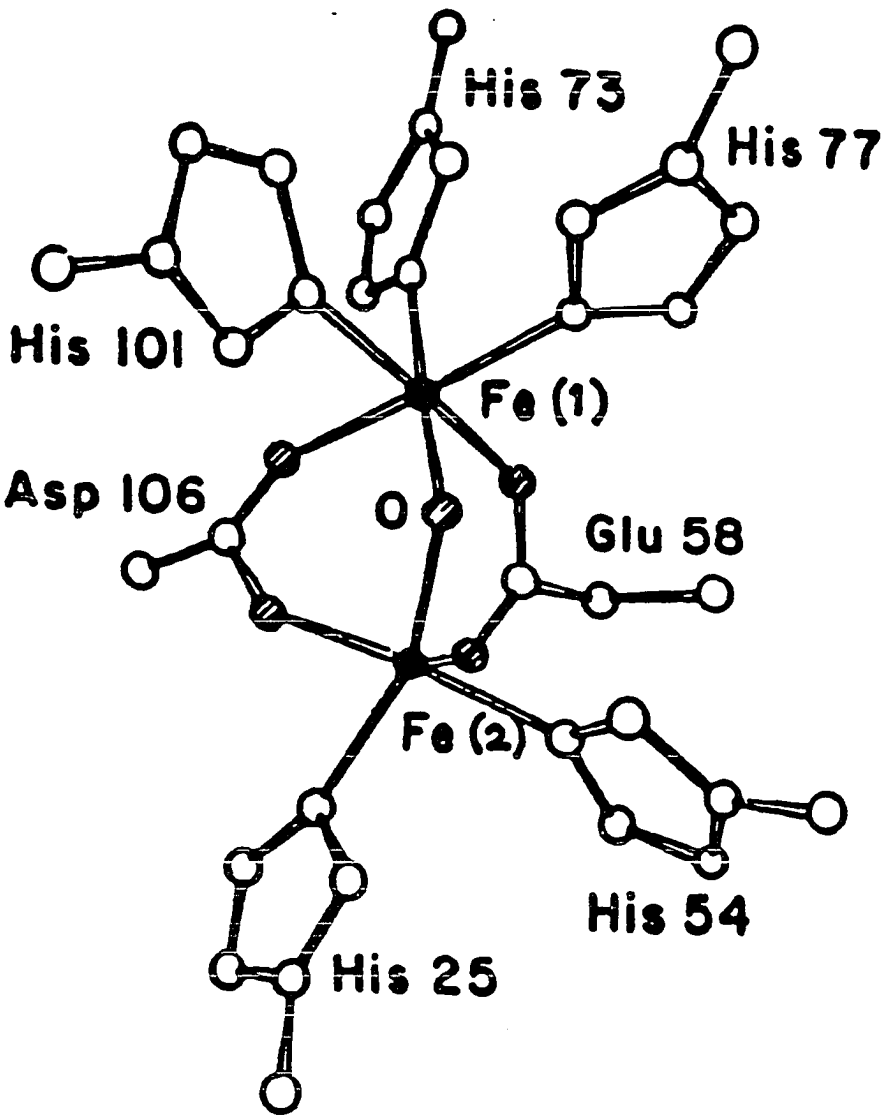
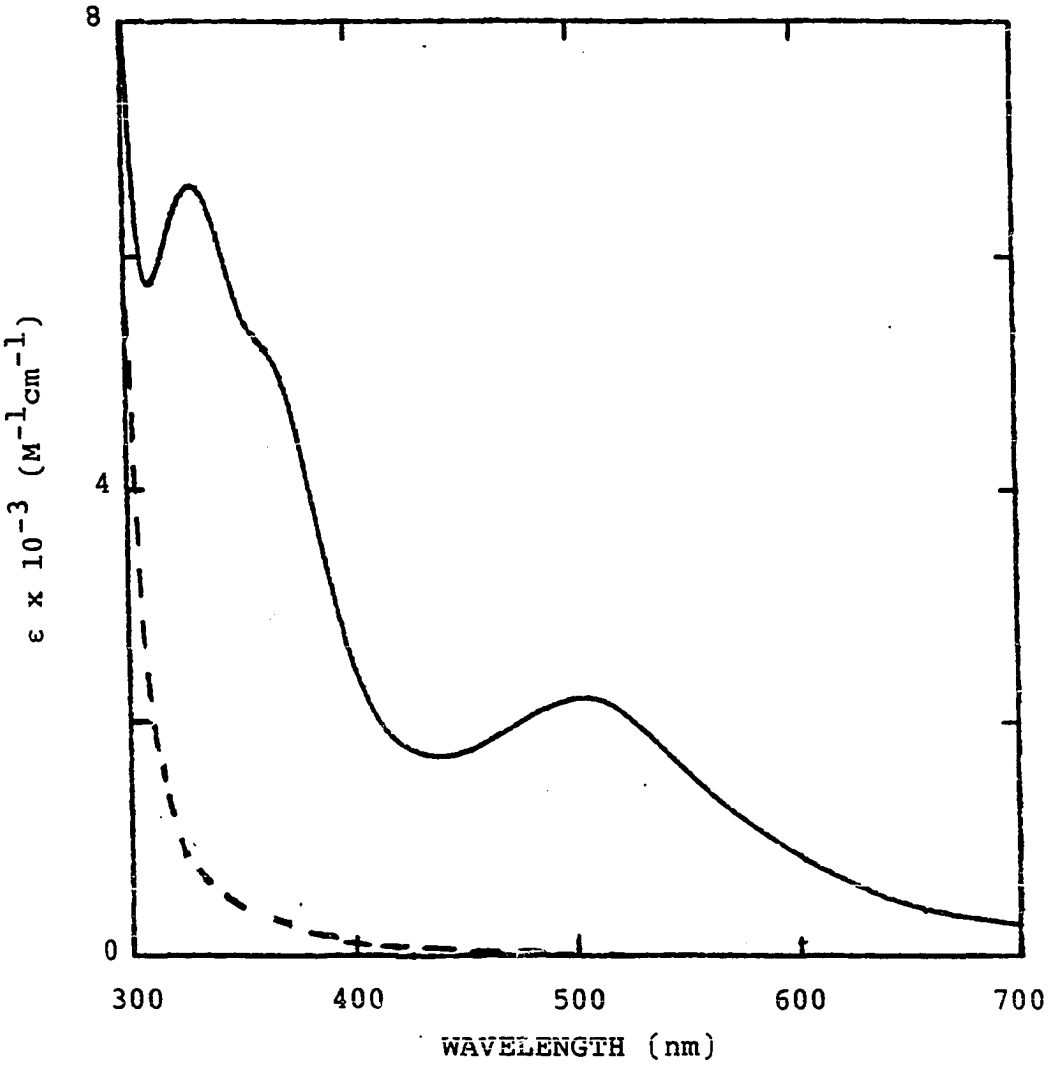


Figure I-3. Active site of methemerythrin

and deoxyHr forms are normally considered to be of greatest physiological importance as they are involved in reversible oxygen binding. OxyHr is violet colored and deoxyHr is colorless. The UV-visible spectra of oxyHr and deoxyHr are shown in Figure I-4. The iron center in metHr is composed of two, high-spin iron atoms bridged by a μ-oxo group and two carboxylate amino acid side chains. The iron atoms are anti-ferromagnetically coupled, $J = -134\ cm^{-1}$ (5). The "outer" iron atom is also coordinated by three histidine imidazoles and the "inner" iron atom is also coordinated by two histidine imidazoles. The sixth coordination position on the "inner" iron atom may be filled by peroxide in oxyHr, or by hydroxide or small anions in metHr. In oxyHr the iron atoms are also anti-ferromagnetically coupled, $J = -77\ cm^{-1}$ (5). This high coupling constant is consistant with a μ-oxo bridge. The two iron atoms are bridged by a hydroxo group along with the carboxylate groups in deoxyHr (6). The magnetic interactions of the iron atoms in deoxyHr, $J \sim -13\ cm^{-1}$, are less than those in the more oxidized forms (7).

MetHr can exist in either of two forms, the acidic or the basic form (8,9). Upon changing pH, there is a slow conversion between the two forms. The acid-base equilibrium has been attributed to the coordination of a hydroxide ion at the active site of the protein in the basic form (10). The acidic and basic forms of metHr can be distinguished by their



(——) OxyHr.
(---) deoxyHr.

Figure I-4. Absorption spectra of oxy and deoxyHr

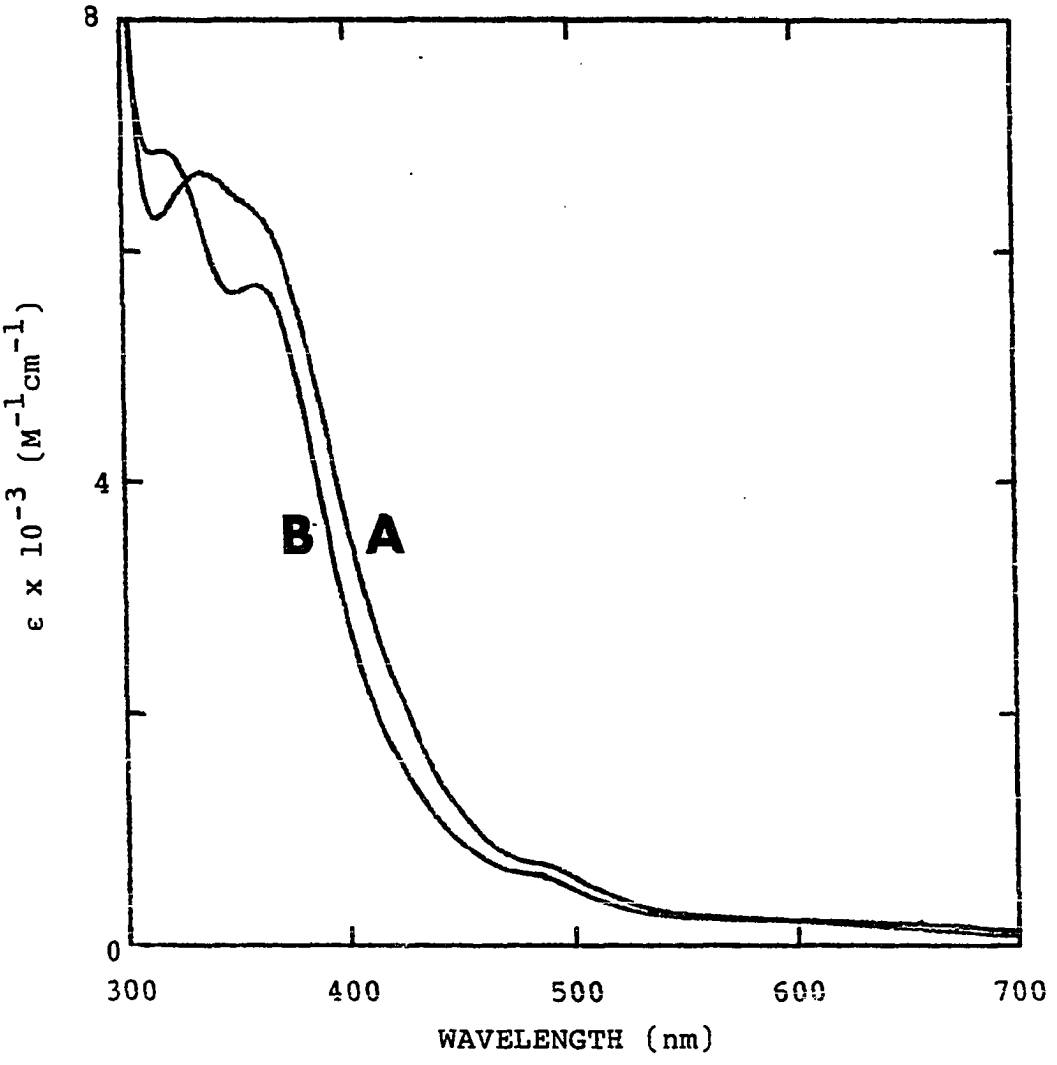
absorption spectra. These spectra are shown in Figure I-5. The pK_a of the iron site is 7.8. The extinction coefficients for the various forms of Hr are listed in Table I-2.

When metHr is mixed with small anions such as azide or thiocyanate, an adduct is formed. The small anions bind to the five coordinate iron. These adducts have increased absorbance in the visible region between 400 and 500 nm. This absorbance has been assigned to a charge transfer transition from the small anion to the iron (8,11).

2. Redox reactions

Oxygen carriers can be considered to be redox active proteins. In Hb, electron density flows from the ferrous ion to oxygen upon oxygenation to give a superoxide ion and a ferric ion. Because of this flow, Hb has been considered a modified oxidase in that the electron flow is reversible. In Hr, a flow of two electrons to oxygen takes place upon oxygenation to give a peroxide ion and two ferric ions.

If Hr is to be active in carrying oxygen, the protein must not be oxidized to the met form because metHr will not bind oxygen. Hemerythrin autooxidizes during storage and presumably in the erythrocytes as well, giving metHr. The half-time for the autooxidation of oxyHr is 18.5 hours at 25 °C with 300 mM Cl^- (12). If the worm is to survive, the metHr must be reduced back to the deoxy level. In this respect



(A) MetHr at pH 6.0, (acidic form).
(B) MetHr at pH 8.0, (basic form).

Figure I-5. UV-visible spectra of methemerythrin

Table I-2. UV-visible absorption spectral data

Species	Wavelength (nm)	ε ($M^{-1}cm^{-1}$)	Reference
NADH	340	6220	13
oxyHr	750sh	200	11
oxyHr	500	2200	11
oxyHr	360sh	5450	11
oxyHr	330	6800	11
metHr-OH^-	597	160	11
metHr-OH^-	480sh	550	11
metHr-OH^-	362	5900	11
metHr-OH^-	320	6800	11
metHr-H_2O	580sh	200	11
metHr-H_2O	400sh	600	11
metHr-H_2O	355	6400	11
metHrN_3^-	446	3700	14
$Fe(CN)_6^{3-}$	420	1026	15
$Fe(CN)_6^{4-}$	420	2	15
Cytochrome b_5 (oxidized)	413	115000	16

also, Hr is a redox active protein. Two different routes for reduction of metHr to deoxyHr can be envisioned, a one- or a two-electron process. The one-electron process would yield semi-metHr, [Fe(III),Fe(II)]. This semi-metHr could then undergo disproportionation to give metHr and deoxyHr, or be further reduced by one electron to give deoxyHr. The two-electron process would yield deoxyHr in one step, without disproportionation or further reduction.

There are two forms of semi-metHr: $(\text{semi-met})_R$, which is formed by the one-electron reduction of metHr, and is apparently in a "metHr-like" conformation; and $(\text{semi-met})_O$, which is formed by a one-electron oxidation of deoxyHr, and is apparently in a "deoxyHr-like" conformation (17). $(\text{Semi-met})_R\text{Hr}$ can be more quickly oxidized to metHr than can $(\text{semi-met})_O\text{Hr}$. $(\text{Semi-met})_O\text{Hr}$ can be more quickly reduced to deoxyHr than can $(\text{semi-met})_R\text{Hr}$. The semi-met forms of Hr are unstable and disproportionate to give deoxyHr and metHr. This disproportionation reaction is clean and complete for both $(\text{semi-met})_R\text{Hr}$ and $(\text{semi-met})_O\text{Hr}$ from T. zostericola. With Hr from P. gouldii, the $(\text{semi-met})_O\text{Hr}$ form is unstable and gradually is converted towards $(\text{semi-met})_R\text{Hr}$. $(\text{Semi-met})_R\text{Hr}$ will then disproportionate, but incompletely, to give metHr and deoxyHr. The rate constant for disproportionation is generally independent of Hr concentration. This observation shows that disproportionation is an intramolecular process

(18).

Semi-metHr is a spin 1/2 system, a coupled high-spin ferric and a high-spin ferrous ion, that gives a characteristic EPR spectrum. Figure I-6 shows the semi-metHr EPR signals. Figure I-7 shows the UV-visible spectra of semi-metHr (17). Only the semi-met forms of Hr display an EPR spectrum near the free electron value, and then only near liquid helium temperatures. $(\text{Semi-met})_O\text{Hr}$ has an axial EPR signal with g-values of 1.94 and 1.68, while $(\text{semi-met})_R\text{Hr}$ has a rhombic EPR signal with g-values of 1.94, 1.86, and 1.66, as shown in Figure I-6 (19). The exact g-values seen in the semi-metHrs are dependent on the pH. Both $(\text{semi-met})_O\text{Hr}$ and $(\text{semi-met})_R\text{Hr}$ will react with azide to form a common semi-metHr azide adduct. This complex is stable towards disproportionation. The semi-metHrN_3^- EPR displays an axial signal with g=1.91 and 1.83 (17).

If the one-electron pathway is involved in the reduction of metHr, an EPR signal corresponding to semi-metHr might be seen if there is a build-up of semi-metHr.

a. Inorganic reducing agents Reduction of metHr by inorganic reducing agents such as dithionite, Cr^{2+}, or $[Cr(15\text{-ane}N_4)(H_2O)_2]^{2+}$ is a complex, multiphasic reaction. The first step is a fast reduction of all the subunits to $(\text{semi-met})_R\text{Hr}$. The $(\text{semi-met})_R\text{Hr}$ subunits are then slowly

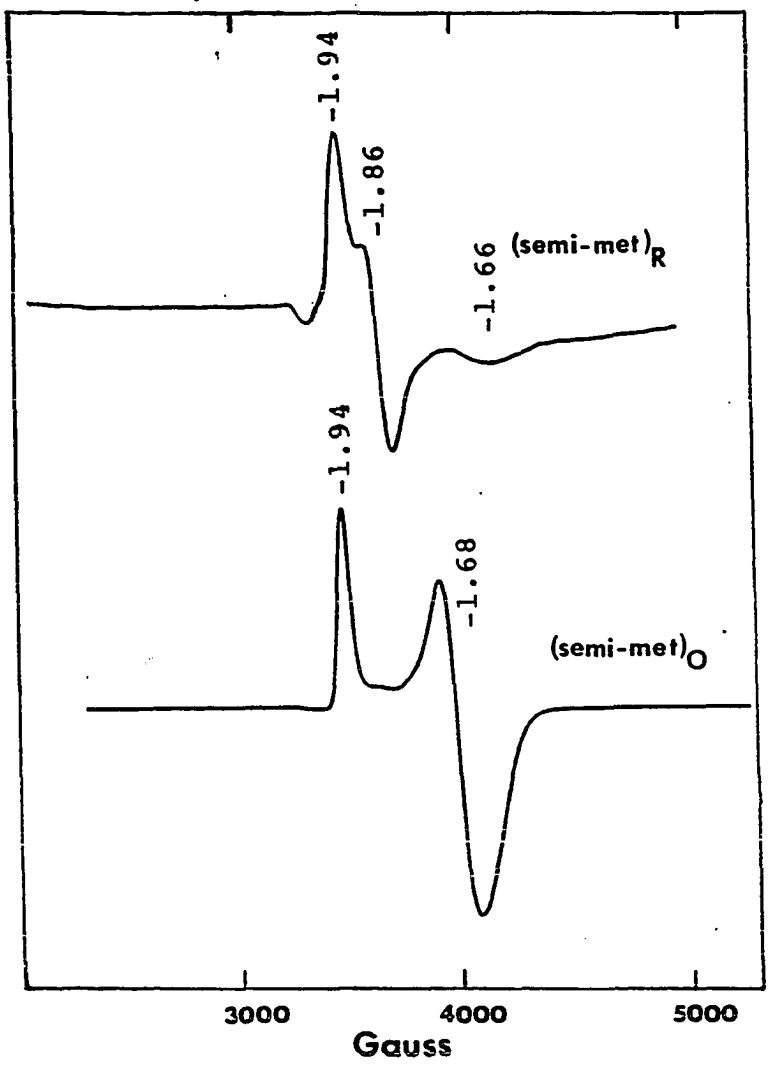
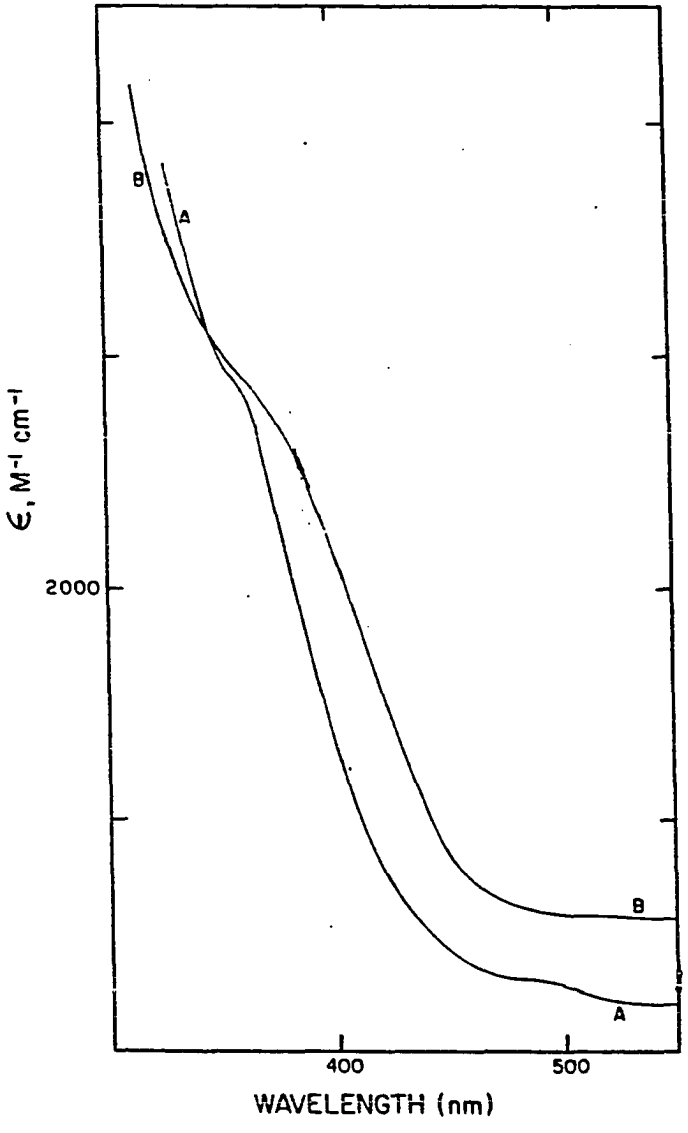


Figure I-6. EPR of P. gouldii $(semi\text{-}met)_R Hr$ and $(semi\text{-}met)_O Hr$



(A) $(\text{Semi-met})_R\text{Hr}$.

(B) $(\text{Semi-met})_O\text{Hr}$.

Figure I-7. Absorption spectra of $(\text{semi-met})_R\text{Hr}$ and $(\text{semi-met})_O\text{Hr}$

converted to the deoxy level. The conversion to the deoxy from $(semi\text{-}met)_R$Hr level has been reported to proceed in two stages, the rate constants for which are independent of reductant and reductant concentration. The reduction rate constants of metHr are given in Table I-3. The mechanism of conversion of semi-metHr to deoxyHr is currently under debate (20,21).

When salyrganic acid is reacted with an octameric Hr, monomeric Hr is formed (22). The metHr monomer is reduced to deoxyHr in a biphasic reaction, and the second phase is independent of dithionite concentration (14). This reaction is much faster than with octameric Hr. Hemerythrin monomers may also be formed by dilution of the octamer, and kinetically behave very similarly to octameric Hr.

The oxidation kinetics of deoxy to metHr with ferricyanide have also been studied (23). The oxidation kinetics are also complex and the mechanism is incompletely understood.

The redox potentials of metHr are included in Table I-4.

b. Riboflavin Irradiated riboflavin has been used to reduce metHr (17). A mixture of EDTA and riboflavin reduced metHr to $(semi\text{-}met)_R$Hr upon irradiation for 60-90 seconds. Further irradiation for 30-45 minutes produces deoxyHr.

When riboflavin is irradiated in a solution containing

Table I-3. Reduction rate constants for P. gouldii metHr[a]

Reducing reagent	k_1 ($M^{-1}s^{-1}$)	$k_2 \times 10^3$ (s^{-1})	$k_3 \times 10^4$ (s^{-1})
Cr^{2+},[b]	34	2.1	1.7
$[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$,[c]	600	3.7	2.3
dithionite [c]	140000	2.3	1.7

[a] Reference 20.

[b] 100 mM cacodylate pH 7.0, T = 20 °C.

[c] 50 mM HEPES pH 7.0, T=20 °C, I = 0.15 M (Na_2SO_4).

Table I-4. Reduction potentials

Reaction	Potential	pH	Reference
$Mb\text{-}Fe^{3+} + e^- = Mb\text{-}Fe^{2+}$	46 mV	7.0	24
cyto $b_5^{3+} + e^- =$ cyto b_5^{2+}	-10 mV	7.0	25
metHr + $2e^-$ = deoxy	230 mV	8.2[a]	26
metHr + e^- = $(semi\text{-}met)_R Hr$	110 mV	8.2[b]	26
$NAD^+ + H^+ + 2e^-$ = NADH	-320 mV	7.0	27
DCIP	140 mV	7.0	28
$Fe(CN)_6^{3-} + e^- = Fe(CN)_6^{4-}$	430 mV	7.0	29
methylene blue	11 mV	7.0	25
Hb α-chain	53 mV	7.0	24
Hb β-chain	113 mV	7.0	24

[a] Themiste zostericola.

[b] Phascolopsis gouldii.

EDTA, the flavin is reduced. EDTA is the electron donor to the flavin. The reduced flavin is then oxidized to the semiquinone by an electron acceptor such as metHr. Two semiquinones disproportionate to give one oxidized and one reduced flavin. The oxidized flavin is then reduced (30).

c. Protein reducing agents Most redox reactions of Hr studied up to this point have involved inorganic redox reagents. Not being a strict electron transport protein, the physiological partner in the reduction of metHr has not previously been isolated.

The reduction of metHr by deoxymyoglobin, a non-physiological reaction, has been studied (31,32). Myoglobin is an oxygen storage protein, and strictly speaking, is not an electron transfer protein. The reaction of deoxymyoglobin and metHr is uniphasic, with a second order rate constant of 1.2 $M^{-1}s^{-1}$ for octameric P. gouldii metHr. Only very small salt or pH effects are observed.

The uniphasic kinetics show that the reduction of semi-metHr to deoxyHr is apparently more rapid than the initial reduction of metHr to semi-metHr. This result is in contrast to the case with inorganic reagents, in which the reduction of metHr to semi-metHr is faster than the reduction of semi-metHr to deoxyHr as given in Table I-3.

3. Other 2Fe proteins

Other proteins contain binuclear iron sites similar to that in Hr and are active in redox reactions. Ribonucleotide reductase from E. coli is one such protein. The function of ribonucleotide reductase is to reduce ribonucleotides to deoxyribonucleotides for subsequent incorporation into DNA. The source of reducing electrons for ribonucleotide reductase is NADPH. The electrons are transferred through thioredoxin reductase to thioredoxin, which in turn reduces the active disulfide bridge of ribonucleotide reductase. An alternate pathway for the reduction of ribonucleotide reductase occurs in both E. coli and mammalian tissue, here the system consists of glutathione reductase, glutathione, and glutaredoxin. Specifically inhibiting electron transfer to glutathione reductase will inhibit DNA synthesis, which is of clincal value in tumor and virus treatment. An important difference between the active site of Hr and ribonucleotide reductase is that in ribonucleotide reductase the iron atoms do not change valence and the redox chemistry occurs only at a disulfide bridge. The iron site apparently acts to stabilize a tyrosine free radical (33).

Uteroferrin also contains a binuclear iron site similar to that of Hr. Low temperature EPR spectra show a rhombic signal. Upon standing, the EPR signal converts to an axial type. This same behavior is seen for semi-metHr (34).

The bacterium Methylcoccus capsulatas contains a methane monooxygenase that catalyzes the conversion of methane to methanol. This enzyme contains two moles of iron per mole of protein and uses NADH as a source of electrons. In the reduced form, it exhibits a $(semi\text{-}met)_R$Hr type EPR signal (35).

4. Model complexes

Model complexes for the iron site of both metHr and deoxyHr have been synthesized (36). These compounds have a bridging oxo- or hydroxo group and two bridging carboxylates. The magnetic couplings of the irons are very similar to those seen in metHr and deoxyHr, respectively.

A model complex for deoxyHr is $[L_2Fe_2(II)(\mu\text{-}OH)(\mu\text{-}CH_3CO_2)_2](ClO_4)\cdot H_2O$. The ligand, L, is N,N',N'',-trimethyl-1,4,7-triazacyclonanane. This model complex has a magnetic coupling constant, J, of -14 cm^{-1}, in good agreement with that for deoxyHr (7). Addition of oxygen to this model complex gives an irreversible oxidation to $[L_2Fe_2(III)(\mu\text{-}O)(\mu\text{-}CH_3CO_2)_2](ClO_4)_2$. The J value of the oxidized compound is -115 cm^{-1}, very similar to the -134 cm^{-1} seen in metHr. The oxidized model complex has been prepared independently (37). Thus, this deoxyHr model complex does not reversibly bind oxygen, only irreversibly oxidizes upon exposure to oxygen. No other redox chemistry has been

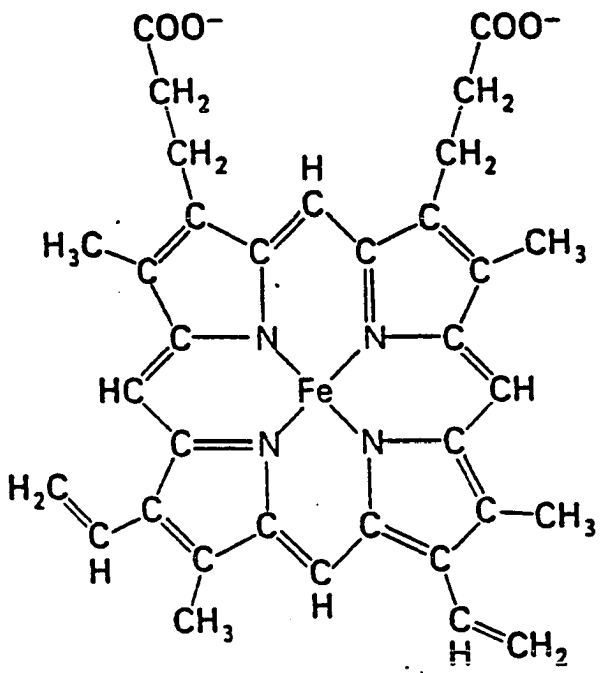
reported for these compounds.

Another series of model complexes for hemerythrin has been isolated but does not mimic the oxygen binding of Hr (38,39). Their formula is $[(HBpz_3)FeO(O_2CR)_2Fe(HBpz_3)]$. R = H, CH_3 or C_6H_5 and $HBpz_3$ = tri-1-pyrazolylborate ion.

B. Hemoglobin

Hemoglobin (Hb) is an oxygen carrying protein that uses heme to bind one mole of diatomic oxygen per subunit. The Hb subunits are arranged into tetramers. The heme is composed of iron and protoporphyrin IX, as shown in Figure I-8. Protoporphyrin IX is one isomer of four pyrrole groups linked with methylene bridges and having four methyl, two vinyl, and two propionate side chains added. A large portion of the amino acids in Hb is arranged in α-helices. The iron atom is ligated by the four pyrrole nitrogens of the protoporphyrin in a square planer conformation. The fifth coordination position is filled by a histidine residue. The sixth coordination position is empty, and it is here where oxygen and other small molecules will bind. Hemoglobin subunits are arranged in a tetramer with the binding of oxygen being cooperative.

DeoxyHb contains Fe(II). OxyHb can be formulated as $Fe(III)O_2^-$. DeoxyHb can be oxidized to metHb Fe(III), which is not functional in oxygen transport.



Heme
(Fe-protoporphyrin IX)

Figure I-8. Structure of Fe-protoporphyrin IX

C. Comparison of the Redox Chemistry of Hemerythrin and Hemoglobin

Differences would be expected in the reduction system of Hb and Hr. Hemoglobin has a partially exposed heme edge which is a good electron transfer point for reducing equivalents, while in metHr the electrons must pass through amino acid side chains. MetHb reduction requires only one electron, whereas metHr reduction to deoxyHr requires two electrons. The reduced electron carriers in the cells, NADH and NADPH, are each capable of donating two electrons. One could envision a mechanism by which both electrons from an electron carrier would be used to fully reduce one molecule of metHr. However, NADH and NADPH do not directly reduce metHr. The stability of semi-metHr and its disproportionation into metHr and deoxyHr make the one-electron pathway a reasonable alternative to the two-electron reduction. NADH also does not directly reduce metHb. Two other proteins, an NADH-cytochrome b_5 reductase and a cytochrome b_5 are required to transfer electrons from NADH to metHb. Both cytochrome b_5 and cytochrome b_5 reductase are soluble proteins in the mature human erythrocyte (40).

There are differences in the structure of the erythrocytes of mammals and those from P. gouldii. Mammalian erythrocytes have no nucleii, while those from P. gouldii are nucleated. Nucleated erythrocytes from birds and reptiles

also contain Hb as an oxygen carrier. The metHb reductase activity in the nucleated erythrocytes of birds and reptiles is membrane bound, while in the non-nucleated erythrocytes, the metHb reductase activity is soluble (41). No physiological reduction system for metHr has yet been isolated.

D. Cytochrome b_5

The hemoprotein cytochrome b_5 was first isolated from liver by Strittmatter in 1955 (42). Cytochrome b_5 is widely distributed in nature, being found in the brain, liver, and erythrocytes of animals, and in plants. Cytochrome b_5 participates in the desaturation of fatty acids, steroid hydroxylation, and the reduction of metHb in erythrocytes. In all known cases, cytochrome b_5 appears to function solely as an electron transfer protein.

The UV-visible absorption spectrum of human erythrocyte cytochrome b_5 is shown in Figure I-9 (43). The EPR spectrum of ferricytochrome b_5 is shown in Figure I-10 (44). The crystal structure of bovine liver cytochrome b_5 shows that the heme group is non-covalently held by hydrophobic interactions in a pocket formed by α-helices on the sides, and a β-pleated sheet on the bottom (45). Two histidine imidazoles ligate the axial positions of the iron. A narrow hydrophobic groove on

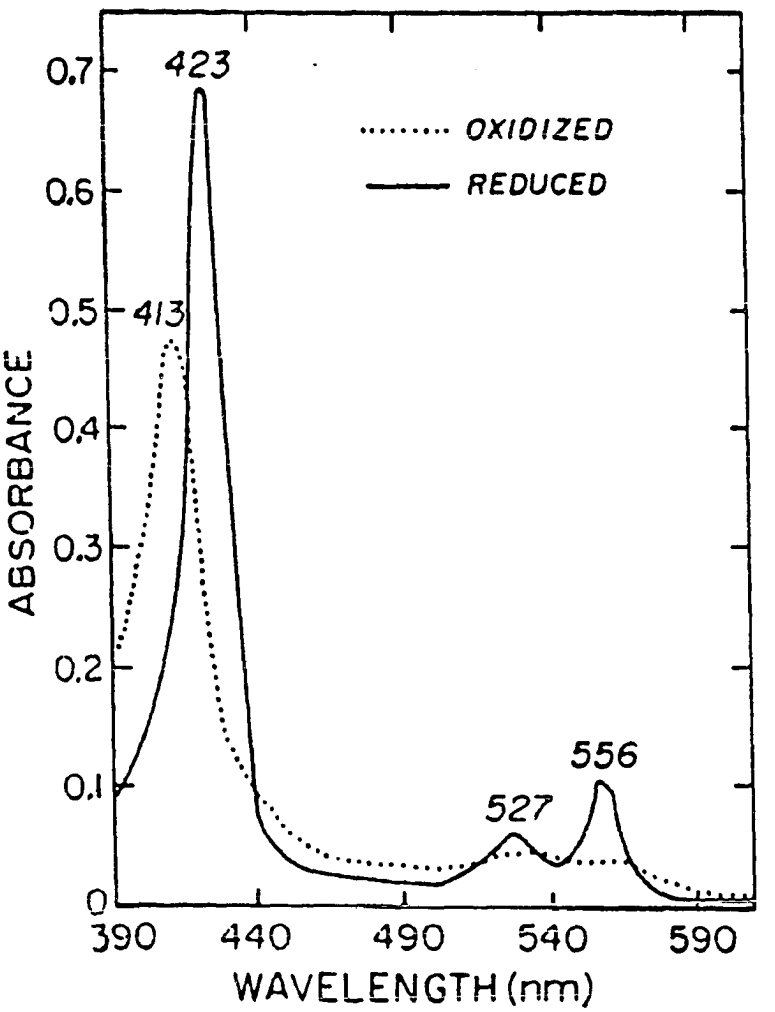


Figure I-9. UV-visible spectra of human erythrocyte cytochrome b_5

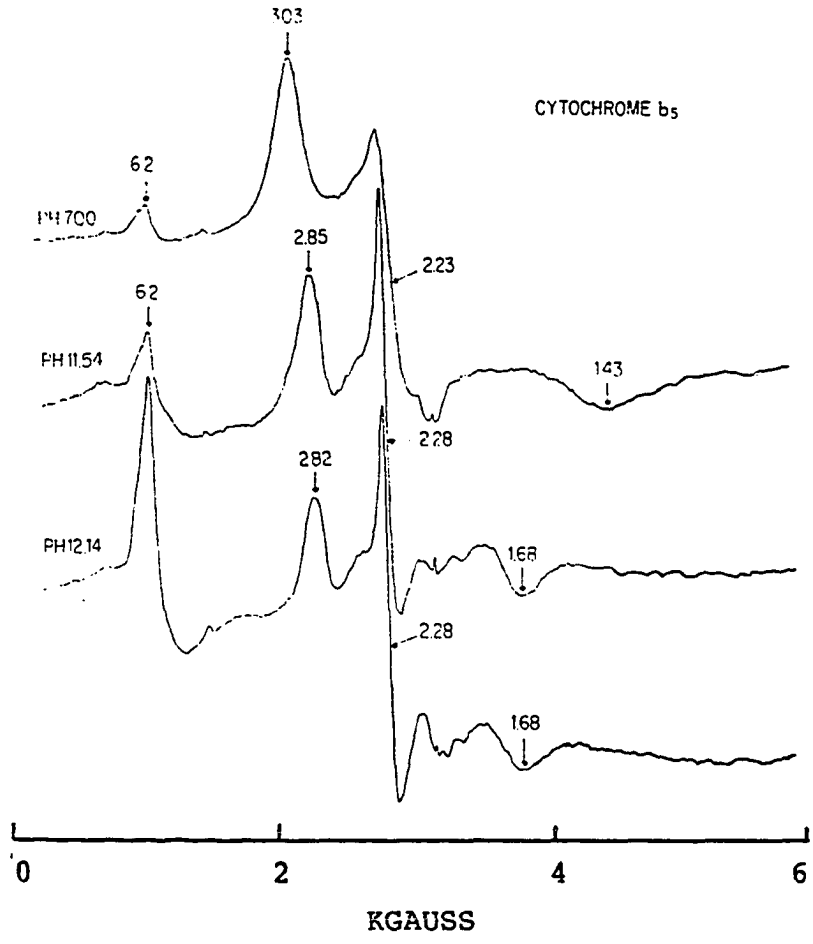


Figure I-10. EPR spectra of oxidized human erythrocyte cytochrome b_5

the surface leads to the heme. The heme group can exist with either Fe^{3+} or Fe^{2+}, and this allows the protein to function as an electron transport protein. Cytochrome b_5 is normally a membrane bound protein in liver, but treatment of the crude liver extract with proteolytic enzymes, such as trypsin, cleaves a 44 amino acid portion from the N-terminus of the protein, leaving a heme containing soluble fragment. This solubilized protein is very similar to the soluble cytochrome b_5 from mature erythrocytes. Rabbit liver cytochrome b_5 can also be solubilized using detergents (46). This protein is larger than the soluble cytochrome b_5 and contains a hydrophobic membrane binding region. In the mature erythrocyte, the protein is naturally soluble with a molecular weight between that of the detergent and the trypsin-solubilized liver cytochrome b_5. In immature red blood cells, the cytochrome b_5 is reported to be membrane bound. As the erythrocyte matures, the cytochrome b_5 is solubilized (47).

A protease that will release soluble cytochrome b_5 and cytochrome b_5 reductase has been isolated from immature erythrocytes (48).

E. Cytochrome b_5 Reductase

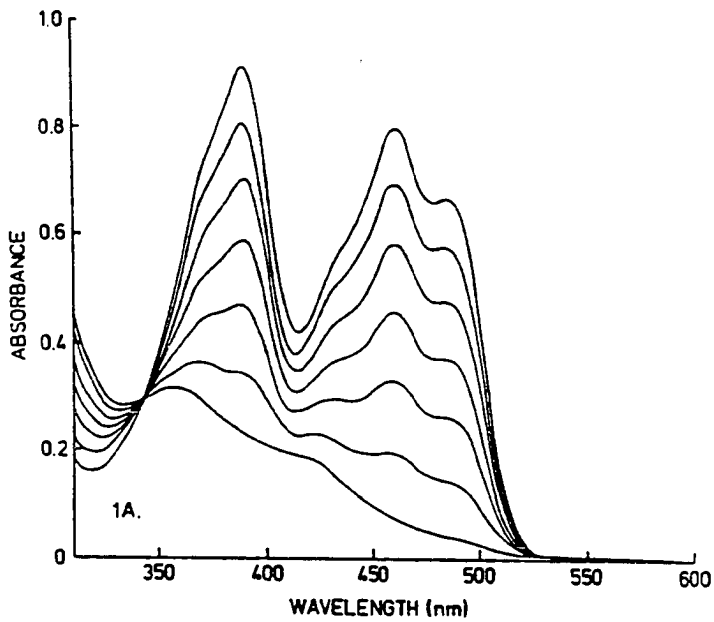
Cytochrome b_5 reductase transfers electrons from NADH to cytochrome b_5. Cytochrome b_5 reductase was first isolated in 1957 from calf liver (49). Although cytochrome b_5 reductase has been studied for almost 30 years, there are still conflicting results on its properties.

Cytochrome b_5 reductase is a flavoenzyme that displays a characteristic visible spectrum. The enzyme can be reduced by two electrons, using either NADH or NADPH, but NADH is more effective. The reduced cytochrome b_5 reductase is oxidized by oxygen, but the NAD^+ bound enzyme is oxidized much more slowly (50).

The visible spectra of both the detergent- and proteolytically-solubilized cytochrome b_5 reductases have maxima at 390 and 461 nm, a minimum at 410 nm, and a shoulder at 490 nm (Figure I-11). This absorbance is bleached upon the addition of NADH. The extinction coefficient at 461 nm is 10,600 $M^{-1}cm^{-1}$ (50).

F. Hemoglobin Reduction Systems

Within erythrocytes, Hb is slowly oxidized to metHb during the normal functioning of the erythrocytes. In a normal adult human male, about 3% of the total Hb is converted



Anaerobic titration of NADH-cytochrome b_5 reductase by sodium dithionite. Upper spectrum is fully oxidized. Lower curve is fully reduced after addition of one equivalent of sodium dithionite.

Figure I-11. Absorption spectra of cytochrome b_5 reductase

to metHb in a day. This metHb then undergoes reduction to deoxyHb at a rate sufficient to keep 99% of the Hb in an oxygen carrying form. Several systems exist in the erythrocyte to keep Hb in the reduced form (51).

The cytochrome b_5 reduction system has recently been reviewed (40). The maximum rate of reduction of metHb in erythrocytes is 1.08 mM/hour (52). Erythrocytes contain a metHb reductase that catalyzes this reduction. This enzyme displays diaphorase activity, i.e., the ability to transfer electrons from NADH to small acceptor molecules such as DCIP or $K_3Fe(CN)_6$. This enzyme is actually a cytochrome b_5 reductase, reducing cytochrome b_5 quickly, but metHb slowly. Cytochrome b_5 in turn reduces metHb relatively quickly.

Soluble cytochrome b_5 occurs in the human erythrocyte at a concentration of 0.22 μM. The midpoint potential is -10 mV, with an isoelectric point of 4.3 (25). Several bands are seen in the isoelectric focusing experiment and are presumably due to loss of the heme during focusing (53). When cytochrome b_5 and metHb are mixed, a difference spectrum forms that has been attributed to a conformational change of the metHb to more closely match that of deoxyHb in a 1:1 complex between metHb and cytochrome b_5. This change would lower the reorganizational energy barrier for electron transfer from cytochrome b_5 to metHb. The complex was most stable between pH 6.0 and 6.2, which is about midway between the pI's of the

two proteins. The complex is less stable at high ionic strengths (54).

The details of the interaction of cytochrome b_5 and metHb have been modeled by computer graphics. This complex is shown in Figure I-12 (55). The crystal structures of both cytochrome b_5 and hemoglobin are known to a high resolution (45,56). There is a group of positively-charged amino acids surrounding the exposed heme edge of Hb. The exposed edge of the heme in cytochrome b_5 is surrounded by negatively-charged amino acid residues that are complementary to the positive ones on Hb. When the two molecules are oriented for maximum contact, the heme groups approach 7-8 Å from each other. In this position, the iron atoms are separated by 16 Å. Two non-ionic interactions bracket the heme contact and exclude water. Propionate groups bridge the hemes and provide a pi conduit for electrons to pass between the heme groups.

Reduced glutathione (GSH) acts as a redox buffer in the erythrocytes. A major function of GSH is to keep the cysteines in the Hb in their reduced form. GSH is also required to maintain the cell membrane. A depletion of GSH leads to cell lysis. In the human erythrocyte, the reduction of metHb by the NADH-metHb reduction system accounts for 95% of the metHb reduction. Ascorbic acid, reduced glutathione, and NADPH dehydrogenase account for the other 5% (51).

Computer graphics fit of cytochrome b_5 and hemoglobin. Upper protein is cytochrome b_5, lower protein is hemoglobin.

Figure I-12. Stereo view of the cytochrome b_5-hemoglobin complex

G. Electron Transfer in Metalloproteins

The investigation of electron transfer in metalloproteins has been of great interest lately. Most previous studies have focused on the interaction between small molecules and protein molecules (57). Lately, Marcus theory has been applied to protein-protein electron transfer reactions (57,58).

The electron transfer mechanism of heme proteins and cytochromes in particular, has been extensively studied. One generality that has emerged is that low-spin hemes have more facile electron transfer than high-spin hemes (59). Apparently, less rearrangement is required for electron transfer from low-spin hemes than from high-spin hemes. Electron transfer between cytochromes is generally postulated to take place through the exposed heme edges. The protein then acts as an insulator. Electron transfer between heme proteins and metal centers in non-heme metalloproteins has been less well studied.

H. Statement of the Problem

Up to this point, studies of the redox reactions of Hr with oxidants and reductants have used either inorganic reagents or non-physiological partner proteins. While these studies have yielded insight into the electron transfer

processes of Hr, the obvious choice for determining what redox reactions actually occur in the erythrocyte is to isolate and characterize any physiological partner proteins or small molecules with which Hr interacts. Such studies would impart physiological relevance to the large body of results on the redox chemistry of Hr.

The redox state of oxygen carrying proteins is of general interest. In humans, an excess of metHb in the blood gives rise to the disease methemoglobinemia. The reduction system for metHb has been well-studied. The electron donor to metHb, cytochrome b_5, is also involved in other redox reactions in the erythrocytes, but it is not known if the evolution of the oxygen carrier Hb simply took advantage of a redox system already present or if the redox system evolved together with the oxygen carrier. P. gouldii, with its completely different oxygen carrier, must also possess a reduction system to counter the autooxidation of Hr. Comparisons of the reduction systems for the Hb-containing and the Hr-containing erythrocytes would provide an interesting contrast.

Model complexes of Hr have no reported redox chemistry (other than irreversible oxidation by O_2) and will not reversibly bind oxygen. The protein matrix must therefore convey some special properties to the iron site. Study of the redox chemistry of the iron site of Hr may shed some light on these properties. Understanding of the redox chemistry of the

iron site of Hr may lead to a better understanding of the mechanisms of some other proteins containing a Hr-like iron center such a ribonucleotide reductase and uteroferrin.

For the above reasons, attempts were made to isolate and characterize the systems responsible for the reduction of metHr in the erythrocytes of P. gouldii. This thesis describes the results of these attempts.

II. EXPERIMENTAL

A. Instrumental

All UV-visible spectra were recorded on either a Perkin-Elmer Model 554 dual-beam scanning spectrophotometer or a Perkin-Elmer Model 8460 diode array spectrophotometer interfaced to a Perkin-Elmer 7300 Series Professional computer. Intact erythrocytes were incubated in a water bath temperature controlled by a Westinghouse chiller to 1 °C. For the kinetic measurements, constant temperature (± 0.1 °C) was maintained by a Brinkman constant temperature bath connected to a thermostated cell holder. The EPR spectra were recorded on an IBM model ER-220D EPR spectrometer equipped with an Oxford Instruments model ESR-9 cryostat to allow spectra to be recorded near liquid helium temperatures. All kinetic rate constants were calculated on a TRS-80 Model III microcomputer with the non-linear least squares program of Duggleby (60). The reported k_{obs} values for oxidation of cytochrome b_5 represent the sum of autooxidation and oxidation by metHr ($k_{obs} = k_{auto} + k[\text{metHr}]$) unless otherwise specified. HPLC was performed on an Altex Spherogel-TSK-300-PW column or Beckman Ultrasphere-ODS C-18 reverse phase column with Beckman 110A pumps and an ISCO V-4 UV-visible detector. Deionized water was obtained from a Millipore water purification system.

For centrifugation at higher acceleration than generated in a clinical centrifuge, a Beckman model J-21B centrifuge with a JA-17 rotor at 16,000 rpm (26,000 g) was used. Isoelectric focusing was performed on an LKB Bromma 2117 Multiphore. A 50-50 mixture of LKB Ampholine pH 3-10 and pH 5-8 was used as the ampholite. The acrylamide gel was 2.6% crosslinked.

B. Protein Isolation

1. Purification of the erythrocytes

Live worms of the species Phascolopsis gouldii were obtained from Marine Biological Laboratory, Woods Hole, Massachusetts. Live worms of the species Themiste zostericola were obtained from Pacific Biomarine Supply in Venice, California. The worms were slit lengthwise and their blood drained into a large porcelain evaporating dish. The erythrocytes were allowed to settle from the coelomic fluid, and the coelomic fluid was removed by decantation. The erythrocytes were filtered through glass wool and then Miracloth to remove debris. Then, the erythrocytes were alternately suspended in artificial sea water and sedimented in a clinical centrifuge. The sea water was discarded. A fatty layer that was present on top of the erythrocytes after sedimenting in a clinical centrifuge was removed with a disposable pipette and discarded. Approximately 100 ml of

packed erythrocytes are obtained from 200 worms.

2. Purification of hemerythrin

P. gouldii Hr was isolated by a modification of Klotz's original procedure (61) that allows co-purification of cytochrome b_5 and cytochrome b_5 reductase.

To isolate Hr, erythrocytes which had been purified as described above were lysed with 1.7 times their volume of deionized water. Assuming the chloride concentration in the intact erythrocytes to be 540 mM (the same as seawater), the lysed erythrocyte solution would have a chloride concentration of 200 mM. The cell debris was removed by centrifugation, first in a clinical centrifuge, then by centrifuging at 26,000 g for one hour. The cell debris was retained for isolation of cytochrome b_5 reductase. Eight-tenths the volume of the intact erythrocytes of deionized water was added to lower the chloride ion concentration to 150 mM. Six hundred grams, wet weight, of Sigma DEAE cellulose cake equilibrated in 10 mM phosphate buffer pH 7.2 was added for every 500 mL of supernatant from the lysed erythrocytes. This mixture was stirred for two hours and the anion exchange cellulose removed from the Hr solution by filtration on a Buchner funnel. The anion exchange cellulose was retained for the isolation of cytochrome b_5.

The Hr solution was centrifuged for one hour at 26,000 g

to remove the fines from the anion exchange cellulose. The supernatant solution was dialyzed against 20% ethanol in water with 0.4% NaCl to crystallize the Hr. The crystals were stored as oxyHr, in 20% ethanol at 4 °C, or dissolved in 50 mM Tris-$SO_4^=$ buffer with 150 mM Na_2SO_4 pH 7.5 and converted to metHr by addition of an excess of $K_3Fe(CN)_6$ at room temperature for two hours. The excess $K_3Fe(CN)_6$ was removed by extensive dialysis against 50 mM Tris-$SO_4^=$ pH 7.5 with 150 mM Na_2SO_4. The metHr was stored as a frozen solution in liquid nitrogen. MetHr in 10 mM phosphate buffer pH 7.5 was passed over a Sephadex A-25 column to remove any traces of cytochrome b_5 or cytochrome b_5 reductase prior to use in reactions with cytochrome b_5 reductase, cytochrome b_5, and NADH.

3. Purification of cytochrome b_5

P. gouldii cytochrome b_5 was isolated by a modification of Kaftory and Hegesh's procedure for the isolation of human cytochrome b_5 (62). The anion exchange cellulose from the Hr purification was washed extensively with 10 mM phosphate buffer pH 7.2 on the filter to remove the Hr. The anion exchange cellulose was then stirred with 500 mM NaCl in 10 mM phosphate buffer pH 7.2 for two hours to remove the bound protein, including cytochrome b_5. A volume of this high salt buffer was used equal to the volume of the supernatant from

the lysed erythrocytes. The high salt buffer was removed from the anion exchange cellulose by suction filtration on a Buchner funnel, and the cellulose washed with 500 mM NaCl in phosphate buffer on the filter to remove the remaining cytochrome b_5. The high salt solutions contained the soluble cytochrome b_5. The cytochrome b_5 solution was dialyzed overnight against several changes of 10 mM phosphate buffer pH 7.2 with 0.5 mM EDTA. After dialysis, the mixture was centrifuged at 26,000 g for one hour to remove a white precipitate. The protein solution was then loaded onto a 2 x 10 cm Sephadex A-25 column equilibrated in 10 mM phosphate buffer pH 7.2 with 0.5 mM EDTA. The cytochrome b_5 stuck to the top of the column, while traces of Hr eluted from the column. The column was washed with 100 mM NaCl and 0.5 mM EDTA in 10 mM phosphate buffer pH 6.0 until the eluate had no absorbance at 280 nm. The column was then eluted with 250 mM NaCl in 10 mM phosphate buffer pH 6.0, and the fractions having Soret absorbances pooled and concentrated in an Amicon ultra-filtration cell with a PM-10 membrane. The pooled fractions were passed over a 1 x 50 cm Sephadex G-50 column that had been equilibrated with 10 mM phosphate pH 7.2 buffer with 500 mM NaCl. The cytochrome b_5 was stored in this buffer frozen at -20 °C until needed.

T. zostericola cytochrome b_5 was purified differently than P. gouldii cytochrome b_5. T. zostericola erythrocytes

from 36 worms that had been deoxygenated by sealing them in one liter plastic bottles for 24 hours in artificial seawater at 16 °C were purified as described earlier and lysed overnight with 1.7 times their volume of deionized water. The cell debris was removed by centrifugation in a clinical centrifuge, and then at 26,000 g for 2 hours. The cell debris was discarded, and the supernatant dialyzed overnight against 10 mM phosphate buffer pH 7.2 to lower the ionic strength. This solution was passed over a 2 x 10 cm Sephadex A-25 column, and the column washed with 10 mM phosphate buffer pH 7.2 until the eluate contained no red color. The column was eluted with 250 mM NaCl. The fractions exhibiting a Soret absorbance were pooled and dialyzed overnight against several changes of 10 mM phosphate buffer pH 7.2. This solution was loaded onto a 0.5 x 5 cm Sephadex A-25 column, and the column flushed for 24 hours with 1% Triton X-100 in 10 mM phosphate buffer pH 7.2 to remove Hr that had precipitated on the A-25 column under the low salt conditions. The Triton was washed from the column with buffer, and the cytochrome b_5 eluted with 250 mM NaCl.

4. Purification of cytochrome b_5 reductase

a. Solubilization Cytochrome b_5 reductase was extracted from the membrane portions of the erythrocytes which had been removed during the preparation of Hr. To this cell

debris (~ 5 ml from 200 worms) was added 30 times its volume of 10 mM Tris-acetate buffer pH 7.7 with 0.1 mM dithiothreitol and 1 mM EDTA. Two volume percent of Triton X-100 was added, and the mixture stirred overnight in a septa-capped 50 ml plastic centrifuge tubes at 4 °C. At this point, the cytochrome b_5 reductase was solubilized. (The criterion for solubilization was that the reductase activity would remain in the supernatant after centrifugation at 26,000 g for two hours.) The reductase activity was stable in this mixture for at least two months at 4 °C in a septum-capped centrifuge tube.

b. Purification The cell debris was removed by centrifugation in a clinical centrifuge before purification of the cytochrome b_5 reductase.

The cytochrome b_5 reductase solution was cleared of any particulate matter by centrifugation at 26,000 g. All traces of salt were removed from the reductase solution by extensive dialysis against 10 mM Tris-acetate buffer pH 7.7 containing 2% Triton, 0.1 mM DTT, and 1 mM EDTA. With this set of conditions, no reductase activity was lost.

The reductase solution was passed over a 0.5 x 5 cm Sephadex A-25 column equilibrated with 10 mM Tris-acetate buffer pH 7.7 containing 2% Triton, 0.1 mM DTT, and 1 mM EDTA. The Hr did not stick to the column, and was flushed out. The

column was washed with the same buffer, and no $K_3Fe(CN)_6$ reductase activity was eluted. The column was eluted with 100 mM Na_2SO_4 in the same buffer, fractions collected, and their activity checked by the ferricyanide assay. Those fractions that were able to catalyze the ferricyanide reduction by NADH were pooled and stored at 4 °C. These fractions could also reduce cytochrome b_5.

C. Assays

1. Hemerythrin concentration

The metHr concentration was determined by adding solid sodium azide (10 mM) to a metHr solution. After a few minutes, the metazide adduct of Hr ($metHrN_3^-$) was fully formed and ε_{447} = 3700 $M^{-1}cm^{-1}$ was used to determine the Hr concentration (11). If the concentration of Hr to be determined was a lysed cell solution, the cell debris was removed by use of a 0.22 μm Millipore filter. The lysed cell solutions contained a low molecular weight impurity with an absorbance maximum at 340 nm that would pass through an Amicon YM-2 membrane, cutoff ~ 1000. This impurity distorted the spectrum of $metHrN_3^-$, but removal of this impurity did not change the absorbance at 447 nm. All protein concentrations are reported on the basis of monomers.

OxyHr concentration was estimated by its absorbance at

500 nm with an extinction coefficient of 2200 $M^{-1}cm^{-1}$ (11).

In lysed cell solutions, it was assumed that oxyHr was the only species with significant absorbance at 500 nm. MetHr has a much lower absorbance at 500 nm than does oxyHr so this probably introduces only a small amount of error. Addition of solid sodium azide slowly converted oxyHr to $metHrN_3^-$, and then A_{447} was used to estimate the total Hr concentration.

In the reconsititued reduction system, oxyHr concentration was determined by the change in A_{500} on exposure to air.

The concentration of semi-metHr was measured by double integration of EPR spectra and comparison to a $CuSO_4$ standard (63).

2. Cytochrome b_5 concentration

The cytochrome b_5 concentration was estimated spectrophotometrically by the absorbance of the Soret peak. ε_{412} = 115,000 $M^{-1}cm^{-1}$ for the Soret peak of oxidized human cytochrome b_5 (16), and this value was adopted for *P. gouldii* cytochrome b_5.

3. Cytochrome b_5 reductase activity

The detection of cytochrome b_5 reductase activity was achieved by one of two non-specific methods, DCIP or ferricyanide reduction. Activity can also be detected by the

specific reduction of cytochrome b_5.

a. Reduction of DCIP Reductase activity was measured by use of 2,6-dichloroindophenol (DCIP) as a two-electron acceptor. Three milliliters of 0.02 mM DCIP in 50 mM Tris-Cl^- pH 7.5 with 0.1 mM EDTA had 10 µL of 10 mM NADH in 50 mM Tris-acetate pH 8.0 added. A sample of the solution to be tested was added, and the absorbance at 600 nm monitored with time. One unit of activity was defined as the activity that would decrease A_{600} by 0.01/minute (64). This assay was not suitable when Triton was present, because the DCIP was insoluble in the presence of Triton.

b. Reduction of ferricyanide Reductase activity was also measured by use of $K_3Fe(CN)_6$ as a one-electron acceptor. Ten µL of 10 mM NADH in 50 mM Tris-acetate pH 8.0 buffer were added to one milliliter of 0.1 mM $K_3Fe(CN)_6$ in 10 mM phosphate buffer pH 7.2 with 0.1mM EDTA. The solution to be assayed was added, and the decrease in absorbance at 420 nm monitored. One unit of activity was defined as the amount of enzyme that would decrease A_{420} by 0.01/minute (48). Using the difference in extinction coefficients between ferricyanide and ferrocyanide of 1024 $M^{-1}cm^{-1}$ at 420 nm, one unit of activity corresponds to 9.8 nmoles of ferricyanide reduced per minute.

c. Reduction of cytochrome b_5 A specific assay for the presence of cytochrome b_5 reductase was the reduction of cytochrome b_5. One-half milliliter of 1 μM P. gouldii cytochrome b_5 in 10 mM phosphate buffer pH 7.2 was mixed with 10 μL of reductase solution and 5 μL of 10 mM NADH in 50 mM Tris-acetate pH 8.0. Reductase activity was determined by the shift of the Soret peak of cytochrome b_5 from the oxidized (412 nm) to the reduced (422 nm) form. Activity was expressed as k_{obs} for the reduction of cytochrome b_5.

D. Experiments on Intact Erythrocytes

Experiments on intact erythrocytes were carried out by incubating either P. gouldii or T. zostericola erythrocytes purified as described above in either a refrigerator at 4 °C, or in a 10 °C water bath. The erythrocytes incubated at 10 °C were kept oxygenated by passing a continuous flow of water-saturated air over the cells. Tenth milliliter volumes of erythrocytes were removed for EPR samples when needed. The EPR samples were transfered to 4 mm O.D. quartz tubes and frozen in liquid nitrogen. The tubes were then evacuated and flame-sealed. The tubes were stored in a liquid nitrogen dewar until their EPR spectra could be recorded. Semi-metHr concentration was determined as described in Section II-C-1. Erythrocytes isolated from worms as received have Hr in the

oxy form. Worms that had been deoxygenated by overnight incubation in sealed plastic jars at 16 °C have Hr in the deoxy form. These erythrocytes reoxygenated upon exposure to oxygen.

E. Succinylation of Hemerythrin

Succinylation of metHr was carried out according to the procedure of Garbett et al. (65). To 0.1 mM metHr in 100 mM sodium bicarbonate buffer pH 8.3 with 100 mM $NaClO_4$ was added 7 mg of solid succinic anhydride per 5 ml of sample. The pH was kept at 8.5 throughout the reaction by the addition of 1 M NaOH. After reaction, the Hr was concentrated and dialyzed extensively to remove the perchlorate. These conditions are reported to modify seven of the twelve amino groups per Hr subunit and yet maintain the octameric structure.

F. Redox Potential of Cytochrome b_5

The redox potential of cytochrome b_5 was determined by anaerobic titration of a mixture of 1 μM cytochrome b_5 and 2 μM methylene blue. Methylene blue was added from a concentrated stock solution made up by weight. Methylene blue has a reduction potential of 0.011 V versus NHE at pH 7.0 and 23 °C, and undergoes a two-electron redox transformation (25). The visible absorption spectrum of methylene blue is shown

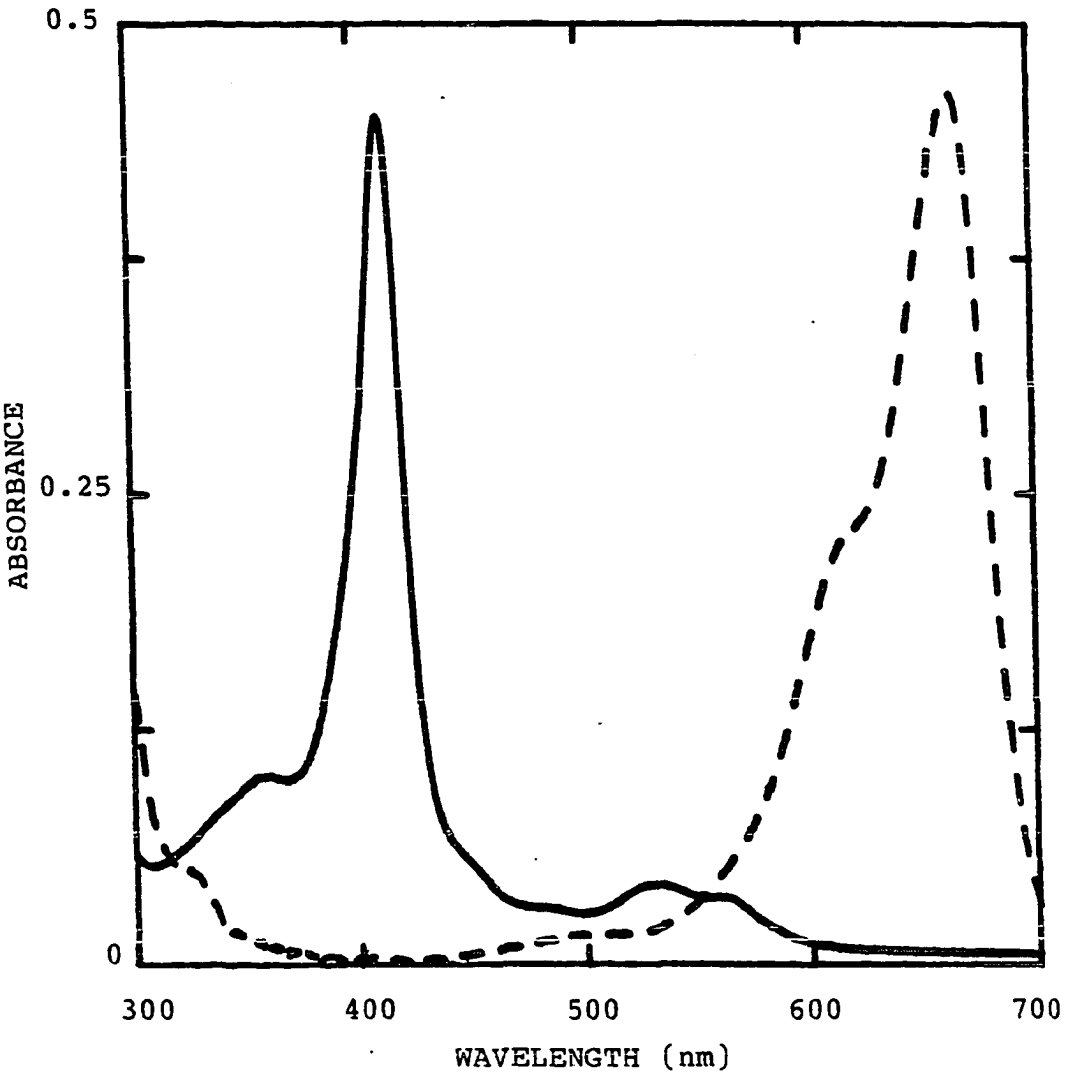
overlaid with the spectrum of oxidized cytochrome b_5 in Figure II-1. The absorbance maximum of methylene blue is 668 nm with no absorbance in the Soret region of cytochrome b_5. The cytochrome b_5 has no absorbance at 668 nm. The absorbance of methylene blue at 668 nm may therefore be used to determine the fraction of oxidized and reduced methylene blue, and the potential of the solution can be determined by the Nernst equation:

$$E = E^{\circ\prime} + (RT/nF)\ln(ox/red)$$

where $E^{\circ\prime}$ is the standard reduction potential, T is the temperature, R is the gas constant, n is the number of electrons, F is the Faraday, ox is the concentration of the oxidized species, and red is the concentration of the reduced species.

The Soret absorbance can be used to calculate the fraction of oxidized and reduced cytochrome b_5. The potential of the solution, E, is changed by adding reductant to the cell, or allowing the solution to autooxidize. The quantity $\ln(ox/red)b_5$ may then be plotted versus the solution potential. At $\ln(ox/red)_{b_5}=0$, the solution potential is at the midpoint potential of cytochrome b_5. The value of n may be calculated from the slope of the line.

The redox titration was carried out in an anaerobic



(——) Oxidized P. gouldii cytochrome b_5.
(---) Methylene blue.

Figure II-1. Visible absorption spectra of oxidized P. gouldii cytochrome b_5 and methylene blue

cuvette under N_2 with solutions of cytochrome b_5 and methylene blue in 100 mM phosphate buffer pH 7.0 with 500 mM NaCl and 1 mM EDTA added (25). Reduction of the solution was accomplished by addition of 0.1 μM riboflavin and successive irradiation of approximatly one second duration with light from a 500 W projector bulb that was filtered through water to remove the IR radiation. Under these conditions, electrons are transferred from EDTA to cytochrome b_5 and methylene blue, with riboflavin functioning as the catalyst (30). Points in the opposite direction were obtained by allowing the solution to autooxidize.

G. Determination of the Molecular Weight of Cytochrome b_5

The molecular weight of cytochrome b_5 was determined by HPLC on a 1 x 30 cm Altex Spherogel-TSK 300-PW column using commercial samples of C. pasteurianum ferredoxin (6000), horse heart cytochrome c (13,000), sperm whale myoglobin (17,800) and deoxyribonuclease I (31,000) as molecular weight markers. The buffer used in these experiments was 10 mM phosphate buffer pH 7.2 with 6 M guanidine hydrochloride. The flow rate was 0.7 ml/min. A 20 μL sample loop was used for sample injection. Retention time was determined by the length of time between sample injection and post column detection at 280 nm. Log(mw) was then plotted versus the retention time. This

standard curve was used to calculate the molecular weight of P. gouldii cytochrome b_5.

H. Oxidation of Cytochrome b_5 by Methemerythrin

Since the reduced cytochrome b_5 is oxidized by oxygen, all kinetics were done under anaerobic conditions. The metHr and the cytochrome b_5 were degassed by alternate cycles of dynamic vacuum and nitrogen gas. Buffer solutions were degassed by bubbling nitrogen through the solution for at least 30 minutes, followed by a short cycle of dynamic vacuum to remove most of the dissolved nitrogen and prevent bubble formation during kinetic runs. Nitrogen was scrubbed free of oxygen by chromous scrubbing towers. All syringes and cuvettes were degassed by repeated cycles of vacuum and nitrogen. The stock cytochrome b_5 solution was diluted to the proper concentration with degassed buffer. The final cytochrome b_5 concentration was ~ 1 μM. The cytochrome b_5 solution, (0.75 ml), with 1 mM EDTA was reduced by the addition of 0.1 μM riboflavin, followed by irradiation with light from a 500 W projector bulb for approximatly one second in an anaerobic semi-micro spectrophotometer cell. The light beam was passed through water to filter out the IR radiation. Alternatively, the cytochrome b_5 was reduced by titration with sodium dithionite. MetHr was added by gas-tight syringe, and

the cuvette inverted several times to mix the solutions. All reactions were carried out under pseudo first-order conditions with an excess of metHr. The reaction was monitored by the decrease in absorbance at 422 nm with time, corresponding to the oxidation of cytochrome b_5.

I. Reductions of Cytochrome b_5 by Cytochrome b_5 Reductase

P. gouldii cytochrome b_5 reductase reductions of P. gouldii cytochrome b_5 were carried out in an anerobic cuvette with NADH acting as the electron source. One-half milliliter of 1 μM anaerobic P. gouldii cytochrome b_5 was mixed with the reductase solution (~ 20 μL) added via a gas-tight syringe through a septum. Ten millimolar NADH (~ 10 μL) was then added through the septum via a gas-tight syringe and the reduction of cytochrome b_5 followed by the change in A_{422}.

J. Reduction of Methemerythrin by the Reductase System

MetHr was reduced to deoxyHr by a reconstituted system consisting of NADH, cytochrome b_5 reductase, and cytochrome b_5. The system consisted of 0.1 to 5 μM cytochrome b_5, 1 to 6 U/ml cytochrome b_5 reductase by the $K_3Fe(CN)_6$ reductase assay, 0.10 mM NADH, and 0.10 mM metHr. Ionic strength of the solution was adjusted by the addition of Na_2SO_4. The pH of

the reaction was controlled by dialyzing both the metHr and cytochrome b_5 into the same buffer. Cytochrome b_5 was reduced by the anaerobic additions of NADH and cytochrome b_5 reductase. MetHr was then added and the reaction followed by UV-visible spectroscopy between 450 and 300 nm. The kinetics could then be measured at any wavelength. The reduction of metHr could also be measured by freezing EPR samples at various times. Semi-metHr, an intermediate, is a spin 1/2 system and can be detected by liquid helium EPR. EPR samples were prepared as described in Section II-C.

K. Concentration of Reduced Glutathione in *P. gouldii* Erythrocytes

The concentration of reduced glutathione in the erythrocytes of *P. gouldii* was determined by the HPLC method of Nishiyama and Kuninori (66). The erythrocytes were isolated from either oxygenated or deoxygenated worms, and then lysed by the addition of two times their volume of deionized water. The cell debris was removed by centrifugation in a clinical centrifuge and subsequent filteration through a 0.22 µm filter. The proteins were denatured in a 100 °C water bath for five minutes with a stream of nitrogen passing through the solution to remove the oxygen. This mixture was then filtered through a 0.22 µm

filter. Twenty microliters of this sample were injected onto a C-18 reverse phase column. The mobile phase of the column was 33 mM phosphate buffer pH 2.2, and the flow rate was 1 ml/minute. The effluent from the column was mixed with a 1.5 mM solution of 6,6'-dithiodinicotinic acid in 200 mM phosphate buffer, pH 7.8 at a flow of 1 ml/minute. The 6,6'-dithiodinicotinic acid reacts with the thiols to form a colored complex which is detected by absorbance at 360 nm.

Standard elution times were determined with the pure compounds by monitoring at 204 nm (a wavelength at which they absorb) without 6,6'-dithiodinicotinic acid. The concentration of the thiols was determined by a standard curve of A_{360} peak height versus the thiol concentration. The concentration of GSH in the erythrocytes was calculated from the dilution of the packed erythrocytes upon lysing. No correction was made for the volume occuppied by the cell membranes and as such the calculated GSH concentration represents a lower limit.

L. Reduction of Methemerythrin with Glutathione

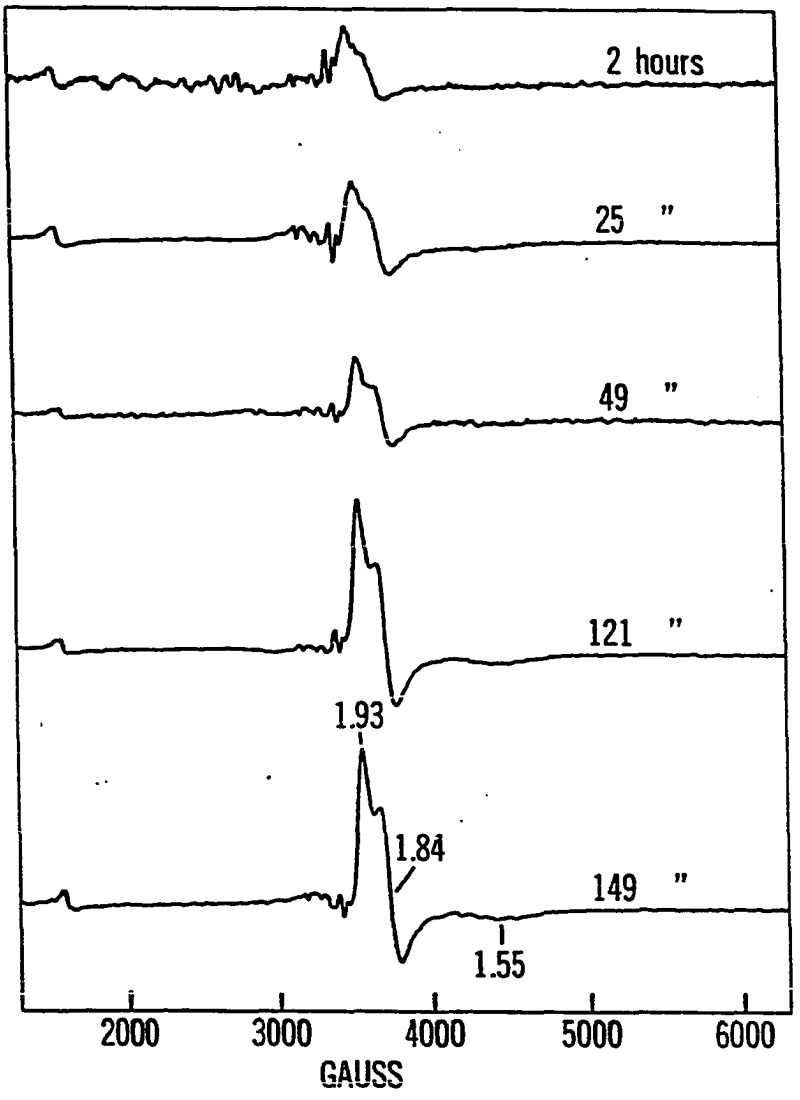
The reduction of metHr was characterized in 50 mM Tris-$SO_4^=$ buffer with 150 mM Na_2SO_4. A stock GSH solution was prepared by measuring the correct amount of solid GSH needed to make a 100 mM solution. One hundred millimolar phosphate

buffer pH 7.2 was then added, and the pH adjusted to the pH of the trial run. Stock metHr solutions were made in buffer, and their concentrations determined by conversion to the azide adduct. The protein and GSH solutions were thoroughly degassed by alternating cycles of vacuum and nitrogen. The GSH was added to the metHr, and the samples incubated at 25 °C. The progress of the reaction was followed by UV-visible spectrometry and EPR.

III. RESULTS

A. EPR of Intact Erythrocytes

EPR was used in an effort to probe the redox states of Hr in the erythrocytes of P. gouldii. Purified erythrocytes (as described in the Experimental section) were incubated aerobically at 4 °C and sampled at various times. The samples were transferred to EPR tubes and frozen in liquid nitrogen until EPR spectra could be recorded. When worms were received in a well oxygenated state, their erythrocytes displayed no semi-metHr EPR signal, even after incubation for 144 hours. Erythrocytes that did display $(\text{semi-met})_R\text{Hr}$ EPR signals were from worms that had been deoxygenated either by shipment in large batches that depleted their oxygen supply, or by overnight incubation in sealed plastic jars at 16 °C. The EPR time course of the aerobic incubation of erythrocytes at 4 °C from worms deoxygenated in the latter fashion is shown in Figure III-1. The results of double integration of the EPR signals are tabulated in Table III-1. When erythrocytes from worms that had been deoxygenated were incubated aerobically at 10 °C, the samples also display an EPR signal with the same g-values as seen in the erythrocytes incubated at 4 °C. These data are also included in Table III-1. The g-values seen in the erythrocytes are 1.935, 1.840, and 1.55. These g-values



Erythrocytes from deoxygenated worms incubated aerobically at 4 °C.
EPR conditions: temperature 4 K; frequency 9.57 GHz; power 0.2 mW; gain 1.6 x 10^5; modulation 16 G at 100 kHz; time constant 0.15 sec.

Figure III-1. EPR spectra of intact P. gouldii erythrocytes

Table III-1. Development of semi-metHr EPR signal in P. gouldii erythrocytes[a]

Time (hr)	[SM] (mM)	$[Hr]_{total}$ (mM)	% SM
Incubation at 4 °C.			
2	----[b]	11.24	----[b]
25	0.044	11.65	0.38
49	0.043	10.22	0.42
121	0.123	10.84	1.13
149	0.143	9.43	1.52
Incubation at 10 °C.			
2	0.0068	14.3	0.047
24	0.037	14.9	0.253
51	0.168	----[b]	----[b]
118	0.203	11.5	1.77
140	0.197	10.8	1.83

[a]Aerobic incubaton of P. gouldii erythrocytes. Erythrocytes from worms that had been previously incubated anerobically overnight.

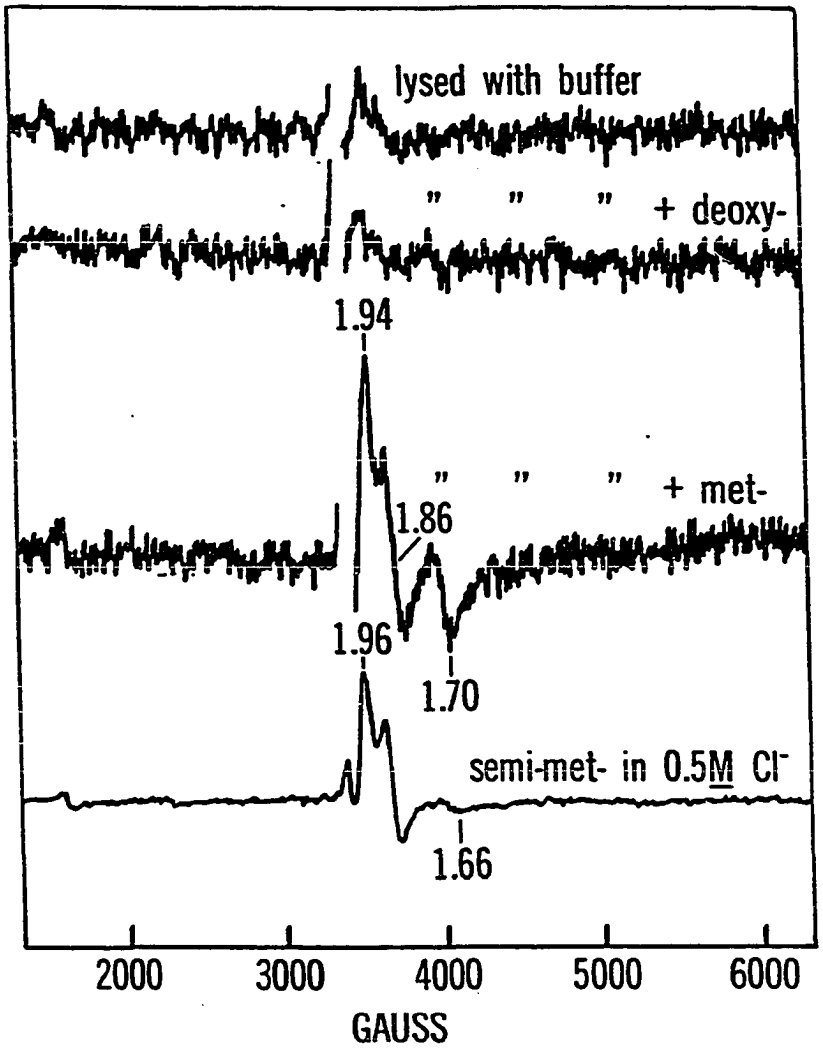
[b]not determined.

are not the same as the g-values seen for the $(semi\text{-}met)_RHr$, or $(semi\text{-}met)_RHrCl^-$ formed in either 500 mM NaCl or artificial seawater by the addition of dithionite. The g-values for $(semi\text{-}met)_RHr$ are 1.94, 1.86, and 1.66. The g-values for $(semi\text{-}met)_RHrCl^-$ are 1.96, 1.86, and 1.66. The differences in the EPR g-values indicate that the semi-metHr seen in the erythrocytes is similar but not identical to $(semi\text{-}met)_RHr$ or $(semi\text{-}met)_RHrCl^-$.

The build-up of semi-metHr in the cells shows that a one-electron redox process does take place inside the cell. The shape of the EPR signal shows that the semi-metHr within the erythrocytes is similar to that of $(semi\text{-}met)_RHr$ (Figure I-6), and therefore is most likely a product of one-electron reduction of metHr.

It is possible that some component in the erythrocyte could oxidize deoxyHr to form $(semi\text{-}met)_OHr$. P. gouldii $(semi\text{-}met)_OHr$ is unstable and spontaneously converts to $(semi\text{-}met)_RHr$. This scenario could explain the formation of $(semi\text{-}met)_R$-type EPR spectrum in the erythrocytes by oxidation of deoxyHr rather than by reduction of metHr.

The results shown in Figure III-2 provide further evidence that the semi-metHr EPR signal seen in the erythrocytes is due to reduction of metHr rather than conversion from $(semi\text{-}met)_OHr$. Twenty microliters of purified aerobic erythrocytes in artificial seawater were added to 100



Oxygenated erythrocytes were lysed in ~ 1.5 mM deoxy- or metHr solutions or in buffer alone (50 mM Tris-Cl^-, pH 8.0) and incubated for 24 hours at 10 °C.

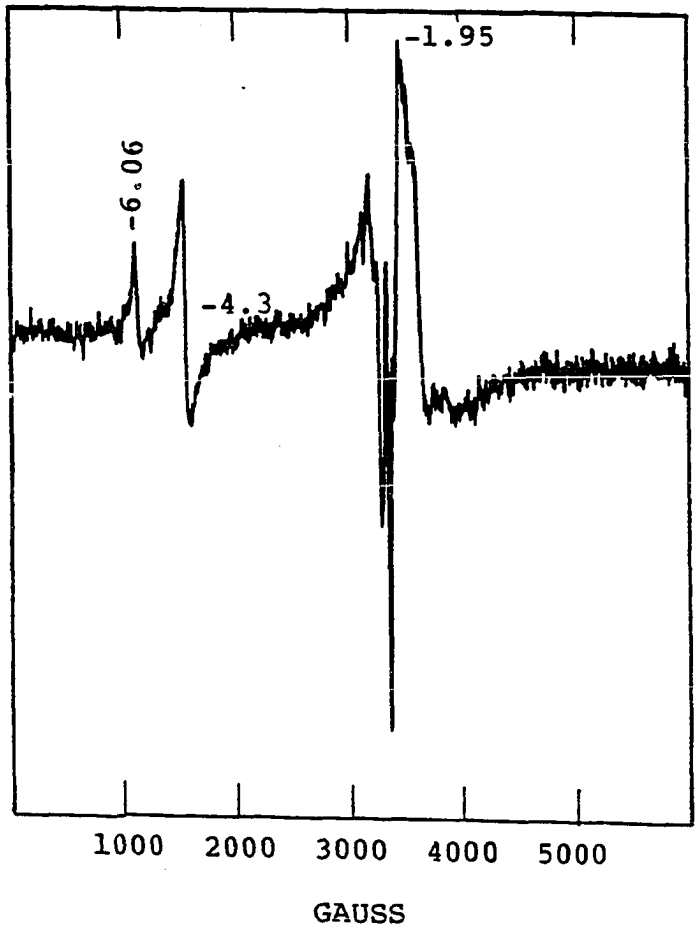
EPR conditions: temperature 4 K; frequency 9.57 GHz; power 0.2 mW; gain 8.0 x 10^6; molulation 16 G at 100 kHz; time constant 0.1 sec. Lowest spectrum is $(semi\text{-}met)_R Hr$ in 50 mM Tris-Cl pH 8.0 with 500 mM NaCl.

Figure III-2. EPR of lysed erythrocytes

μL of 1.5 mM anaerobic metHr, deoxyHr, or buffer alone (50 mM Tris-Cl^- pH 8.0). The lower ionic strength of these latter three solutions causes the erythrocytes to lyse. The samples were incubated for 24 hours at 10 °C and then frozen for EPR. A semi-metHr EPR signal is seen in only the solution with added metHr. The g-values are 1.94, 1.86, and 1.7. This $(semi\text{-}met)_R Hr$ EPR signal is presumably due to the reduction of the metHr. The lack of semi-metHr EPR signals from erythrocytes lysed with buffer shows that the EPR signals seen when the erythrocytes are lysed in metHr are not solely due to components from the erythrocytes. The lack of a $(semi\text{-}met)_R Hr$ or a $(semi\text{-}met)_O Hr$ EPR signal when the erythrocytes are lysed with deoxyHr shows that the semi-metHr signal seen in the erythrocytes is probably not coming from the oxidation of deoxyHr.

The EPR spectrum of $(semi\text{-}met)_R Hr$ produced by dithionite reduction of metHr in the presence of 500 mM NaCl is also shown in Figure III-2 for the purpose of comparing g-values.

Erythrocytes from the sipunculid T. zostericola that had been deoxygenated by overnight incubation in sealed plastic jars at 16 °C were purified and incubated at 4 °C for 48 hours at which time an EPR sample was frozen. The EPR spectrum of this sample is shown in Figure III-3. A $(semi\text{-}met)_R Hr$ signal may be seen from this sample at g = 1.95, showing that in T. zostericola, as well as in P. gouldii, a one electron



T. zostericola erythrocytes deoxygenated overnight and subsequently incubated aerobically at 4 °C for 48 hours. Positive signal near 3000 G is due to the EPR cavity. EPR conditions: temperature 4 K; frequency 9.42 GHz; power 201 μW; gain 2.5 x 10^5; modulation 16 G at 100 kHz; time constant 50 ms.

Figure III-3. EPR of T. zostericola erythrocytes

reduction pathway for metHr exists. T. zostericola $(semi\text{-}met)_O Hr$ is unlikely to convert to $(semi\text{-}met)_R Hr$ (26), strongly suggesting that the EPR spectrum of T. zostericola erythrocytes is due to the reduction of metHr rather than oxidation of deoxyHr.

These EPR results strongly suggest that there is indeed a one-electron pathway for the reduction of metHr in sipunculan erythrocytes. The two-step reduction of metHr to deoxyHr in the erythrocytes implied by these results is the same behavior seen with inorganic reducing agents, in which the reduction of metHr proceeds through $(semi\text{-}met)_R Hr$.

B. Reducing Components in the Membrane

After providing evidence for a one-electron reduction pathway for metHr in the erythrocytes of P. gouldii, the next step was to determine the location of reducing components. These components could conceivably be located in the cytoplasm, a membrane, or distributed between both.

The location of one of the reducing components was determined to be in the membrane fraction in the following manner. All steps were carried out aerobically. P. gouldii erythrocytes were lysed by addition of one volume of deionized water and the cell membranes were separated by centrifugation for one hour at 26,000 g. One-half of the supernatant was

removed and the pellet of cell debris was resuspended in the remaining half of the supernatant. The samples were incubated for 24 hours at 10 °C with the reasoning that the oxyHr present in the supernatant would slowly autooxidize to give metHr. The metHr would be reduced back to deoxyHr if all the components necessary for reduction were present. At the end of the incubation period, the samples with no cell debris consisted of 80% oxyHr. The total Hr concentration was determined by A_{447} after conversion to the azide derivative. The sample with resuspended cell debris contained 95% oxyHr. This result suggests that at least one part of the system necessary for reduction of metHr is membrane bound. Addition of 10 mM NADH to the solutions had no effect on the amount of oxyHr present. Table III-2 summarizes these results.

No semi-metHr EPR signals were observed from any of the samples of Table III-2. The lack of semi-metHr signals does not mean a one-electron reduction of metHr is not involved in keeping the concentration of metHr low, because semi-metHr is rapidly disproportionated by oxygen (17).

The DCIP reductase activity assay was used to detect diaphorase activity in the erythrocytes. Fresh oxygenated erythrocytes lysed with one volume of deionized water and checked for activity without removing the cell debris have a reductase activity of 190 U/ml. When this solution is centrifuged at 26,000 g for one hour, 90% of the diaphorase

Table III-2. OxyHr concentration in supernatants from lysed *P. gouldii* erythrocytes[a]

	no cell debris		cell debris	
$[NADH]_{added}$ (mM)	0	10	0	10
[oxyHr] (mM)	4.53	4.29	5.45	5.63
$[Hr]_{Total}$ (mM)	5.68	5.34	5.63	5.98
% oxyHr	79.7	80.3	96.8	94.1

[a]Cells lysed with one volume of deionized water. Cell debris removed by centrifugation and resuspended in one-half of the supernatant. Samples incubated for 24 hours at 10 °C.

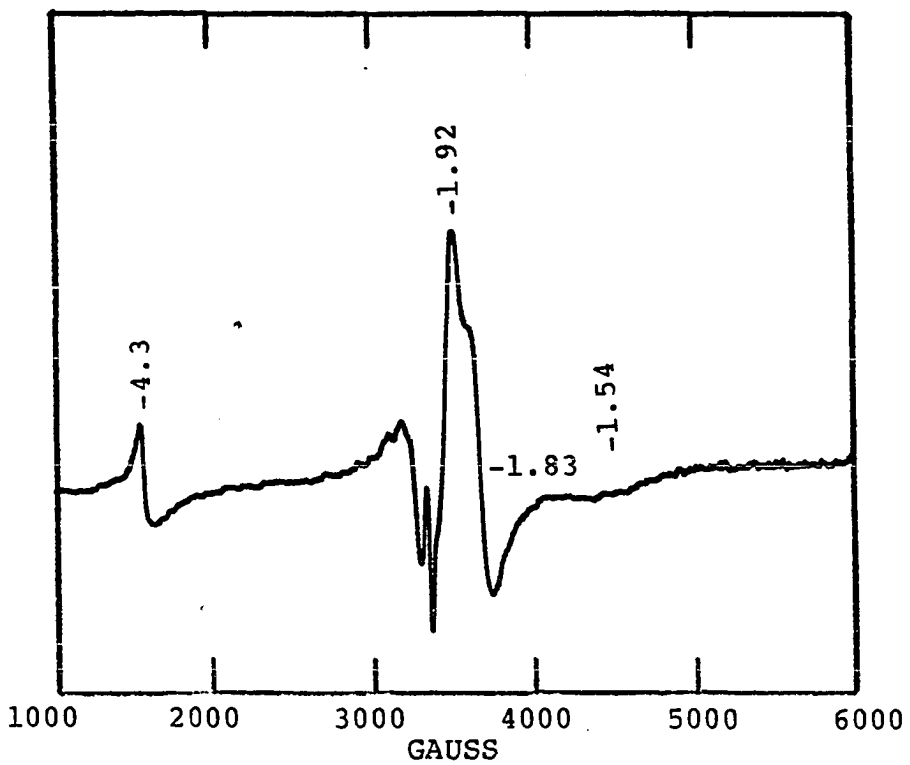
activity is retained in the pellet. This result provides further evidence that a component of the reduction system is membrane bound and that NADH is the source of electrons. When NADPH is used in place of NADH, no reduction of DCIP was detected.

Because of the detection of diaphorase activity in the membrane fractions from the erythrocytes of P. gouldii, the membrane fractions were frozen for EPR. This EPR spectrum is shown in Figure III-4. A semi-metHr signal is seen with the same g-values as those seen in the intact erythrocytes. This observation is consistent with the fact that some component in the membrane is involved in metHr reduction. The concentration afforded by placing only membranes in the EPR tube allows the semi-metHr signal to be seen at a much earlier time.

C. Isolation of Cytochrome b_5 from P. gouldii Erythrocytes

Since Hr is a soluble protein, it is reasonable to assume that some component of the metHr reduction system is soluble. The result of the search for this component was the development of an isolation procedure for P. gouldii cytochrome b_5.

The major protein in the erythrocytes of P. gouldii is Hr, at a concentration of ~ 10 mM. Cytochrome b_5 is present



P. gouldii cell debris frozen for EPR after cell lysis. Cell debris was collected immediately after lysis by centrifugation at 26,000 g for 1 hour and a 0.1 ml sample transfered to an EPR tube by suspension in a small amount of 10 mM phosphate buffer pH 7.2. EPR conditions: temperature 4 K; frequency 9.42 GHz; power 201 μW; gain 2.5 x 10^5; modulation 16 G at 100 kHz; time constant 200 ms.

Figure III-4. EPR of P. gouldii cell debris

in much smaller amounts. Batch anion exchange is used to separate the cytochrome b_5 from the Hr because of their widely different isoelectric points. Hemerythrin has an isoelectric point of 7.8 and cytochrome b_5 has an isoelectric point below 4. The erythrocytes are lysed with 1.7 times their volume of deionized water, and the cell debris is removed by centrifugation. Assuming 540 mM Cl^- in the erythrocytes, the concentration of Cl^- in the lysed cell solution is 200 mM. Lysing the erythrocytes with a larger volume of water such that the Cl^- concentration drops below 200 mM results in the loss of cytochrome b_5 reductase activity in the cell debris. Eight-tenths the volume of the erythrocytes is added to the supernatant to lower the ionic strength of the cell lysate solution to 150 mM Cl^-. The pH of the centrifuged cell lysate solution is approximately 7. Six hundred grams of swollen bulk DEAE cellulose per 500 ml of lysed cell supernatant is added to the solution and the slurry is stirred for 2 hours. The anion exchange cellulose used in the batch extraction was previously washed thoroughly in phosphate buffer pH 7.2, so its addition to the cell lysate would not drastically affect the pH of the lysed cell solution. At this pH, the cytochrome b_5 is negatively charged and absorbs on the anion exchanger, while the positively charged Hr does not absorb. The relatively high ionic strength of this solution, 150 mM Cl^-, prevents proteins with only a slight negative charge at this

pH from absorbing on the anion exchanger. The chloride also increases the solubility of Hr.

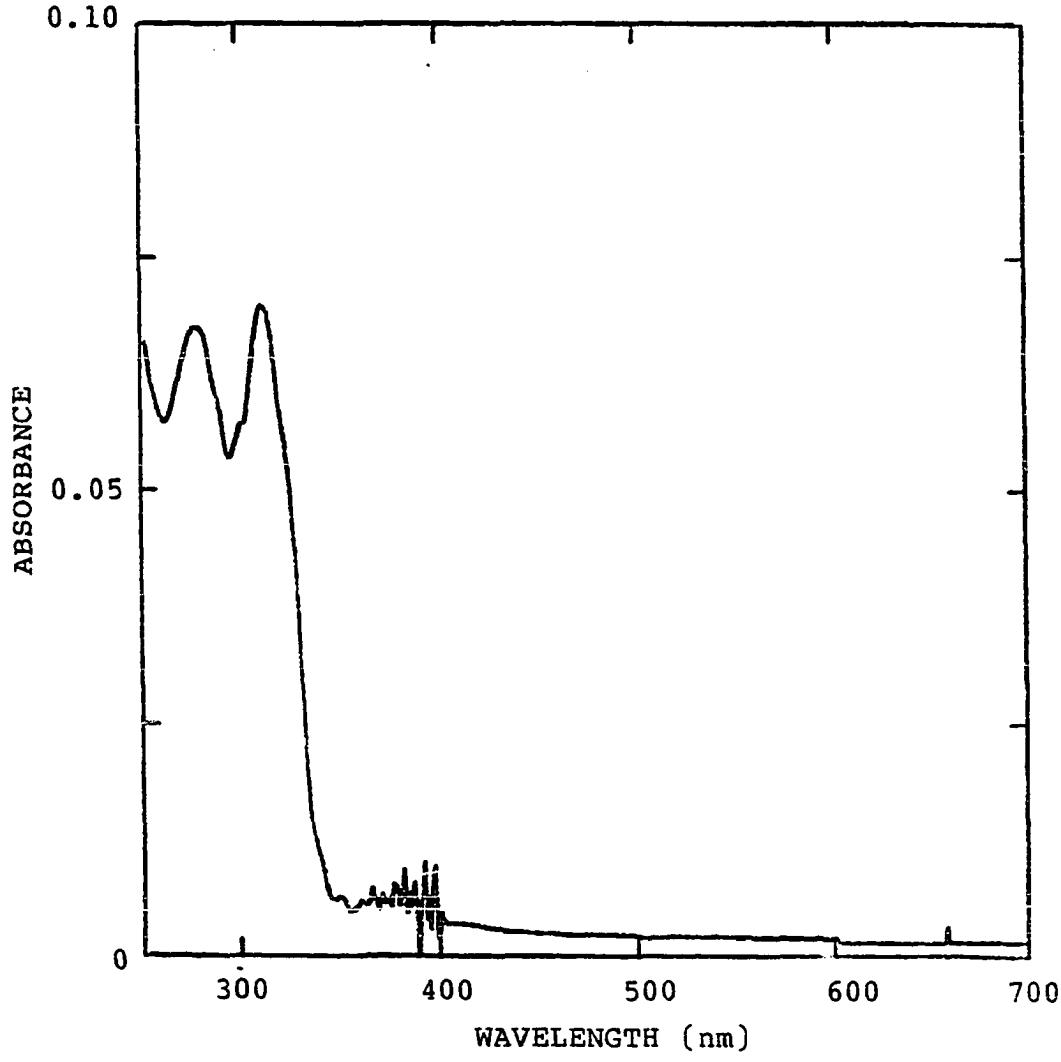
The batch ion-exchange is the most critical step of the cytochrome b_5 isolation. There is approximately 33,000 times more hemerythrin than soluble cytochrome b_5 in the P. gouldii erythrocyte, and therefore the efficient removal of Hr is very important. Both Whatmann DE-52 and bulk unswollen DEAE cellulose work well for the batch anion extraction, and neither can be recycled. The bulk unswollen DEAE cellulose is cheaper, so its use has been adopted. A column was found not to be a viable alternative to the batch anion exchange extraction. The column would quickly plug with small fat particles that had not been removed by centrifugation of the cell lysate, thereby greatly reducing the flow rate.

Most of the Hr can be removed from the anion exchange resin by suction filtration and subsequent washing of the cake with 10 mM phosphate buffer pH 7.2. The cytochrome b_5 is then removed by stirring the anion exchange cellulose with 500 mM NaCl in 10 mM phosphate buffer pH 7.2 for two hours. The wash containing the cytochrome b_5 is removed by filtration, and the cake is then washed on the filter with 10 mM phosphate buffer pH 7.2 containing 500 mM NaCl to remove the remaining cytochrome b_5. The 500 mM NaCl wash contains the cytochrome b_5 and is dialyzed against 10 mM phosphate, pH 7.2, 0.5 mM EDTA to remove the NaCl. As the ionic strength is decreased,

a white precipitate forms. This precipitate is removed by a high-speed centifugation.. After the batch anion exchange step, the Soret peak of the cytochrome b_5 can be seen emerging from the background of Hr and other contaminants in the absorption spectrum of the eluate.

The next step consists of loading the dialyzed cytochrome b_5 solution on a Sephadex A-25 anion exchange column equilibrated in 10 mM phosphate buffer pH 7.2 with 0.5 mM EDTA. Traces of Hr and other contaminants elute through the column while the cytochrome b_5 sticks to the top of the column. The column is then washed with 10 mM phosphate buffer pH 6.0 with 0.5 mM EDTA and 100 mM NaCl until no more A_{280} is detected in the wash. Some fractions elute from this column with an absorbance maximum at 312 nm. The absorption spectrum of such a fraction is shown in Figure III-5. This species has not been identified. The cytochrome b_5 is then eluted with the same buffer containing 250 mM NaCl. The fractions having a Soret absorption are collected and concentrated with an Amicon PM-10 ultrafiltration membrane.

DEAE cellulose is not a good substitute for the Sephadex A-25 in this step. The volume of solution to be pumped over the column is large, approximately one liter from a batch of 200 worms. Flow rates of up to 200 ml/min can be sustained in a 1 cm x 10 cm column of A-25. These conditions allow loading of the column in a reasonable time, and concentrate the



Fraction eluting from a Sephadex A-25 column with 10 mM phosphate buffer pH 6.0 with 0.5 mM EDTA and 100 mM NaCl.

Figure III-5. UV-visible absorption spectrum of A_{312} species

cytochrome b_5 on the relatively small column. The same size DEAE cellulose column will not withstand the high flow rate. Hemerythrin would also adsorb to the cellulose and then elute at 250 mM NaCl. This behavior of Hr prevents its removal from cytochrome b_5 by the DEAE cellulose.

The concentrated cytochrome b_5 solution is finally chromatographed on a Sephadex G-50 column in 10 mM phosphate pH 7.2 with 500 mM NaCl and 0.5 mM EDTA. The cytochrome b_5-containing fractions with $A_{412}/A_{280} > 4.0$ are pooled for use. The other cytochrome b_5-containing fractions are pooled with cytochrome b_5 that is to be recycled. Cytochrome b_5 that has $A_{412}/A_{280} > 4.0$ elutes as one peak when chromatographed on an HPLC molecular weight column and monitored at 280 nm or 412 nm. Table III-3 summarizes the cytochrome b_5 purification.

P. gouldii cytochrome b_5 that has been used in reactions with metHr, cytochrome b_5 reductase, and NADH can be repurified to the same quality as fresh cytochrome b_5. The used cytochrome b_5 is stored frozen in the reaction mixture at -20 °C. When the solution is thawed for repurification, it is treated as if it has just been batch eluted from the DEAE cellulose.

Five-tenths mM EDTA is required throughout the preparation to remove trace metal ions which otherwise showed up as sharp EPR signals around g=2.

The total yield of cytochrome b_5 is only 25 nmoles (0.35

Table III-3. Summary of P. gouldii cytochrome b_5 isolation and purification[a]

step	volume (ml)	$[b_5]$ (μM)
washed packed erythrocytes	100	0.3 (calc)
lysed erythrocytes	270	---
solution for batch extraction	350	---
after extraction	800	---
After Sephadex A-25 column	25	1.2
After Sephadex G-50 column	5	5.0

[a] 200 P. gouldii worms, final yield: 25 nmoles or 0.35 mg of cytochrome b_5.

mg) from 200 worms. The present cost for P. gouldii worms is $1.60 each or $1000 worms/mg cytochrome b_5.

It is not possible to directly determine the concentration of cytochrome b_5 in the erythrocytes or immediately after lysis of the erythrocytes due to the large amount of Hr present. An estimate of concentration of cytochrome b_5 was made by assuming 100% yield in the purification of cytochrome b_5. This estimate is effectively a lower limit of concentration of cytochrome b_5. This lower limit is 0.3 μM, an amount comparable to the 0.22 μM reported to be present in the human erythrocyte (53).

Deoxygenation of the worms prior to isolating cytochrome b_5 increases the yield of cytochrome b_5 by a factor of about two. This practice was not commonly employed since the quality of Hr isolated from deoxygenated erythrocytes was lower.

Electron microsopy has been carried out on the coelomic fluid of P. gouldii (67). Three types of cells were detected: prohemocytes (presumably immature erythrocytes), erythrocytes, and leucocytes. No indication of the relative amounts of these different cells was given in that work. A reasonable estimate of the fraction of prohemocytes in the mature worm is < 10%. The packed erythrocytes most likely contain all three types of cells.

An important question is: from which type of cell is the

cytochrome b_5 isolated? No reports have been made of cytochrome b_5 from leucocytes, so it is unlikely that the P. gouldii cytochrome b_5 is isolated from leucocytes. If the cytochrome b_5 is isolated only from the prohemocytes, the concentration of cytochrome b_5 would have to be > 3 μM in these prohemocytes. This concentration would be an order of magnitude higher than in other systems with a soluble cytochrome b_5 making this possibility unlikely. Also, by analogy with Hb-containing erythrocytes, prohemocytes would not contain soluble cytochrome b_5 but rather a membrane bound cytochrome b_5. The procedure used to isolate cytochrome b_5 does not isolate a membrane bound protein. This line of reasoning suggests that the majority of P. gouldii cytochrome b_5 is isolated from mature erythrocytes.

D. Properties of P. gouldii Cytochrome b_5

1. Molecular weight

The molecular weight of cytochrome b_5 was determined by comparison to C. pasterianum ferredoxin (6K), horse heart cytochrome c (13K), sperm whale myoglobin (17.8K), and deoxyribonuclease I (31K). These commercial proteins were chromatographed under the same conditions as P. gouldii cytochrome b_5 on an Altex Spherogel-TSK-300-PW size exclusion column with a mobile phase of 10 mM phosphate buffer pH 7.2

with 6 M guanidine hydrochloride at a flow rate of 0.7 ml/min. Their retention times were measured and the data were fitted to the function:

$$\text{retention time} = a \log (mw) + b$$

where retention time is the time needed to elute the peaks, mw is the molecular weight, and a and b are experimentally determined constants. The experimental data is given in Table III-4. The plot of the experimental data is shown in Figure III-6. The best fit equation was determined by linear least squares to be:

$$\text{retention time} = -2.5274 \log(mw) + 20.888$$

The molecular weight of the P. gouldii cytochrome b_5 was determined to be 14,000 ± 500 D. The molecular weight is within experimental error of the molecular weight determined for human erythrocyte cytochrome b_5 (13,700) (25).

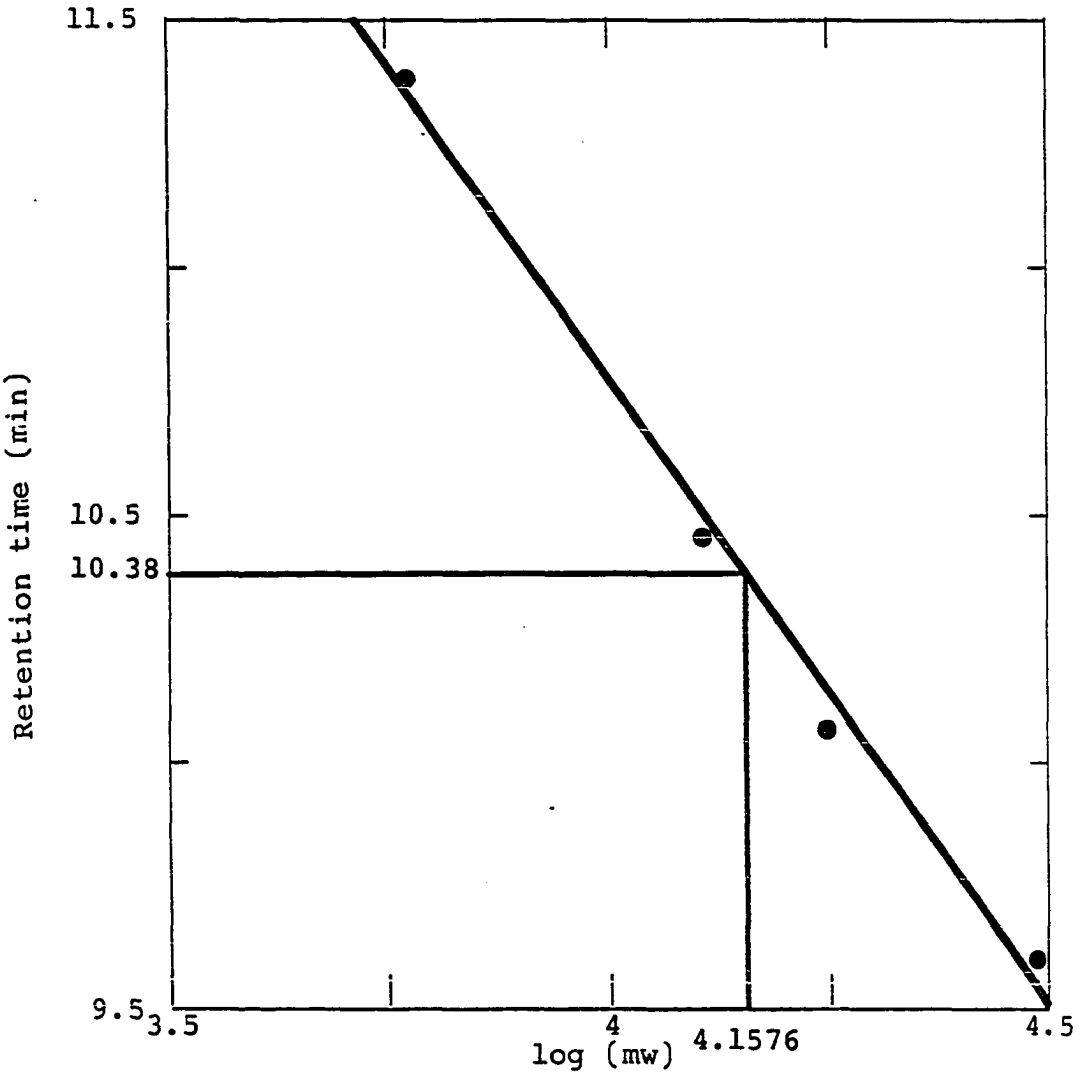
2. Isoelectric point

The isoelectric point of cytochrome b_5 was determined to be between 3.7 and 3.9 by isoelectric focusing. This would give the protein a net negative charge at physiological pH. The determined isoelectric point of P. gouldii cytochrome b_5

Table III-4. Molecular weight calibration for HPLC of *P. gouldii* cytochrome b_5[a]

Protein	mw	log(mw)	retention (min)
Standards-			
C. p. ferredoxin	6,000	3.7782	11.38
cytochrome c	13,000	4.1139	10.46
myoglobin	17,800	4.2504	10.07
deoxyribonuclease I	31,000	4.4914	9.60
Unknown-			
P. g. cytochrome b_5	13,800	4.1576	10.38

[a]10 mM phosphate buffer pH 7.2, 6 M guanidine hydrochloride, 0.7 ml/min, Altex Spherogel-TSK 300-PW molecular weight column.



Standards: C. p. ferredoxin (6K), cytochrome c (13,000K), myoglobin (17.8K), and deoxyribonuclease I (31K). 6 M guanidine hydrochloride on an Altex Spherogel-TSK 300-PW column. Experimental values for P. gouldii cytochrome b_5 are shown by the vertical and horizontal lines.

Figure III-6. Molecular weight calibration for P. gouldii cytochrome b_5

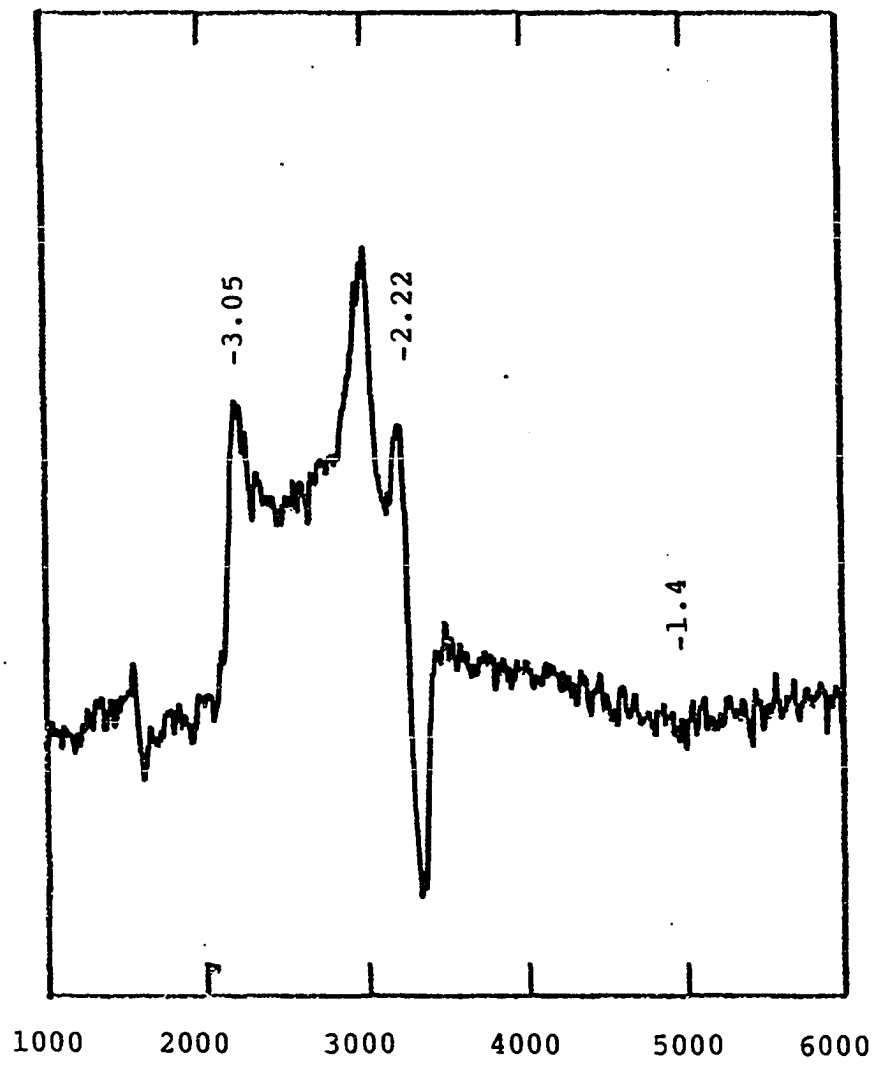
is slightly lower than the value of 4.3 reported for human erythrocyte cytochrome b_5 (25). Several minor bands were seen in the isoelectric focusing experiment. These bands could by due to isozymes of the cytochrome b_5 or due to apo-cytochrome b_5 where the heme is lost during isoelectic focusing as is the case for human erythrocyte cytochrome b_5 (53).

3. EPR spectrum

The EPR spectrum of the P. gouldii cytochrome b_5 is shown in Figure III-7. The EPR shows a rhombic signal with g-values of 3.05, 2.22, and 1.4. These g-values are very close to those seen for both human erythrocyte cytochrome b_5 and microsomal cytochrome b_5. The g-values reported for human erythrocyte cytochrome b_5 are 3.03, 2.21, and 1.39 (44). The EPR spectrum is typical of a low-spin heme Fe(III) system, indicating strong axial bis-imidazole ligation (68).

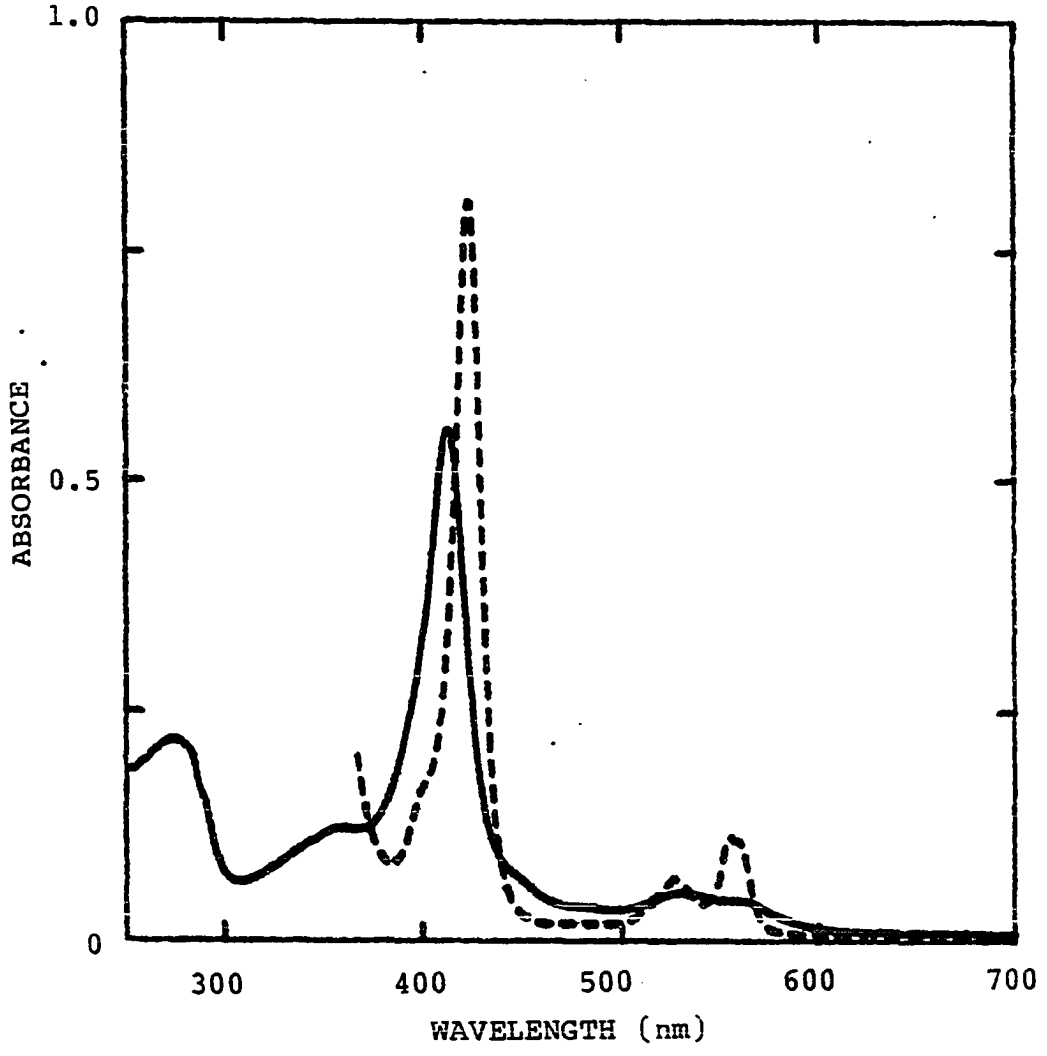
4. UV-Visible spectra

The UV-visible spectra of cytochrome b_5 from the erythrocytes of P. gouldii are shown in Figure III-8. The oxidized protein exhibits a Soret band at 412 nm. In the dithionite reduced form the Soret band maxima is at 422 nm and the α-band has a maxima at 555 nm, the β-band at 526 nm. Human cytochrome b_5 has a Soret maxima at 412 nm in the oxidized form and 423 nm in the reduced form. Reduced human



Approximately five micromolar P. gouldii cytochrome b_5 in 10 mM phosphate buffer pH 7.2.
EPR conditions: Temperature 4 K; frequency 9.42 GHz; power 20 μW; gain 5.0 x 10^5; modulation 16 G at 100 kHz; time constant 0.20 sec; 10 scans.

Figure III-7. EPR spectrum of oxidized P. gouldii cytochrome b_5



10 mM phosphate buffer pH 7.2; oxidized form (solid line); dithionite reduced form (dashed line)

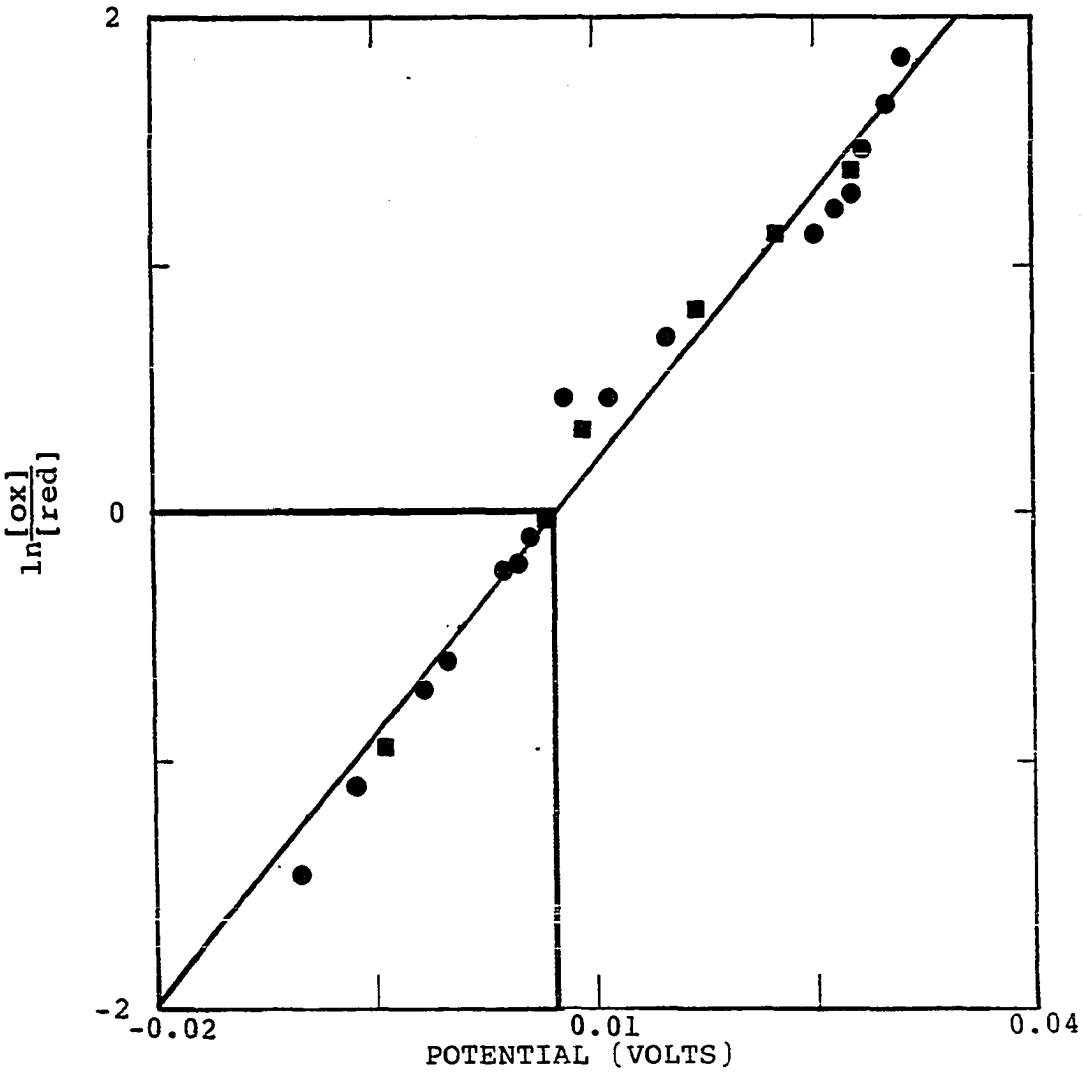
Figure III-8. UV-visible spectra of P. gouldii cytochrome b_5

cytochrome b_5 has an α-band maximum at 556 nm and a β-band maximum at 526 nm (43).

5. Midpoint reduction potential

Figure III-9 shows a plot of the redox titration of P. gouldii cytochrome b_5 and methylene blue. The midpoint reduction potential of the cytochrome b_5 was determined to be 7 ± 2 mV versus NHE with a one-electron transfer in 100 mM phosphate buffer pH 7.0 with 500 mM NaCl. Without NaCl present the titration was not reversible and an interaction occurred between the methylene blue and cytochrome b_5. This interaction was shown by the inability to remove methylene blue from the cytochrome b_5. The value of n, the number of electrons transferred by cytochrome b_5 in changing oxidation states, was determined to be 0.95 by the slope of the line, showing that the redox center transfers one electron. The P. gouldii cytochrome b_5 reduction potential is close to the 0 ± 3 mV reported for human cytochrome b_5 when corrected to I = 500 mM. Human cytochrome b_5 has a reported midpoint potential of -10 mV in 100 mM phosphate buffer pH 7.0 (25). The reduction potential of trypsin solublized bovine liver cytochrome b_5 changes with ionic strength and the equations developed to fit this ionic strength dependence were used here to correct the value for human erythrocyte cytochrome b_5 (69).

Cytochrome b_5 was reduced during the redox titration by



100 mM phosphate buffer pH 7.0, 500 mM NaCl, 23 °C, reduction by irradiated riboflavin/EDTA. (●) reduction, (■) autooxidation.

Figure III-9. Redox titration of P. gouldii cytochrome b_5

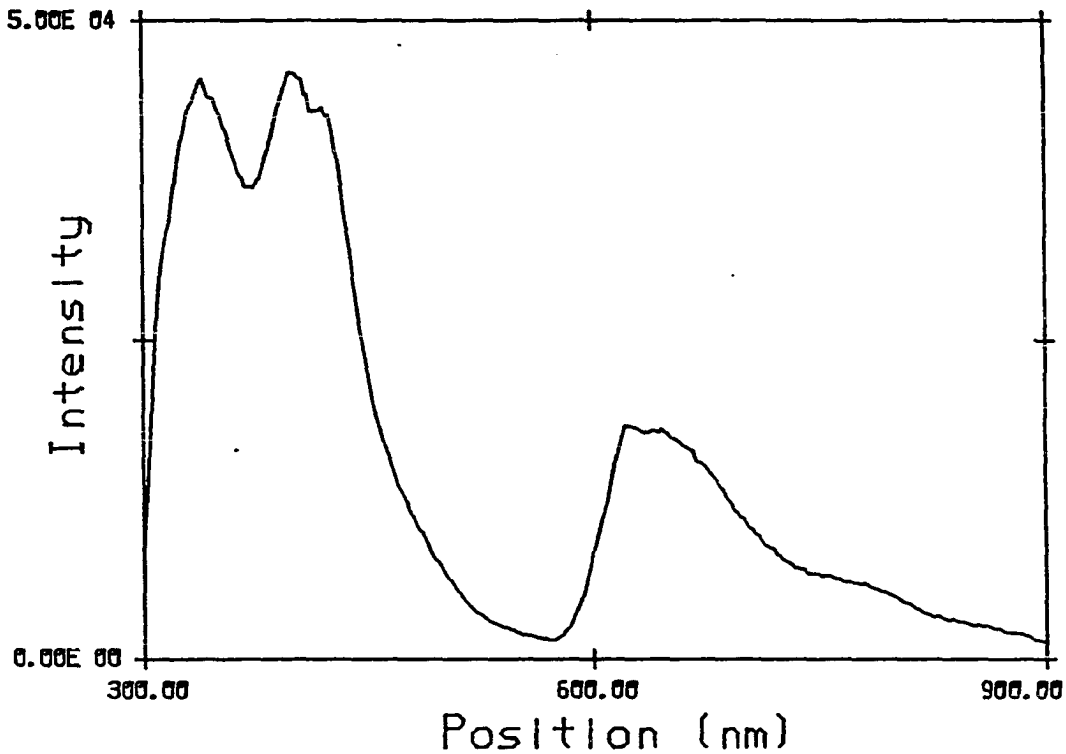
irradiation of the cytochrome b_5 in a mixture of EDTA and riboflavin. Upon irradiation, both the methylene blue and cytochrome b_5 were reduced. To determine if the system was at equilibrium, the system was allowed to autooxidize. The plot for oxidation overlaid the plot for reduction as shown in Figure III-9.

6. Fluorescence spectrum

The fluorescence spectrum of oxidized P. gouldii cytochrome b_5 was measured. Excitation at 280 nm gives the emission spectrum shown in Figure III-10. Excitation at wavelengths other than 280 nm produces only very weakfluorescence.

7. Reaction of cytochrome b_5 with CO and CN^-

Reaction of heme proteins with CO and CN^- provides information about the coordination of the heme iron atom. Heme proteins with an open coordination site at the iron, such as hemoglobin or myoglobin, react with CO and CN^- and generate a characteristic spectrum. These small molecules will not react with heme proteins that have strong axial ligation. Addition of either 10 mM CN^- or gasous CO to solutions of either oxidized or reduced P. gouldii cytochrome b_5 results in no spectral change, indicating no binding of the small molecule to the iron. This result is in agreement with that seen for other cytochromes b_5, and indicates strong axial



1.4 μM P. gouldii cytochrome b_5 in 10 mM phosphate buffer pH 7.2, excitation at 280 nm.

Figure III-10. Fluorescence spectrum of P. gouldii cytochrome b_5

ligation of the iron.

8. Crystal structure of cytochrome b_5

The ultimate structural characterization of P. gouldii cytochrome b_5 would be to solve its crystal structure. Professor Richard Honzatko of the Biochemistry Department at Iowa State University has been supplied with 1 mg of pure (by HPLC) P. gouldii cytochrome b_5. At this point, no crystals suitable for X-ray diffraction have been grown, although some micro crystals have been obtained. Higher purity protein may be required for suitable crystal growth. Isoelectric focusing may be required to produce protein of sufficient purity.

E. Isolation of Cytochrome b_5 from T. zostericola Erythrocytes

Cytochrome b_5 was also isolated from the erythrocytes of T. zostericola. The isolation of T. zostericola cytochrome b_5 was more complex and had a much lower yield than the isolation of P. gouldii cytochrome b_5.

The T. zostericola erythrocytes are purified from worms that had been deoxygenated by incubation in sealed plastic jars for 24 hours at 16 °C. The erythrocytes are lysed overnight by 1.7 times their volume of deionized water. The lysed cell solution is centrifuged in a clinical centrifuge,

and then at 26,000 g for 2 hours to remove all cell debris. The supernatant is dialyzed overnight in 6,000 molecular weight cutoff dialysis tubing against 5 times its volume of 10 mM phosphate buffer pH 7.2 and loaded onto a Sephadex A-25 column. Most of the hemerythrin passed through the column but a significant portion precipitated in the column. The column was washed with 10 mM phosphate buffer pH 7.2 until the eluate was no longer red-colored, and then eluted with 300 mM NaCl in the same buffer. The fractions with Soret absorbances were dialyzed overnight against five times their volume of 10 mM phosphate buffer pH 7.2 and subsequently loaded on another A-25 column. The column was flushed overnight with 10 mM phosphate buffer pH 7.2 with 2 % Triton X-100 to solublize and remove the Hr that precipitated at low salt concentration in the column, and which would elute off the column with the cytochrome b_5 when the salt concentration is increased. The Triton was washed out of the column and the cytochrome b_5 eluted with 300 mM NaCl. At this time a suitable UV-visible spectrum could be obtained so no further purification was attempted.

The yield of T. zostericola cytochrome b_5 is approximately 0.8 nmoles from 50 worms, much lower than the yield of P. gouldii cytochrome b_5. No cytochrome b_5 was obtained if the worms were not deoxygenated overnight.

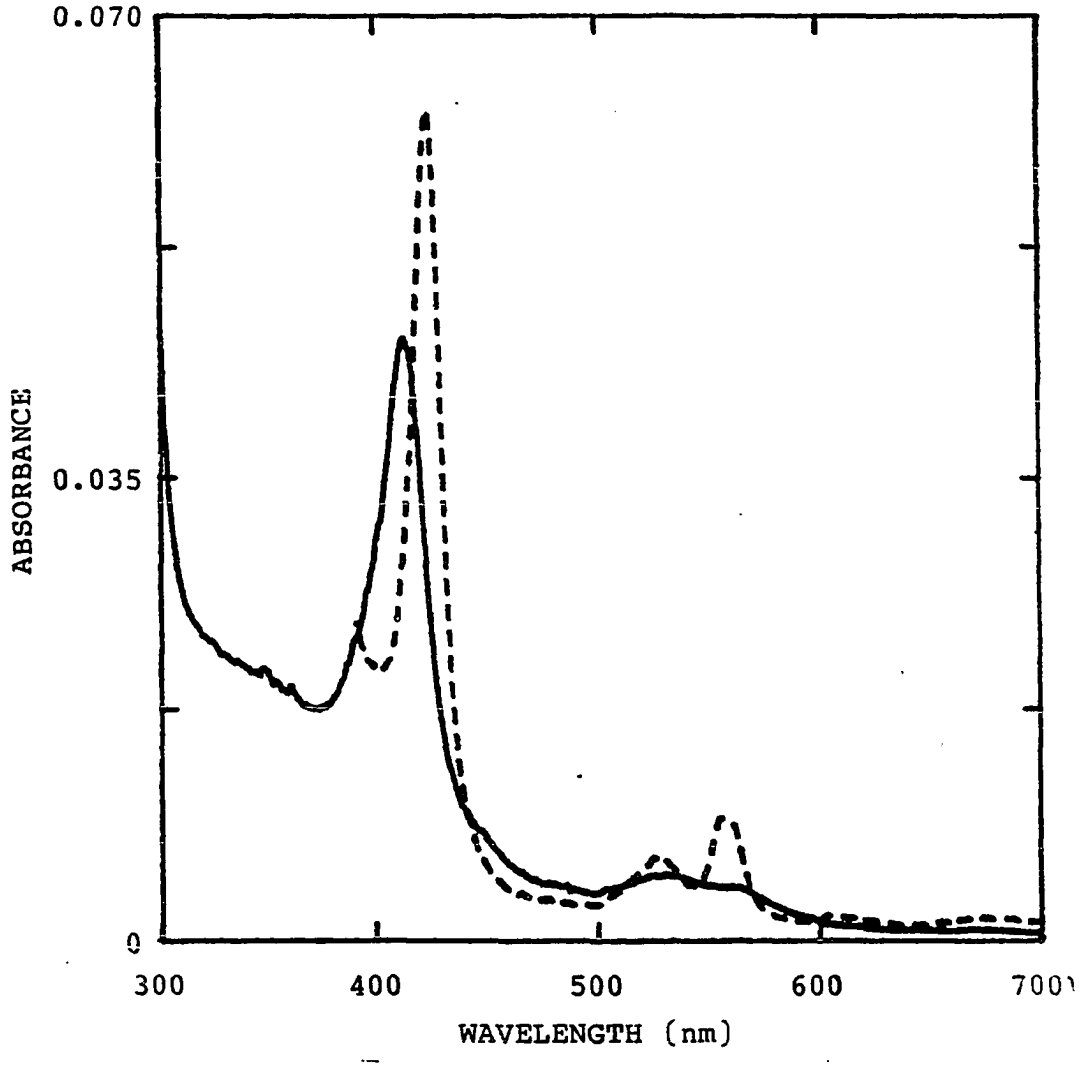
The UV-visible spectra of cytochrome b_5 from T.

zostericola erythrocytes is shown in Figure III-11 and Table III-5 shows that those spectra are very similar to human and P. gouldii cytochrome b_5. This result shows that the presence of cytochrome b_5 in hemerythrin-containing erythrocytes is not just a peculiarity of P. gouldii, even though there is apparently much less cytochrome b_5 in the erythrocytes of T. zostericola than in those of P. gouldii.

F. Oxidation of Reduced Cytochrome b_5 by MetHr

P. gouldii cytochrome b_5 may be reduced either by dithionite or by irradiation with a 500 W projector lamp in an EDTA/riboflavin mixture. It is much more convenient to reduce the cytochrome b_5 by exposure to light than by titration with a dithionite solution. Because of the ease of reduction with EDTA/riboflavin, its use was adopted for most of the reactions.

When metHr is added anaerobically to an anaerobic solution of P. gouldii cytochrome b_5 reduced by sodium dithionite or irradiated EDTA/riboflavin, the cytochrome b_5 is oxidized. The oxidation of cytochrome b_5 may be followed by the decrease in A_{422}. As the cytochrome b_5 is oxidized, the Soret maxima shifts from 422 nm to 412 nm, causing a decrease in A_{422}. The reaction is first order with respect to both metHr and cytochrome b_5. The observed rate constants for this



10 mM phosphate buffer pH 7.2, oxidized form (solid line), dithionite reduced form (dashed line).

Figure III-11. UV-visible spectra of T. zostericola cytochrome b_5

Table III-5. Spectroscopic properties of *T. zostericola* cytochrome b_5

	T. zostericola	*P. gouldii*	Human
Soret band			
oxidized	412 nm	412 nm	413 nm
reduced	423 nm	422 nm	423 nm
α-band	555 nm	555 nm	556 nm
β-band	527 nm	526 nm	527 nm

reaction are given in Table III-6 and plotted in Figure III-12. The second order rate constant for this reaction is 650 ± 50 $M^{-1}s^{-1}$ in 10 mM phosphate buffer pH 7.2 with 0.5 mM EDTA at 20 °C.

Table III-7 shows the observed rate constants for oxidation of reduced cytochrome b_5 by metHr in 10 mM phosphate buffer pH 7.5 with 150 mM Na_2SO_4 and 0.5 mM EDTA at 20 °C. The observed rate constants for this reaction are plotted versus metHr concentration in Figure III-13. Under these conditions, the oxidation of cytochrome b_5 by metHr is first order in both metHr and cytochrome b_5. The second order rate constant for this reaction is 110 $M^{-1}s^{-1}$.

The non-zero intercepts in both Figure III-12 and III-13 are due to the autooxidation of P. gouldii cytochrome b_5.

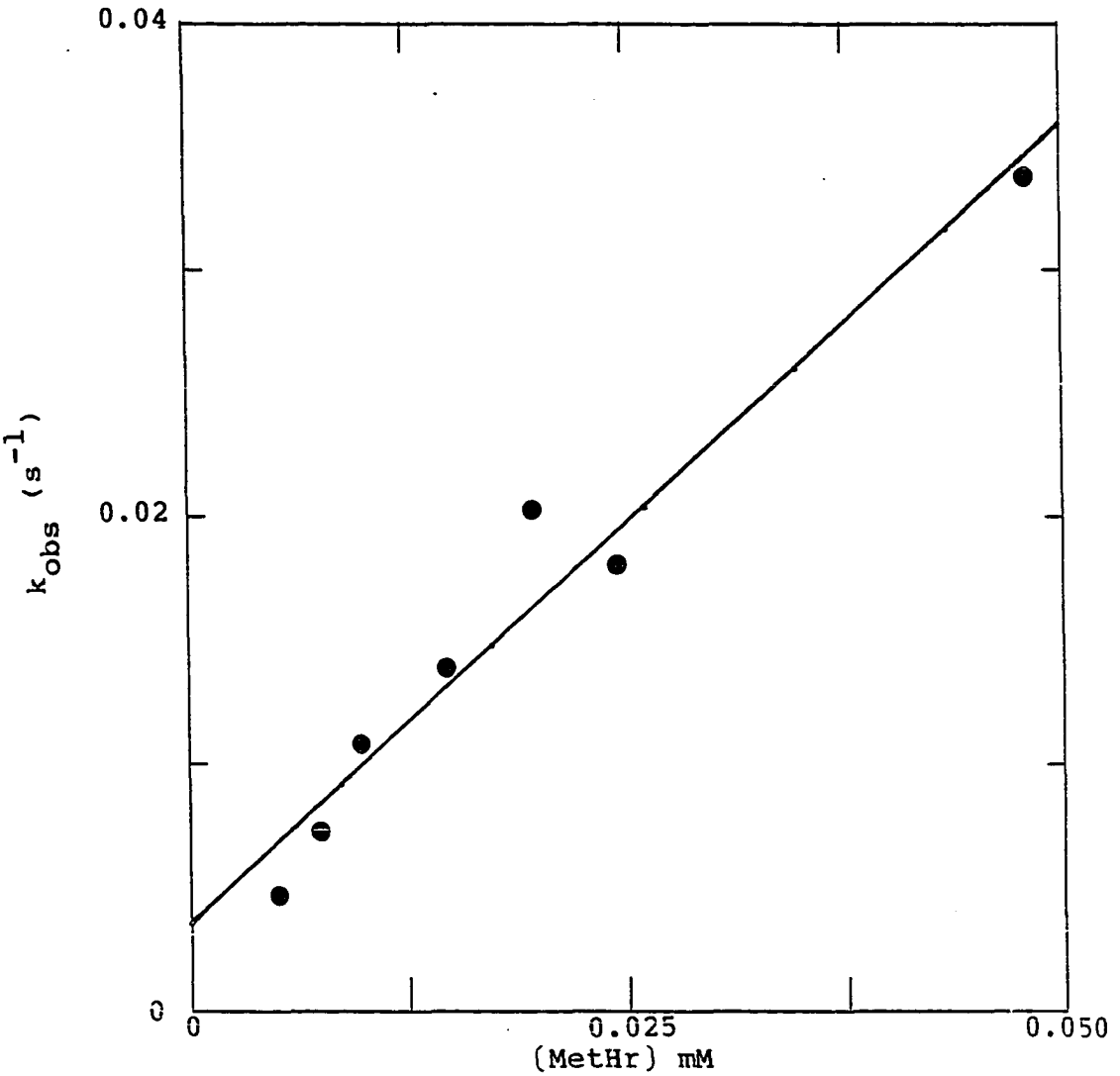
The conclusion that the oxidation of P. gouldii cytochrome b_5 by metHr is first order with respect to cytochrome b_5 is based on the excellent one exponential fits of the change in A_{420} with time. Figure III-14 shows an example of such a fit The reaction is also first order with respect to metHr, as shown by the linear fit of k_{obs} with respect to the metHr concentration as shown in Figures III-12 and III-13.

The reduction of metHr by reduced P. gouldii cytochrome b_5, discussed above, gives $(semi\text{-}met)_RHr$, not deoxyHr. This conclusion is based on the following results. When reduced P.

Table III-6. Observed first order rate constants for the oxidation of P. gouldii cytochrome b_5 by metHr[a]

[metHr] (μM)	k_{obs} (s^{-1})
5.00	0.00464
7.52	0.00714
10.0	0.0107
14.9	0.0138
19.8	0.0202
24.6	0.0181
48.0	0.0336

[a]20 °C, 1.2 μM cytochrome b_5, 10 mM phosphate buffer pH 7.2, 0.5 mM EDTA, reduction of cytochrome b_5 by dithionite addition.



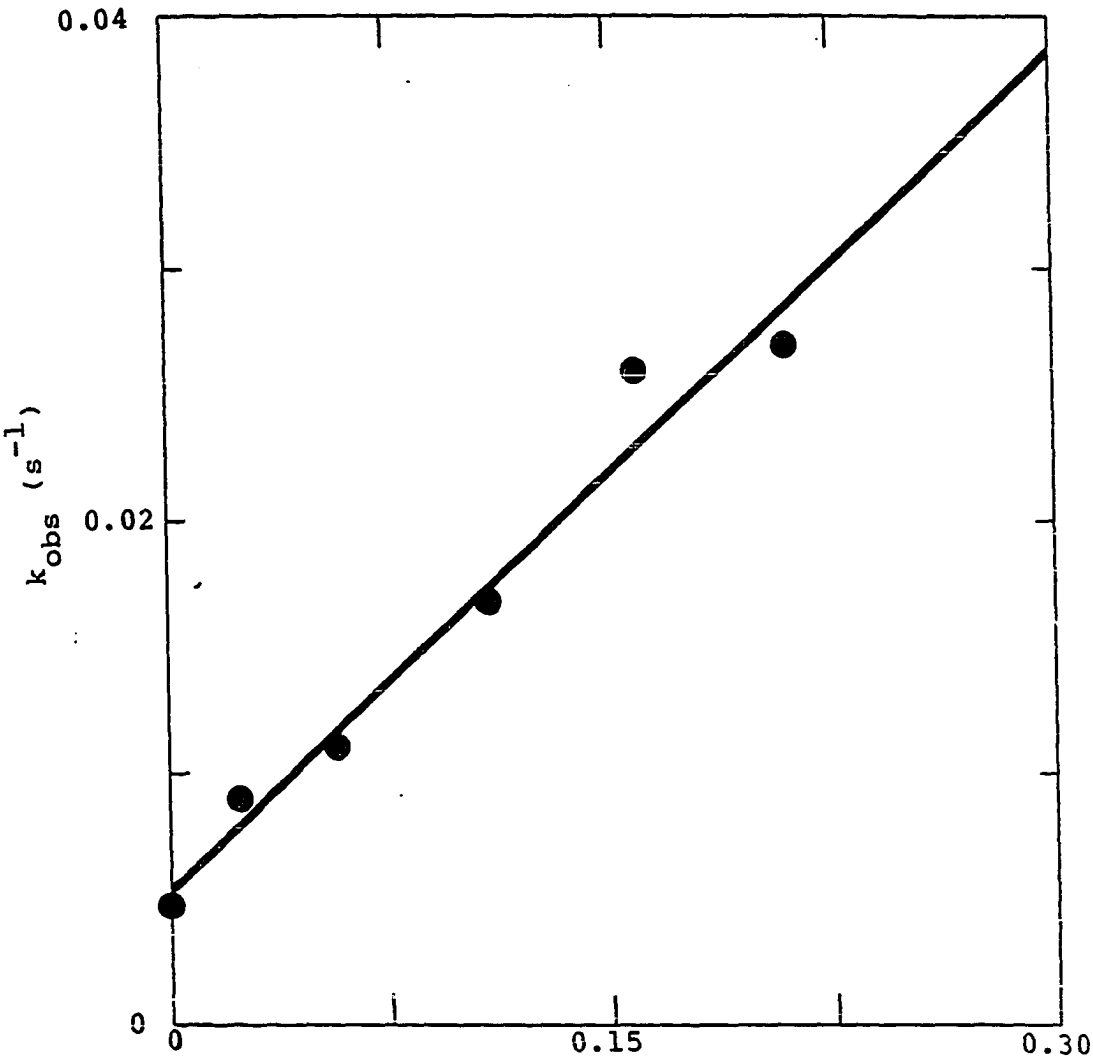
Observed first order rate constants for the oxidation of dithionite reduced P. gouldii cytochrome b_5 by metHr. Ten mM phosphate buffer pH 7.2; 0.5 mM EDTA; 20 °C.

Figure III-12. Dependence of the rate of oxidation of P. gouldii cytochrome b_5 on the concentration of metHr

Table III-7. Observed first order rate constants for the oxidation of P. gouldii cytochrome b_5 by metHr[a]

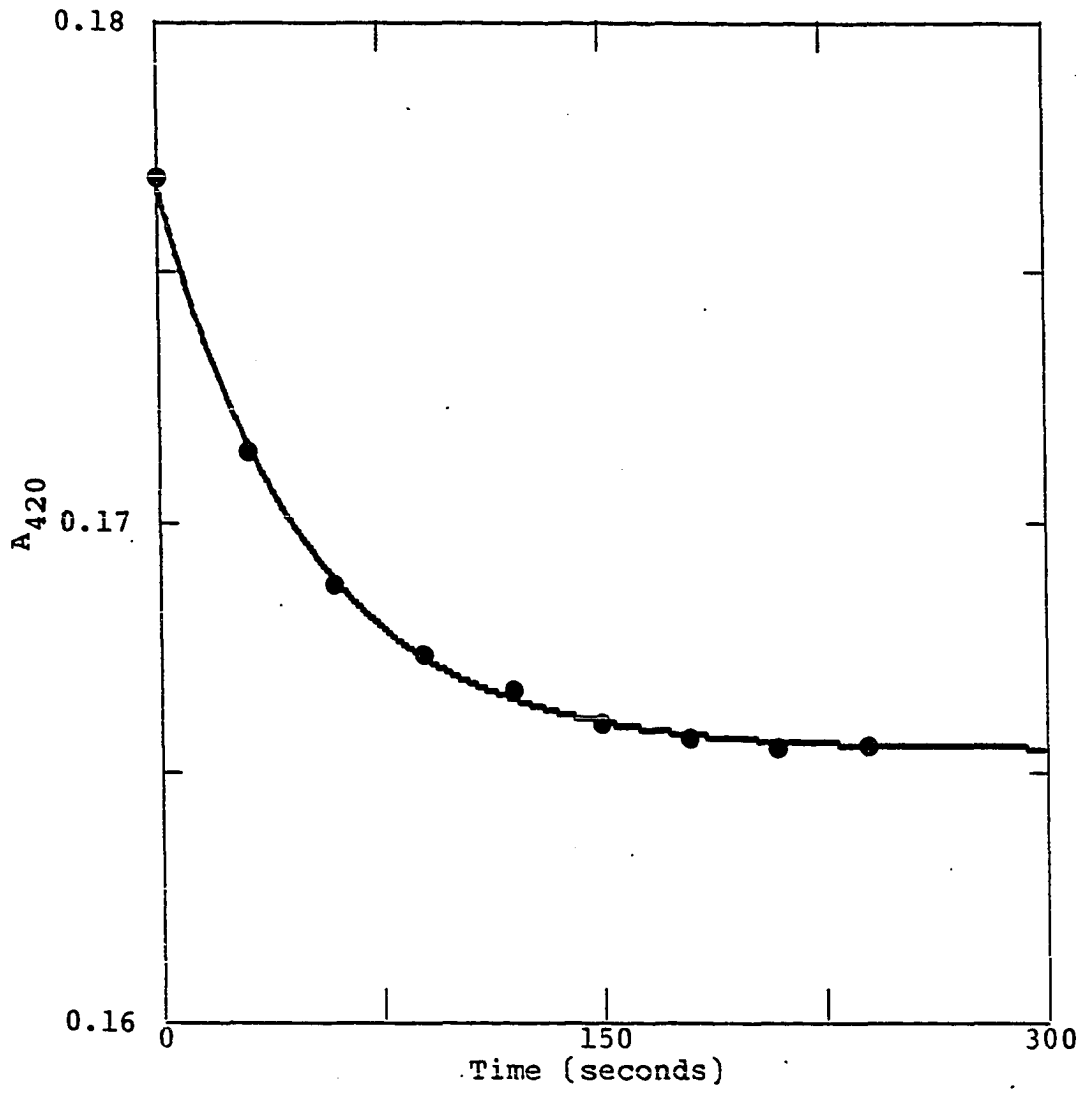
[metHr] (μM)	k_{obs} (s^{-1})
0	0.0050
24	0.0091
58	0.011
110	0.017
160	0.026
210	0.027

[a]20 °C, 1.2 μM cytochrome b_5, 10 mM phosphate buffer pH 7.5, 150 mM Na_2SO_4, 0.5 mM EDTA, photoreduction of cytochrome b_5 in the presence of EDTA and riboflavin.



Observered first order rate constants for the oxidation of riboflavin reduced P. gouldii cytochrome b_5 by metHr. Ten mM phosphate buffer pH 7.5; 0.5 mM EDTA; 150 mM Na_2SO_4; 20 °C.

Figure III-13. Dependence of the rate of oxidation of P. gouldii cytochrome b_5 on the concentration of metHr

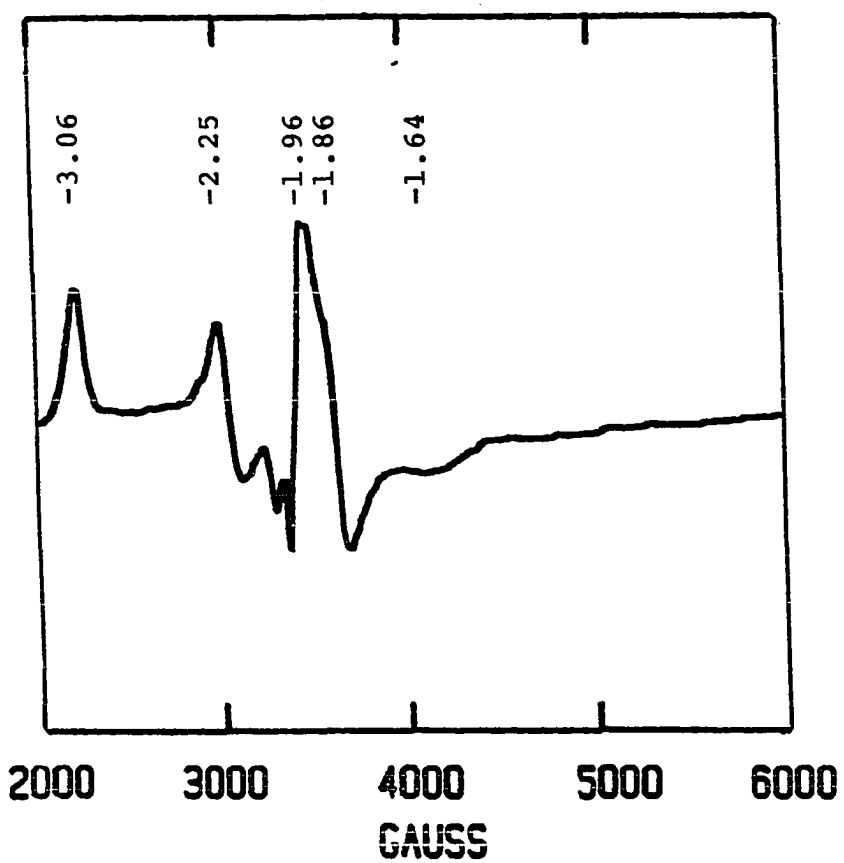


Oxidation of 1.4 μM dithionite reduced P. gouldii cytochrome b_5 by 19.8 μM metHr in 10 mM phosphate buffer, pH 7.2, 20 °C.

Figure III-14. One exponential fit of A_{420} versus time during the oxidation of P. gouldii cytochrome b_5 by metHr

gouldii cytochrome b_5 is mixed with metHr, allowed to react for two minutes, and then frozen for EPR, the resulting solution displays both an oxidized cytochrome b_5 and a $(semi\text{-}met)_R Hr$ EPR signal. Figure III-15 shows the resultant EPR spectrum formed upon the addition of 200 μM metHr to 170 μM riboflavin/EDTA reduced P. gouldii cytochrome b_5 in 10 mM phosphate buffer pH 6.5 with 150 mM Na_2SO_4. The sample was incubated at room temperature for two minutes and then frozen for EPR. The intensity of the oxidized cytochrome b_5 signal and the $(semi\text{-}met)_R Hr$ signal are comparable, showing the 1:1 stoichiometry.

Another line of evidence that the reduction of metHr by P. gouldii cytochrome b_5 proceeds to $(semi\text{-}met)_R Hr$ is the oxidation of approximately half the cytochrome b_5 (19 μM) when half a molar equivalent of metHr (10 μM in iron dimer) is added anaerobically to reduced cytochrome b_5 in 10 mM phosphate buffer pH 7.2. If cytochrome b_5 reduced metHr to deoxyHr, all the cytochrome should oxidize, but in fact, only 38% is oxidized after two minutes as determined by the decrease in A_{555}, suggesting that the end product is $(semi\text{-}met)_R Hr$. The autooxidation of cytochrome b_5 makes it difficult to determine the exact stoichiometry of this reaction. Determination of the exact stoichiometry requires the deconvolution of the autooxidation rate from the oxidation of cytochrome b_5 by metHr.



200 μM metHr in 10 mM phosphate buffer pH 7.2 reduced by 170 μM P. gouldii cytochrome b_5. EPR conditions: temperature 4 K; frequency 9.42 GHz; power 6.3 mW; gain 5.0 x 10^4; modulation 16 G at 100 kHz; time constant 50 msec; 5 scans.

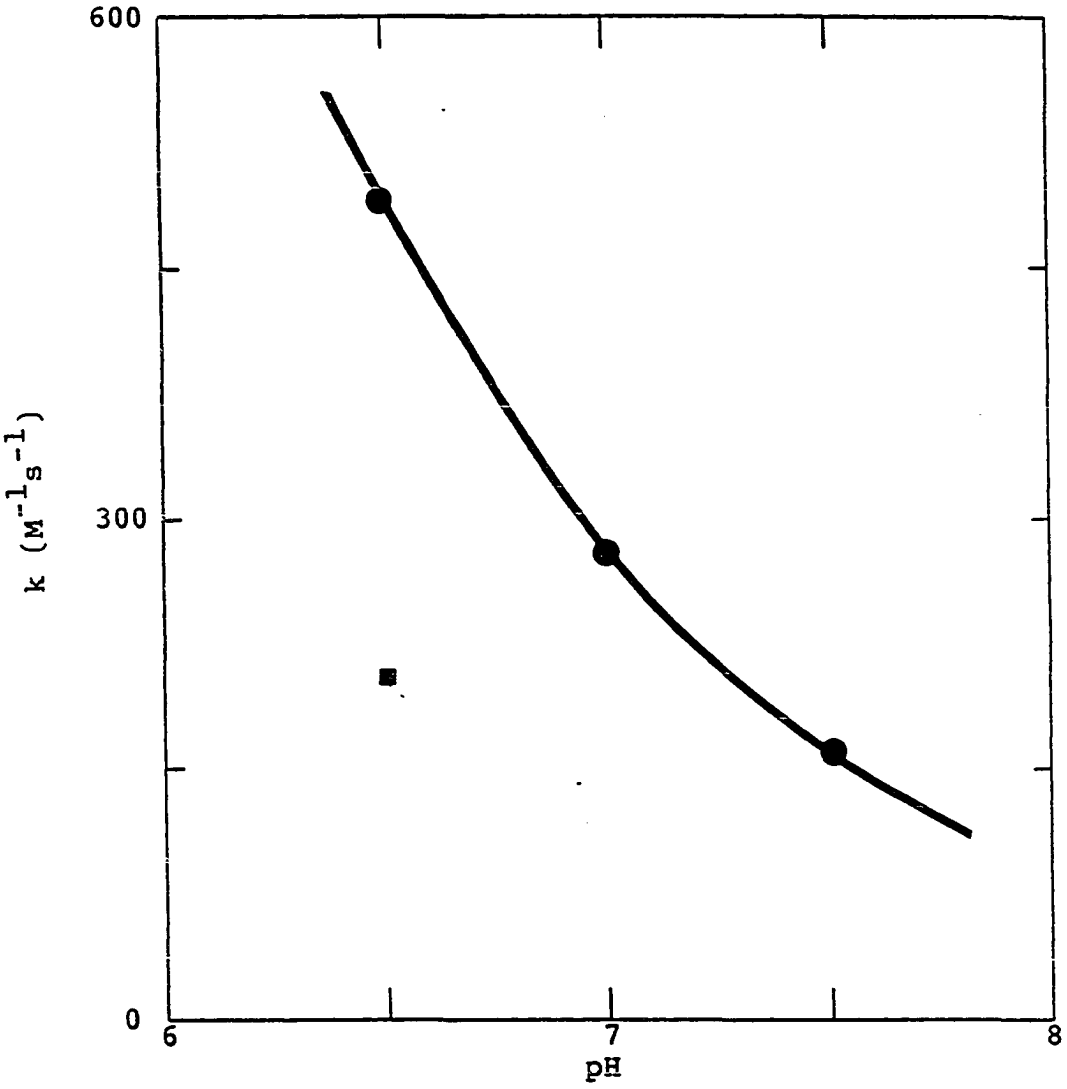
Figure III-15. EPR spectrum of a mixture of metHr and reduced P. gouldii cytochrome b_5

1. Effect of pH

Figure III-16 shows the effect of pH on the rate of reaction of reduced P. gouldii cytochrome b_5 with metHr. The reactions were carried out in an anaerobic cuvette with 0.75 ml of 1.3 μM cytochrome b_5 in the desired buffer with 1 mM EDTA. Ten microliters of 10 μM riboflavin was added to the cuvette and the solution reduced by irradiation from a 500 W projector lamp. Twenty-five microliters of metHr was then added and the solutions mixed by rapidly inverting the cuvette several times. The reaction was followed by the decrease in A_{422}.

The pH dependence was studied between pH 6.5 and 7.5. At pH's lower than 6.5, the reaction was too fast to monitor on a conventional spectrophotometer. At pH's above 7.5, the autooxidation of cytochrome b_5 was faster than the oxidation of cytochrome b_5 by metHr.

As can be seen from the data in Table III-8 and from Figure III-16, the rate of reaction is increased by a factor of three at pH 6.5 as opposed to pH 7.5. This increase could be due either to conditions more favorable for complex formation between the two proteins, or to an effect on the acid-base equilibrium at the iron site of metHr. The electrostatic interactions between metHr and cytochrome b_5 are due to ionizable amino acids on the surface of the protein. Upon changing the pH of the protein solution, the amino acids



(●) effect of pH on the oxidation of EDTA/riboflavin reduced cytochrome b_5. 150 mM Na_2SO_4; 0.5 mM EDTA. (■) metHr equilibrated at pH 7.5, see text for details.

Figure III-16. Effect of pH on the second order rate constant for oxidation of reduced P. gouldii cytochrome b_5 by metHr

Table III-8. Effect of pH on the rate constants for oxidation of reduced P. gouldii cytochrome b_5 by metHr[a]

pH	k_{obs}[b] (s^{-1})	[met] (μM)	k ($M^{-1}s^{-1}$)	buffer
6.5	0.047	96	490	50 mM MES
7.0	0.027	98	280	50 mM HEPES
7.5	0.0095	61	160	50 mM Tris-sulfate
6.5[c]	0.012	61	204	

[a]25 °C, 150 mM Na_2SO_4, 1.3 μM cytochrome b_5, which had been photoreduced in the presence of 1 mM EDTA and riboflavin.

[b]Corrected for autooxidation.

[c]Using metHr initially at pH 7.5, see text for details.

on the surface of the proteins should be titrated instantaneously. On the other hand, the change of the iron site between high and low pH forms takes several minutes (70).

The origin of the pH effect was explored by adding 25 μL of metHr at pH 7.5 to 0.75 ml of reduced cytochrome b_5 at pH 6.5. The amino acids on the surface of metHr should immediately titrate to pH 6.5 upon changing of the buffer pH. The electrostatic interactions should then be the same as if both proteins were equilibrated at pH 6.5 overnight. The only difference in reaction rate should then be due to a change in the state of the iron site or a conformational change in the protein backbone. The second order rate constant under these conditions is 200 $M^{-1}s^{-1}$. This rate constant is closer to that seen at pH 7.5 (160 $M^{-1}s^{-1}$), which is the pH at which the metHr had been equilibrated, than to the rate constant at pH 6.5 (490 $M^{-1}s^{-1}$), the pH where the reaction is actually carried out. This result shows that most of the increase in rate at low pH comes from the acid-base equilibrium of the iron site in metHr or a conformational change rather than increased cytochrome b_5-metHr interaction.

2. Effect of ionic strength

The effect of ionic strength on the rate of oxidation of reduced P. gouldii cytochrome b_5 by metHr was investigated by adjusting the ionic strength with Na_2SO_4 or NaCl. The ionic

strength dependence on the rate of reaction of reduced cytochrome b_5 and metHr yields information about the contribution to the rate of electrostatic interactions between the two proteins. If the net charges on the two proteins have the same sign, the rate should increase at higher ionic strength. If the signs of the charges are opposite, the reaction should decrease at higher ionic strength.

The ionic strength of the solution may be calculated by the following relationship:

$$\mu = 1/2 \sum [i] z_i^2$$

where μ is the ionic strength, $[i]$ is the concentration of the i^{th} ion, and z_i is the charge on the i^{th} ion.

The effect on the rate of reaction by ionic strength is due to the change in activity of the ions. The observed rate is related to the rate constant in an ideal solution, where the activity coefficients, γ, would be equal to one, by the equation:

$$k_{actual} = k_{ideal}(\gamma_A \gamma_B)/\gamma^{\ddagger}$$

γ_A and γ_B are the activity coefficients of reactant A and B respectively. $\gamma^{\ddagger} = 1$ for reference to an ideal solution.

The Debye-Huckel law:

$$\log_{10}\gamma_i = Az_i^2\mu^{1/2}/(1+\mu^{1/2})$$

relates the activity coefficient of an ion to the ionic strength of the solution where γ_i is the activity coefficient for the i^{th} ion, A is a collection of physical constants equal to 0.509 for water at 298 °C, z_i is the charge on the i^{th} ion and μ is the ionic strength.

Substitution of the activity coefficient into the equation relating k_{actual} to k_{ideal} gives:

$$\log k = \log k_{ref} + 2Az_Az_B\mu^{1/2}/(1+\mu^{1/2})$$

From this equation it can be seen that a plot of log k versus $\mu^{1/2}/(1+\mu^{1/2})$ should have a slope of the product of the charges on the two reactants multiplied by 2A (71).

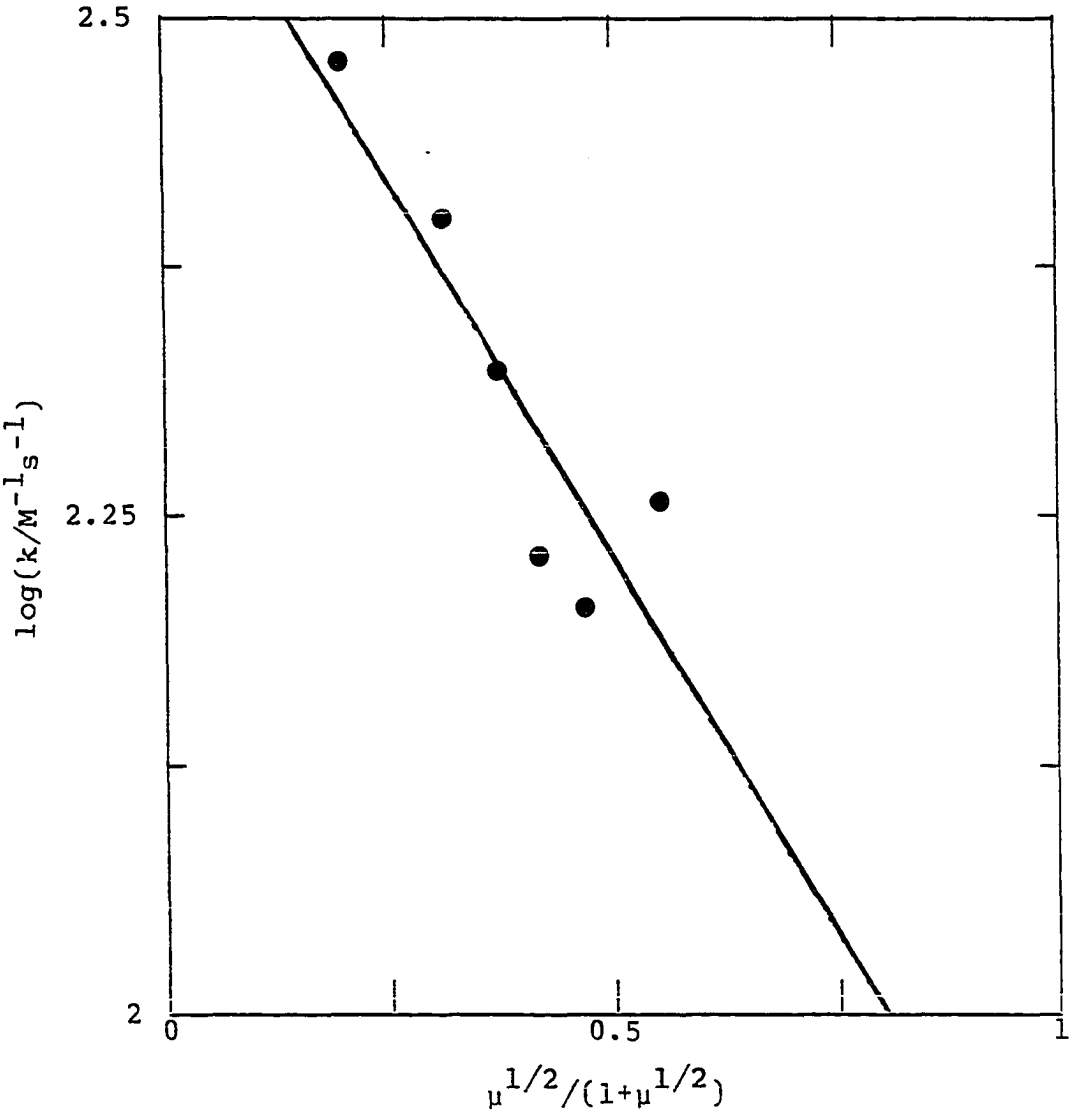
Table III-9 shows the data for the ionic strength dependence of the rate constants for the reduced P. gouldii cytochrome b_5-metHr reaction. Figure III-17 shows that a plot of log k versus $\mu^{1/2}/(1+\mu^{1/2})$ is in fact linear with the rate of reaction decreasing with increasing ionic strength as expected for oppositely charged proteins. At $\mu > 1$ no decrease in rate is seen with increasing ionic strength indicating that the charge effects have been cancelled out.

Table III-9. Effect of ionic strength on the rate constants for oxidation of reduced P. gouldii cytochrome b_5 by metHr[a]

μ (M)	$\mu^{1/2}/(1+\mu^{1/2})$	k_{obs}[b] (s^{-1})	k ($M^{-1}s^{-1}$)	log k
0.06	0.197	0.018	300	2.4771
0.21	0.314	0.015	250	2.3979
0.36	0.375	0.013	210	2.3222
0.51	0.417	0.010	170	2.2304
0.81	0.473	0.0098	160	2.2041
1.56	0.555	0.011	180	2.2561

[a]10 mM phosphate buffer pH 7.5, 25 °C, ionic strength adjusted with Na_2SO_4, 1.2 μM cytochrome b_5 was photoreduced in the presence of 1 mM EDTA and riboflavin, 61 μM metHr.

[b]Corrected for autooxidation.



1.2 μM P. gouldii cytochrome b_5; 61 μM metHr; ten mM phosphate buffer pH 7.5; 25 °C. Ionic strength adjusted by the addition of Na_2SO_4.

Figure III-17. Effect of ionic strength on the second order rate constant for the oxidation of reduced P. gouldii cytochrome b_5 by metHr

The best equation for these data is:

$$\log(k) = -0.754\ \mu^{1/2}/(1+\mu^{1/2}) + 2.60$$

The product of the charges on the proteins is then calculated to be -0.74 at pH 7.5. This low net charge is consistent with an isoelectric point of 7.8 for metHr.

Chloride forms an adduct with metHr and this adduct is the expected form of metHr in the erythrocytes that contain 500 mM Cl^-. Therefore, it is important to determine whether or not reduced P. gouldii cytochrome b_5 will reduce $metHrCl^-$. The rate constant for the reduction of metHr in 10 mM phosphate buffer pH 7.5 with 500 mM NaCl (μ = 560 mM) is 120 $M^{-1}s^{-1}$, compared to 190 $M^{-1}s^{-1}$ when the ionic strength is adjusted to the same value with Na_2SO_4 in 10 mM phosphate buffer pH 7.5 at 25 °C. These results show that $metHrCl^-$ is reduced by reduced cytochrome b_5, but at a rate only approximately two-thirds that expected if the only effect of NaCl is to change the ionic strength.

The addition of chloride to metHr changes the isoelectric point of Hr as well as binding to the iron site (8). The isoelectric point of Hr in 50 mM Cl^- is ~ 6. This change in isoelectric point would give metHr a net negative charge at pH 7.5. With net negative charges on both cytochrome b_5 and metHr, the rate of reaction would be expected to decrease.

3. Effect of temperature

The temperature dependence of the rate constant for the oxidation of reduced P. gouldii cytochrome b_5 by metHr was studied in 10 mM phosphate buffer pH 7.5 with 1 mM EDTA. The cytochrome b_5 (1.2 μM) was reduced by irradiation in the presence of riboflavin and EDTA. MetHr was then added to oxidize the cytochrome b_5. Reactions were carried out at 15, 20, and 25 °C. The resulting data are listed in Table III-10 and plotted as $\ln(k_{obs}/T)$ versus 1/T in Figure III-18. The best fit line is:

$$\ln(k_{obs}/T) = -4293 \times (1/T) + 5.88$$

This fit gives $\Delta H^{\ddagger} = 35.7 \pm 0.1$ kJ/mol and $\Delta S^{\ddagger} = -36 \pm 8$ J/(mol K).

4. Effect of lysine modification

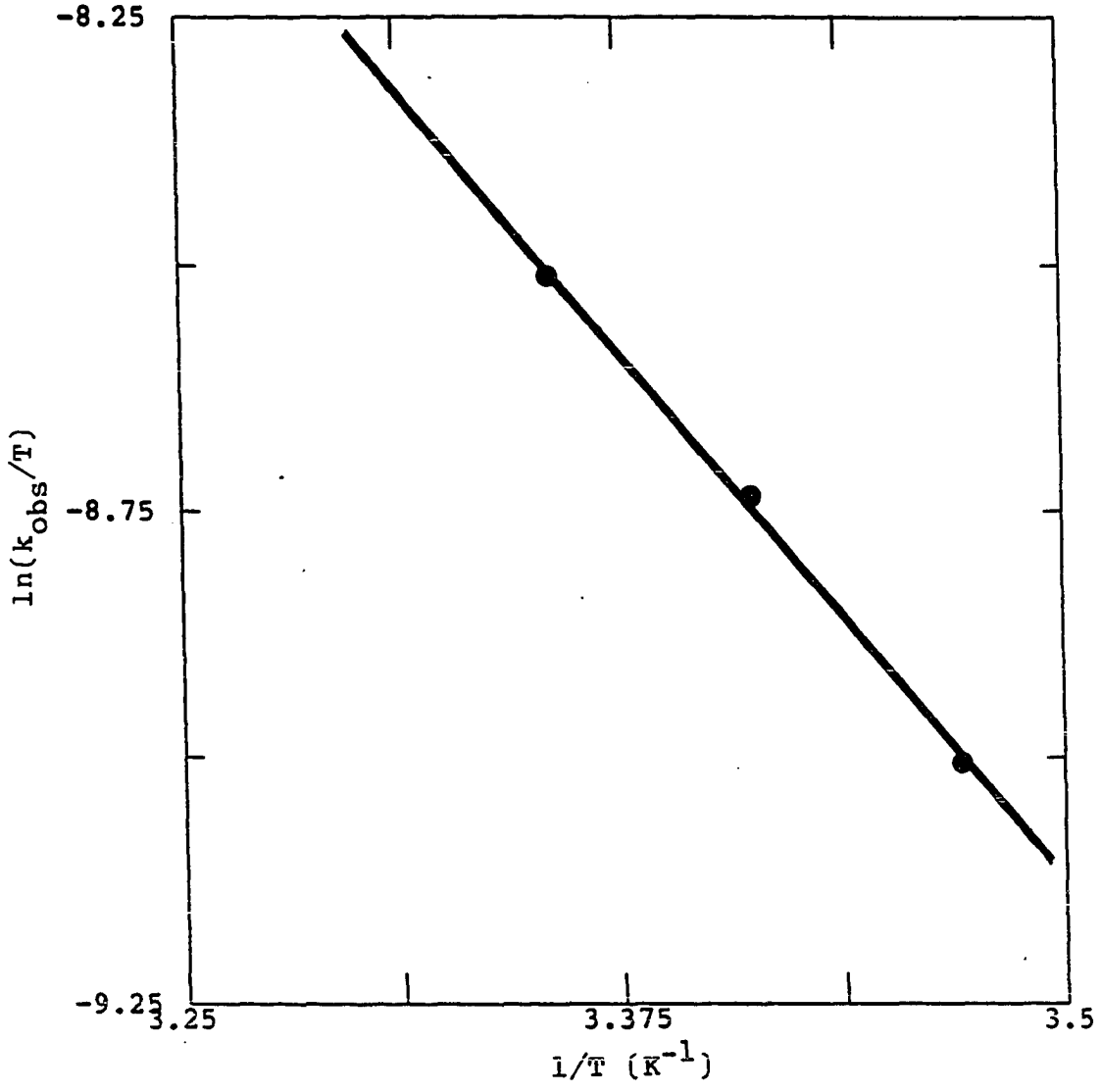
It is possible that the lysine residues on the surface of metHr help to orient the cytochrome b_5 for the transfer of electrons or help funnel the electrons through the ligating histidine residues to the iron atoms. Seven lysine residues are conserved in the sequences of three different hemerythrins (72). Of these seven, three, Lys 74, 75, and 103 are in the vicinity of His 73, 77, and 101 which ligate the "outer" iron atom as shown in Figure III-19. These lysine residues are

Table III-10. Temperature dependence of rate constants for oxidation of reduced P. gouldii cytochrome b_5 by metHr[a]

Temp (K)	1/T (K^{-1})	k_{obs}[b] (s^{-1})	$\ln(k_{obs}/T)$	[metHr] μM	k ($M^{-1}s^{-1}$)
288	0.003472	0.0353	-9.0068	92	384 ± 20
293	0.003413	0.0470	-8.7378	92	511 ± 20
298	0.003356	0.0600	-8.5105	92	650 ± 20

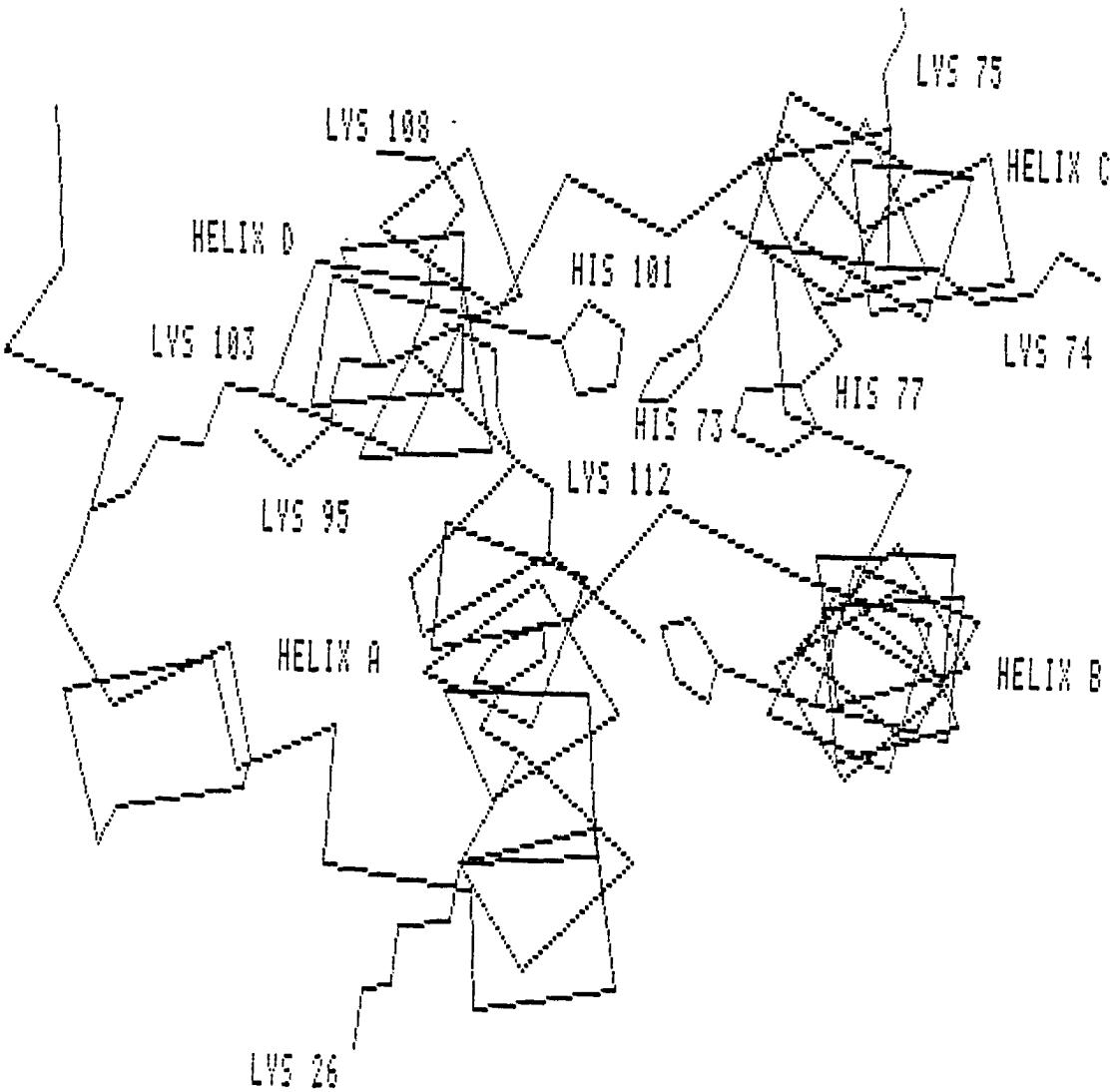
[a]10 mM phosphate pH 7.5, 1.2 μM cytochrome b_5 which had been photoreduced in the presence of 0.5 mM EDTA and riboflavin.

[b]Corrected for autooxidation.



Ten mM phosphate buffer pH 7.5; 150 mM Na_2SO_4; 0.5 mM EDTA; 92 µM metHr; 1.2 µM cytochrome b_5.

Figure III-18. Temperature dependence of rate constant for oxidation of reduced P. gouldii cytochrome b_5 by metHr



View down the proposed oxygen binding channel of metHr. Alpha carbon atoms and side chains of conserved lysine and histidine residues are shown. The figure was generated by a computer graphics program. Coordinates were obtained from the Brookhaven Protein Data Bank.

Figure III-19. Positions of conserved lysine residues in the structure of the T. dyscritum metHr subunit

exposed to the solvent and are probably easily modified by chemical methods. The lysine residues of Hr can be modified by reaction with succinic anhydride. MetHr was succinylated under conditions which are reported to modify 7 lysine residues per subunit (63). The rate of oxidation of reduced P. gouldii cytochrome b_5 by the modified metHr was studied. Table III-11 shows the results of this reaction. These data are also plotted in Figure III-20.

The modification of the lysine side chains lowers the rate of oxidation of reduced cytochrome b_5. Under the same reaction conditions, native metHr will oxidize cytochrome b_5 with a second order rate constant of 160 $M^{-1}s^{-1}$, whereas the observed second order reaction rate constant with succinylated metHr is 30 $M^{-1}s^{-1}$.

The decrease in rate shows that some lysine residues on P. gouldii metHr are involved in its reduction by P. gouldii cytochrome b_5 in some way.

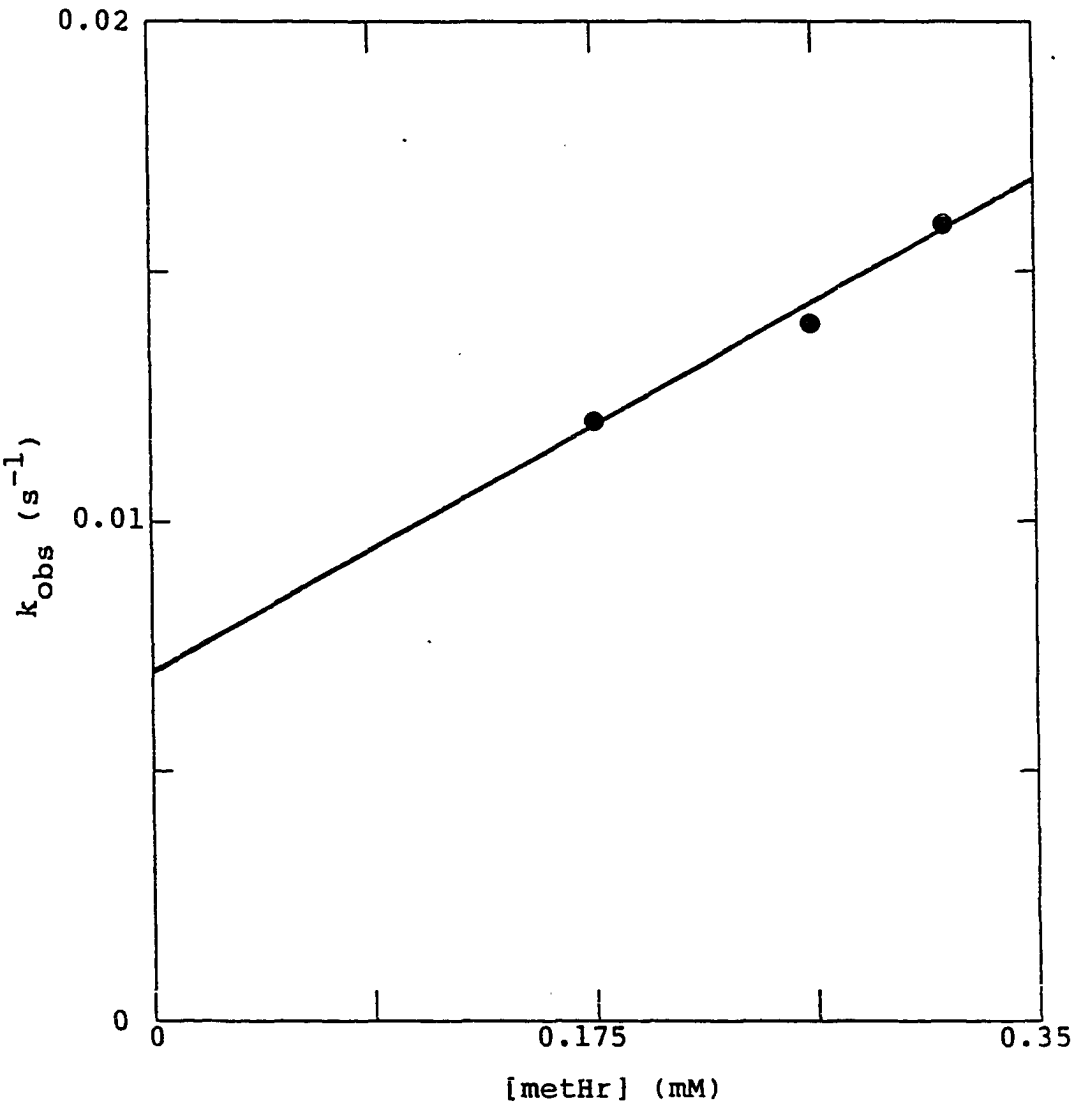
G. Isolation of Cytochrome b_5 Reductase

Cytochrome b_5 reductase was isolated from the erythrocytes of P. gouldii. The isolation of P. gouldii cytochrome b_5 reductase is not so straightforward as the isolation of cytochrome b_5 reductase from human erythrocytes since the reductase is membrane bound in P. gouldii, but

Table III-11. Effect of lysine modification of metHr on the rate constants for the oxidation of reduced P. gouldii cytochrome b_5[a]

[Hr] (μM)	k_{obs} (s^{-1})
176	0.012 ± 0.001
262	0.014 ± 0.001
316	0.016 ± 0.001

[a] 1.4 μM photoreduced cytochrome b_5, 10 mM phosphate buffer pH 7.5, 0.5 mM EDTA, 150 mM Na_2SO_4.



The oxidation of 1.4 μM riboflavin reduced cytochrome b_5 by succinylated metHr. Ten mM phosphate buffer pH 7.5; 0.5 mM EDTA; 150 mM Na_2SO_4; 25 °C.

Figure III-20. Dependence of k_{obs} for the oxidation of reduced P. gouldii cytochrome b_5 on the concentration of succinylated metHr

soluble in human and other non-nucleated Hb containing erythrocytes.

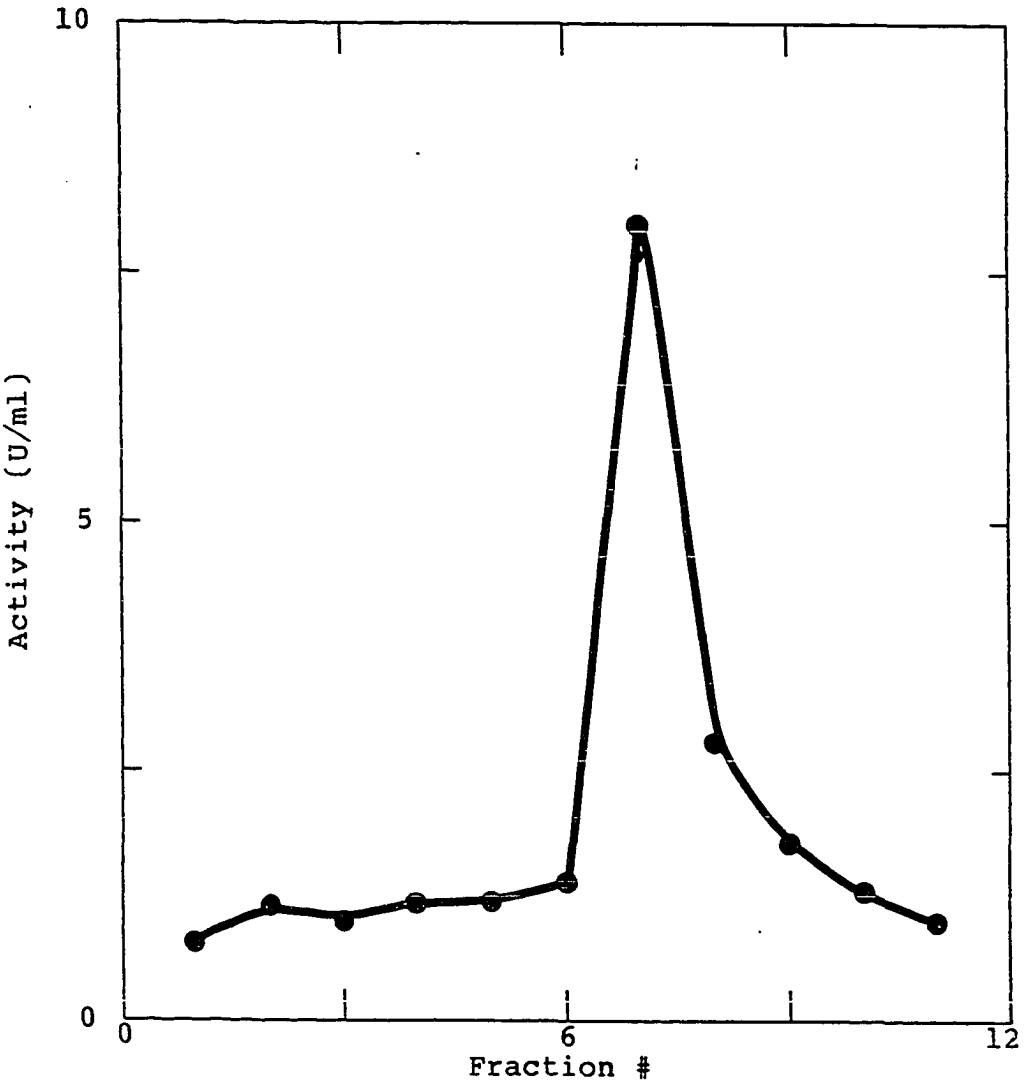
Because P. gouldii cytochrome b_5 reductase is membrane bound, solublization is the first step in its isolation from the erythrocytes. The solubilization is accomplished by suspending the cell debris in 30 times its volume of 10 mM Tris-acetate buffer pH 7.7, 2% Triton X-100 (a non-ionic detergent), with 1 mM EDTA and 0.1 mM DTT. This treatment solubilizes approximately half of the reductase (by the $K_3Fe(CN)_6$ reductase activity assay) in 24 hours. The solution loses most of its reductase activity unless it contains 0.1 mM DTT and 1 mM EDTA. The criterion for soluble reductase activity is that the activity is not sedimented after centrifugation at 26,000 g for two hours.

When the reductase activity has been solubilized, the excess cell debris is removed by centrifugation at 26,000 g for one hour. The reductase solution is then dialyzed anaerobically against 10 mM Tris-acetate buffer pH 7.7 with 0.1 mM DTT and 1.0 mM EDTA to remove the salt from the reductase solution. The solution must have been completely freed of particulate matter prior to this dialysis, or reductase activity will be lost during dialysis. This loss of reductase activity may be due to small particulate matter providing a favorable environment for the reductase to precipitate on as the salt is removed. The small particles

are then lost either by sticking to the dialysis tubing, or by being trapped at the top of the A-25 column during the next step. The dialyzed reductase solution is next passed over a small (0.5 x 5 cm) Sephadex A-25 column which has been equilibrated in the dialysis buffer plus 2% Triton. The contaminating Hr elutes from the column without being retained. The column is flushed with 10 mM phosphate buffer pH 7.7 containing 2% Triton, 0.1 mM DTT, and 1 mM EDTA until the eluate has the same A_{280} as the buffer. The reductase activity is then eluted from the column with 100 mM Na_2SO_4 in 10 mM phosphate buffer pH 7.7 with 2% Triton, 0.1 mM DTT and 1mM EDTA. The elution profile of the cytochrome b_5 reductase is shown in Figure III-21.

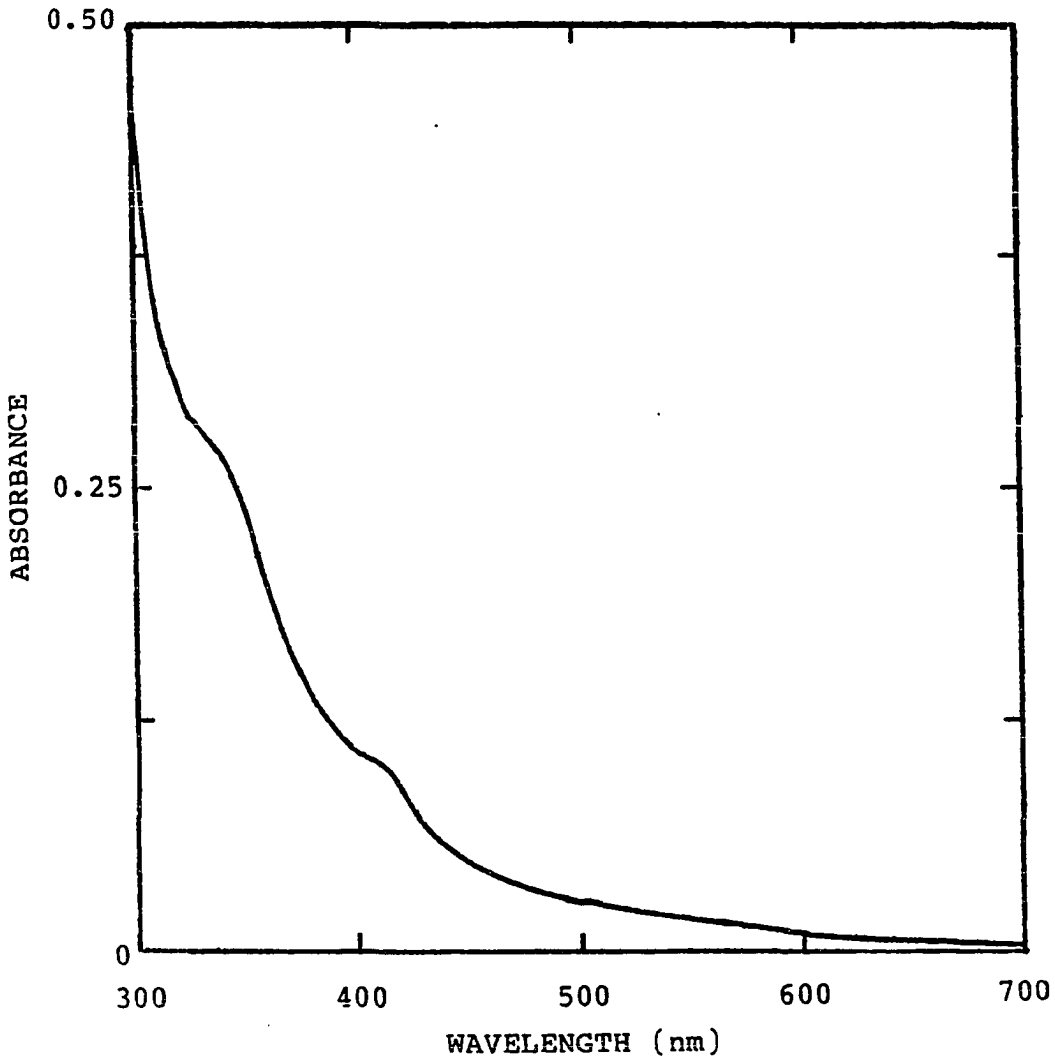
Cytochrome b_5 reductase was found to be sensitive to oxidative inactivation. In the absence of DTT, the enzyme is rapidly inactivated. In anaerobic reaction mixtures containing cytochrome b_5, NADH, and cytochrome b_5 reductase, the reductase is still active, even after 24 hours. Cytochrome b_5 reductase can be stored for several months in a centrifuge tube sealed to exclude oxygen at 4 °C in 10 mM phosphate buffer pH 7.7 with 100 mM Na_2SO_4, 0.1 mM DTT, 1 mM EDTA, and 2% Triton.

The visible absorption spectrum of P. gouldii cytochrome b_5 reductase is shown in Figure III-22. This spectrum is similar to that of the reduced flavin cytochrome b_5 reductase



$K_3Fe(CN)_6$ assay for reductase activity of fractions of solublized cytochrome b_5 reductase eluted from a 0.5 x 5 cm Sephadex A-25 column with 100 mM Na_2SO_4 in 10 mM phosphate buffer pH 7.7 with 0.1 mM EDTA, 1 mM EDTA and 2 % Triton. Two milliliter fractions collected.

Figure III-21. Elution profile of P. gouldii cytochrome b_5 reductase from a Sephadex A-25 column



75 U/ml of cytochrome b_5 reductase activity by the $K_3Fe(CN)_6$ activity assay, 2% Triton, 10 mM phosphate buffer pH 7.7, 100 mM Na_2SO_4, 1 mM EDTA, 0.1 mM DTT.

Figure III-22. Visible spectrum of P. gouldii cytochrome b_5 reductase

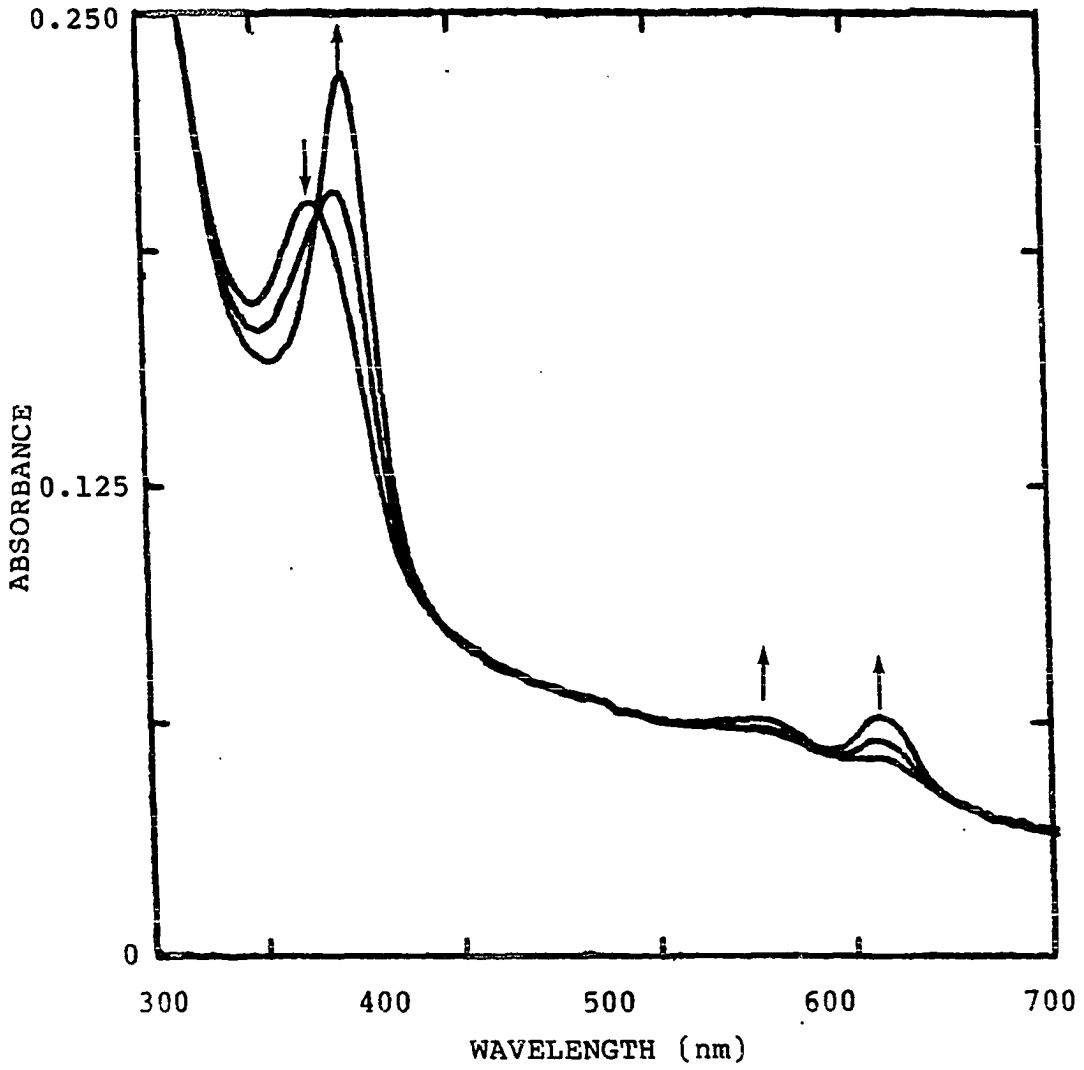
typified in Figure I-11 (49). The reduced cytochrome b_5 reductase has a shoulder at ~ 415 nm and a peak at ~ 350 nm. P. gouldii cytochrome b_5 reductase appears to have the same general features superimposed on a rising background.

H. Catalytic Properties of Cytochrome b_5 Reductase

Cytochrome b_5 reductase is a necessary component of the metHr reduction system both in vivo and in vitro. In vivo, the reductase is the link between NADH and cytochrome b_5. In vitro, the reductase is necessary to maintain the cytochrome b_5 in the reduced form over the time course of the reduction of metHr to deoxyHr.

P. gouldii cytochrome b_5 reductase catalytically reduces P. gouldii cytochrome b_5 using NADH as the electron donor. Use of NADPH in the place of NADH does not result in the reduction of cytochrome b_5. NADH does not reduce cytochrome b_5 in the absence of cytochrome b_5 reductase, even over a time course of many hours. The reduction of cytochrome b_5 by NADH in the presence of cytochrome b_5 reductase is shown in Figure III-23.

The rate of reduction of P. gouldii cytochrome b_5 by P. gouldii cytochrome b_5 reductase was studied with varying concentrations of NADH. Over the range of 2.4 to 1100 μM NADH, the observed first order rate constant for reduction of



One micromolar cytochrome b_5, 0.8 U/ml cytochrome b_5 reductase, 100 µM NADH, 10 mM phosphate buffer pH 7.5, 150 mM Na_2SO_4, 25 °C, 1 trace every 30 seconds. Arrows indicate direction of absorbance change with time.

Figure III-23. Reduction of P. gouldii cytochrome b_5 by P. gouldii cytochrome b_5 reductase

cytochrome b_5 is 0.06 ± 0.01 s^{-1}, as shown in Table III-12. These data are plotted in Figure III-24. This relative independence of the rate of reduction of cytochrome b_5 with respect to NADH concentration means that the reductase activity under these conditions remains essentially constant as NADH is depleted.

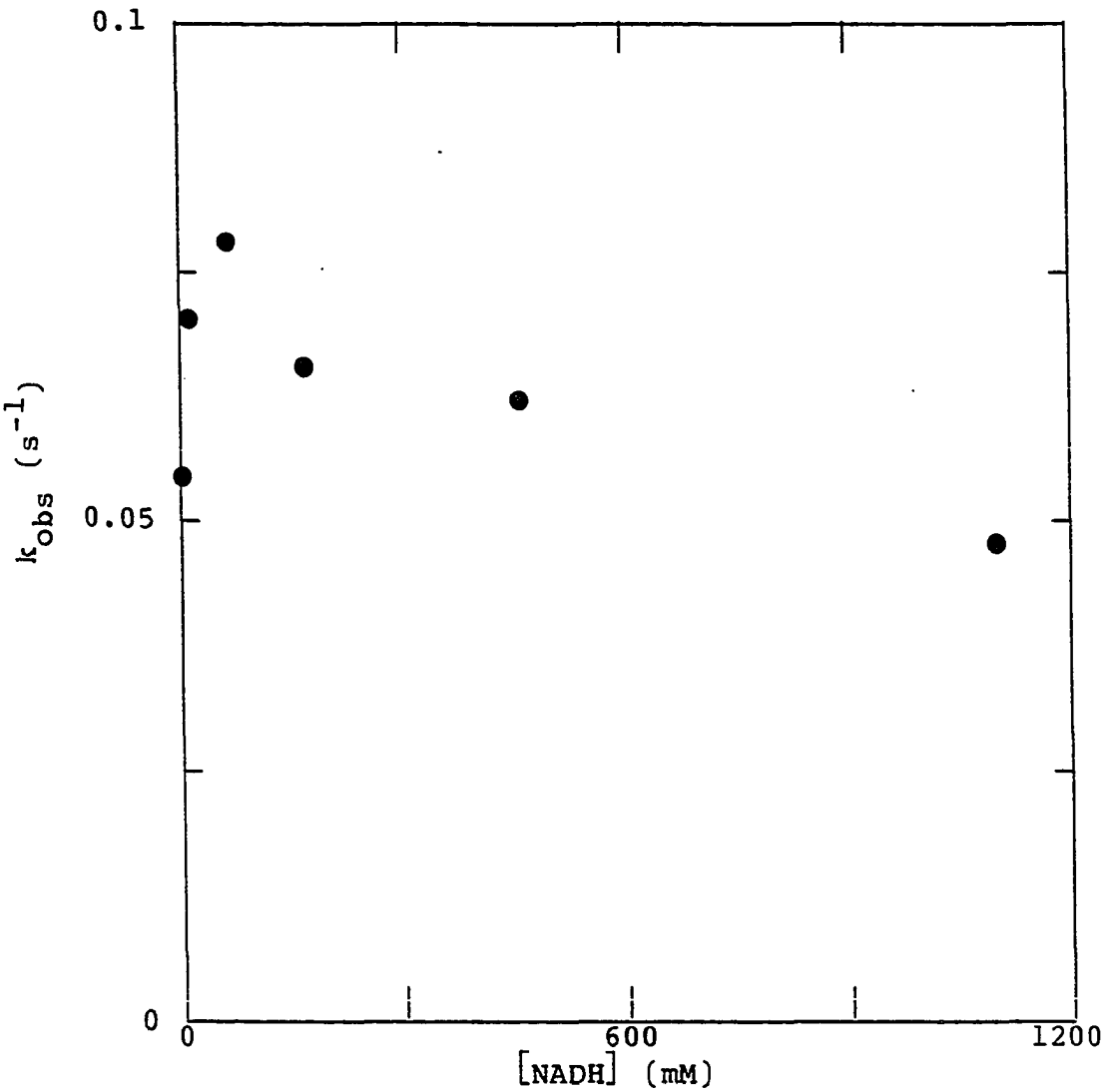
Cytochrome b_5 in the range of 1-6 μM is reduced by a 1 U/ml reductase-NADH solution with very good first order kinetics. Figure III-25 shows the change in A_{422} due to reduction of cytochrome b_5 fit to one exponential, k_{obs} = 0.035. This result shows that even at 6 μM cytochrome b_5 the reductase is not saturated. The relative independence of the rate constant for cytochrome b_5 reduction on the concentration of NADH, and the non-saturation of the reductase even at 6 μM cytochrome b_5 concentration allows the reduction of cytochrome b_5 by cytochrome b_5 reductase to be treated as a second order reaction in the presence of excess NADH.

The rate of reduction of both $K_3Fe(CN)_6$ and P. gouldii cytochrome b_5 by the same cytochrome b_5 reductase solution was determined for comparison purposes. Under conditions of the standard $K_3Fe(CN)_6$ assay, the $K_3Fe(CN)_6$ saturated the enzyme, as shown by the linear decrease in A_{420} over the first portion of the reaction. Over the range of 1 to 4 U/ml of cytochrome b_5 reductase activity (as measured by the standard $K_3Fe(CN)_6$ reductase assay), the first order rate constant for reduction

Table III-12. Dependence on NADH concentration of observed first order rate constants for the reduction of *P. gouldii* cytochrome b_5 by *P. gouldii* cytochrome b_5 reductase[a]

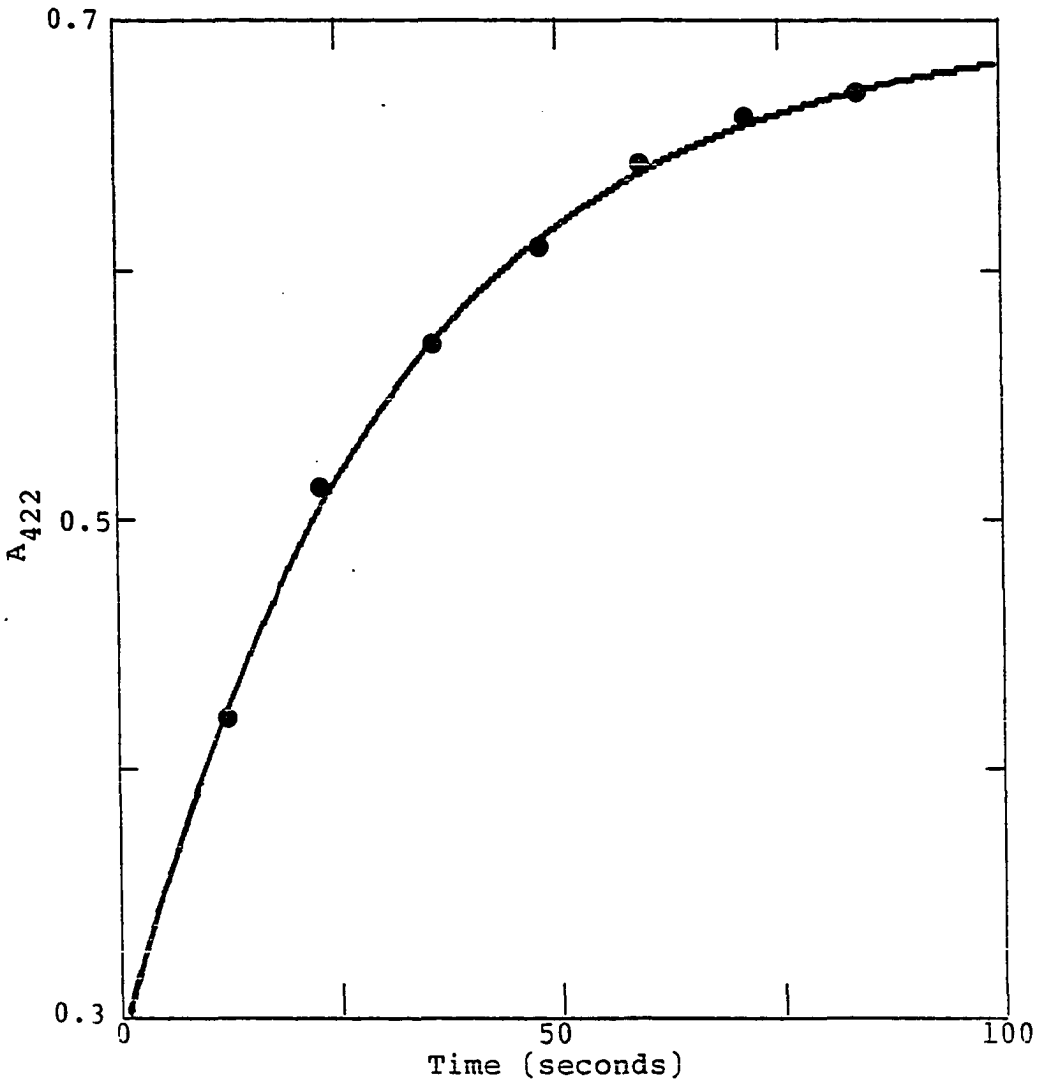
[NADH] (μM)	k_{obs} (s^{-1})
2.4	0.054
12	0.070
68	0.078
170	0.065
460	0.062
1100	0.048

[a] 1.2 μM cytochrome b_5, 4 U/ml cytochrome b_5 reductase, 10 mM phosphate buffer pH 7.2, 150 mM Na_2SO_4, 25 °C.



Observed rate of reduction of 1.2 μM cytochrome b_5 by NADH in the presence of 4 U/ml cytochrome b_5 reductase in 10 mM phosphate buffer pH 7.2, 150 mM Na_2SO_4, 25 °C. k_{obs} calculated from one exponential fits of ΔA_{422} versus time.

Figure III-24. Dependence on concentration of NADH of k_{obs} for reduction of P. gouldii cytochrome b_5 by P. gouldii cytochrome b_5 reductase



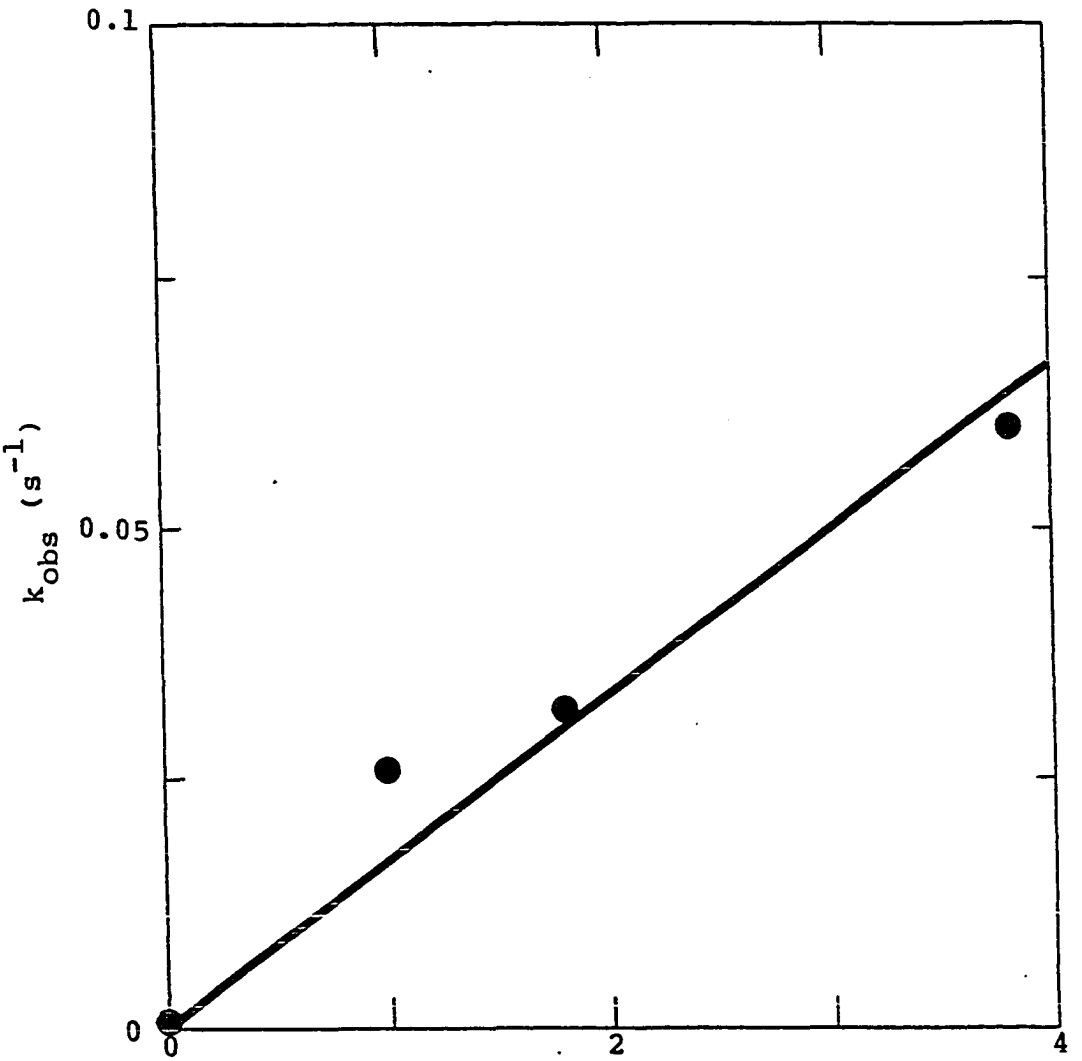
Reduction of 6 μM cytochrome b_5 by 100 μM NADH in the presence of 1.8 U/ml cytochrome b_5 reductase in 10 mM phosphate buffer pH 7.5, 150 mM Na_2SO_4, 25 °C, k_{obs} = 0.035 s^{-1}.

Figure III-25. One exponential fit of the change in A_{422} due to reduction of P. gouldii cytochrome b_5 by P. gouldii cytochrome b_5 reductase

of cytochrome b_5 is linear with cytochrome b_5 reductase activity as shown in Figure III-26. These data are tabulated in Table III-13. These data yield a second order rate constant for reduction of cytochrome b_5 by cytochrome b_5 reductase and NADH to be 0.019 $s^{-1}(U/ml)^{-1}$. This rate constant may be used to calculate the rate of cytochrome b_5 reduction by a known concentration of cytochrome b_5 reductase as measured by the $K_3Fe(CN)_6$ reductase assay.

I. Reduction of MetHr by the Reductase System

For two reasons it was necessary to reconstitute a system to reduce metHr using NADH, a biological electron donor as the source of electrons. First, demonstrating that the isolated proteins, cytochrome b_5 and cytochrome b_5 reductase, could be combined to form a reconstituted system for the reduction of metHr to deoxyHr, would show that a functional metHr reduction system has been isolated. This reduction system could presumably function in the erythrocyte. Second, cytochrome b_5 reductase and NADH are required to maintain P. gouldii cytochrome b_5 in the reduced form long enough to reduce metHr to deoxyHr. The reduction of metHr to $(semi\text{-}met)_R Hr$ by reduced cytochrome b_5 is fast but the subsequent reduction of $(semi\text{-}met)_R Hr$ to deoxyHr is slow compared to the rate of autooxidation of P. gouldii cytoochrome b_5. The cytochrome b_5



1.2 μM cytochrome b_5, 10 mM phosphate buffer pH 7.2, 150 mM Na_2SO_4, 100 μM NADH, 25 °C. k_{obs} calculated from the one exponential fits of ΔA_{422} versus time.

Figure III-26. k_{obs} for reduction of P. gouldii cytochrome b_5 versus P. gouldii cytochrome b_5 reductase concentration

Table III-13. k_{obs} for reduction of P. gouldii cytochrome b_5 versus P. gouldii cytochrome b_5 reductase concentration[a]

[reductase] (U/ml)	k_{obs} (s^{-1})
0	0
1.0	0.026
1.8	0.032
3.8	0.060

[a] 1.2 μM cytochrome b_5, 10 mM phosphate buffer pH 7.2, 150 mM Na_2SO_4, 25 °C, 100 μM NADH.

is kept reduced for long periods of time by NADH and cytochrome b_5 reductase so that the reduction of $(semi\text{-}met)_R Hr$ to deoxyHr can be studied. Such a system for the anaerobic reduction of metHr to deoxyHr is composed of NADH, cytochrome b_5 reductase, and cytochrome b_5.

The reduction of metHr by NADH through cytochrome b_5 reductase and cytochrome b_5 may be followed by UV-visible spectrophotometry. MetHr, NADH, $(semi\text{-}met)_R Hr$, and cytochrome b_5 all contribute to the absorbance in some part of the visible and near UV spectrum. MetHr and $(semi\text{-}met)_R Hr$ absorb at all wavelengths below 700 nm. NADH absorbs at wavelengths less than 400 nm (λ_{max} 340 nm). The Soret band of cytochrome b_5 absorbs between 400 and 430 nm depending on the oxidation state of cytochrome b_5.

The reduction rate constants were calculated from absorbance changes at 450 nm for the following reasons: First, the region between 400 and 430 nm is not suitable because the cytochrome b_5 Soret band dominates there and the metHr contribution is a small portion of the total absorbance. Second, the region between 300 and 400 nm is not suitable due to the fact that the disappearance of both NADH and metHr would be monitored and some of the NADH disappearance is due to countering the autooxidation of cytochrome b_5. Third, at wavelengths less than 300 nm, the proteins absorb strongly and do not reflect the oxidation state of the metals. Fourth,

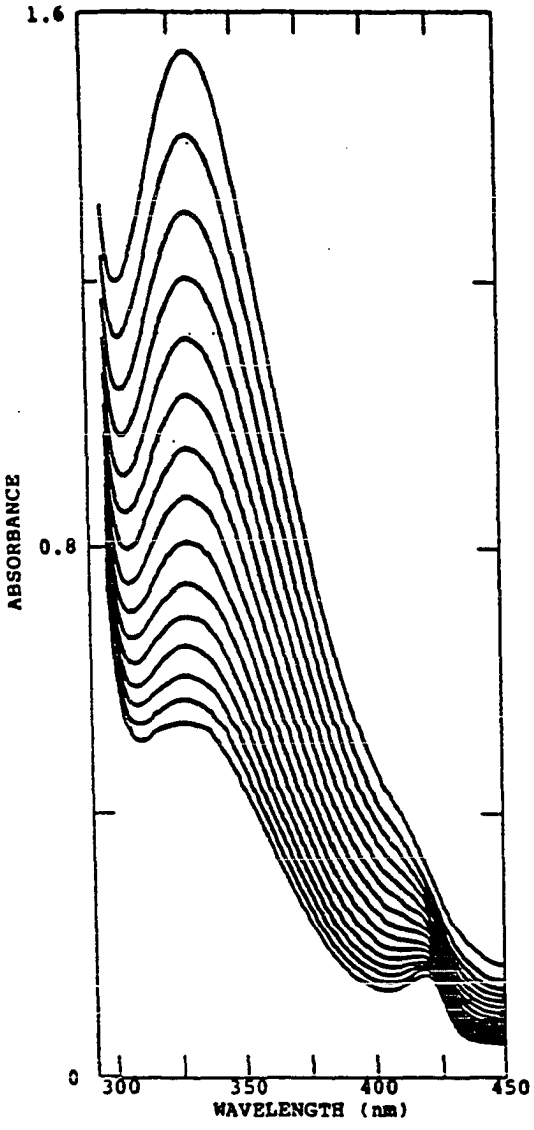
only metHr absorbs significantly at 450 nm and characteristic absorbance changes occur in each step of the reduction of metHr.

The reductions of metHr by the reductase system were carried out in an anerobic cuvette. Three-fourths milliliter of oxidized P. gouldii cytochrome b_5 was placed in the cuvette and its concentration determined. Ten microliters of 10 mM NADH and 20 µL reductase solution were added. The cytochrome b_5 was allowed to reduce and then 100 µL of metHr was added. The solution was mixed by inversion and the reaction followed by spectrophotometry. The final Triton X-100 concentration was 0.045%.

Figure III-27 and III-28 show the time course of the reduction of metHr by NADH, cytochrome b_5 reductase, and cytochrome b_5. At the end of the reaction, deoxyHr had been formed as evidenced by the increase in absorbance at 500 nm when it was mixed with air, shown in Figure III-29. The yield of oxyHr was 100% as determined by ΔA_{500} upon exposure to oxygen.

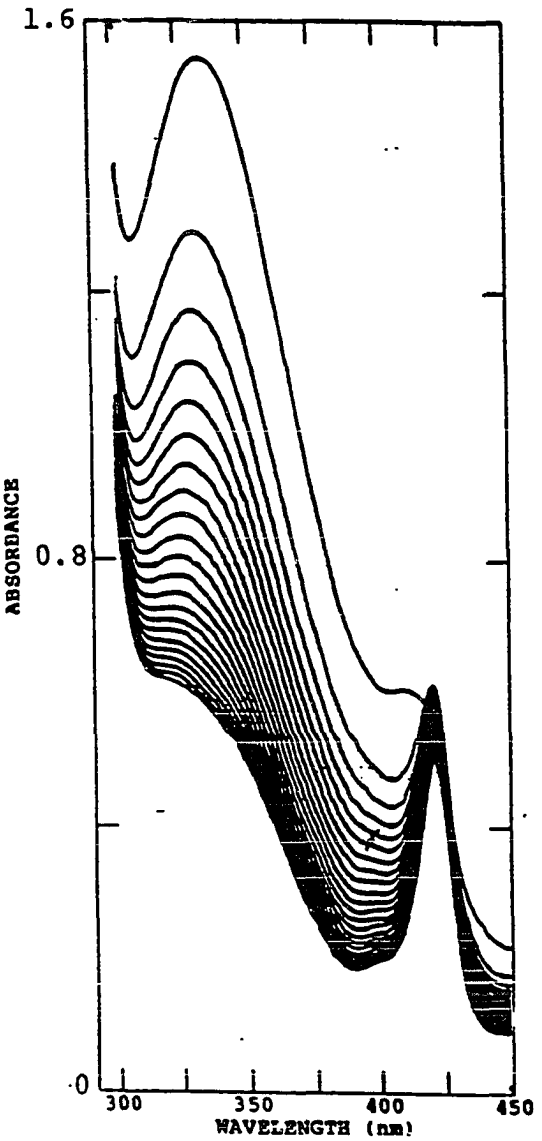
1. Effect of cytochrome b_5 concentration

The reduction of metHr by the reductase system is a biphasic reaction. A trace of A_{450} versus time fit to two exponentials is shown in Figure III-30. The first phase is linearly dependent on cytochrome b_5 concentration, as shown in



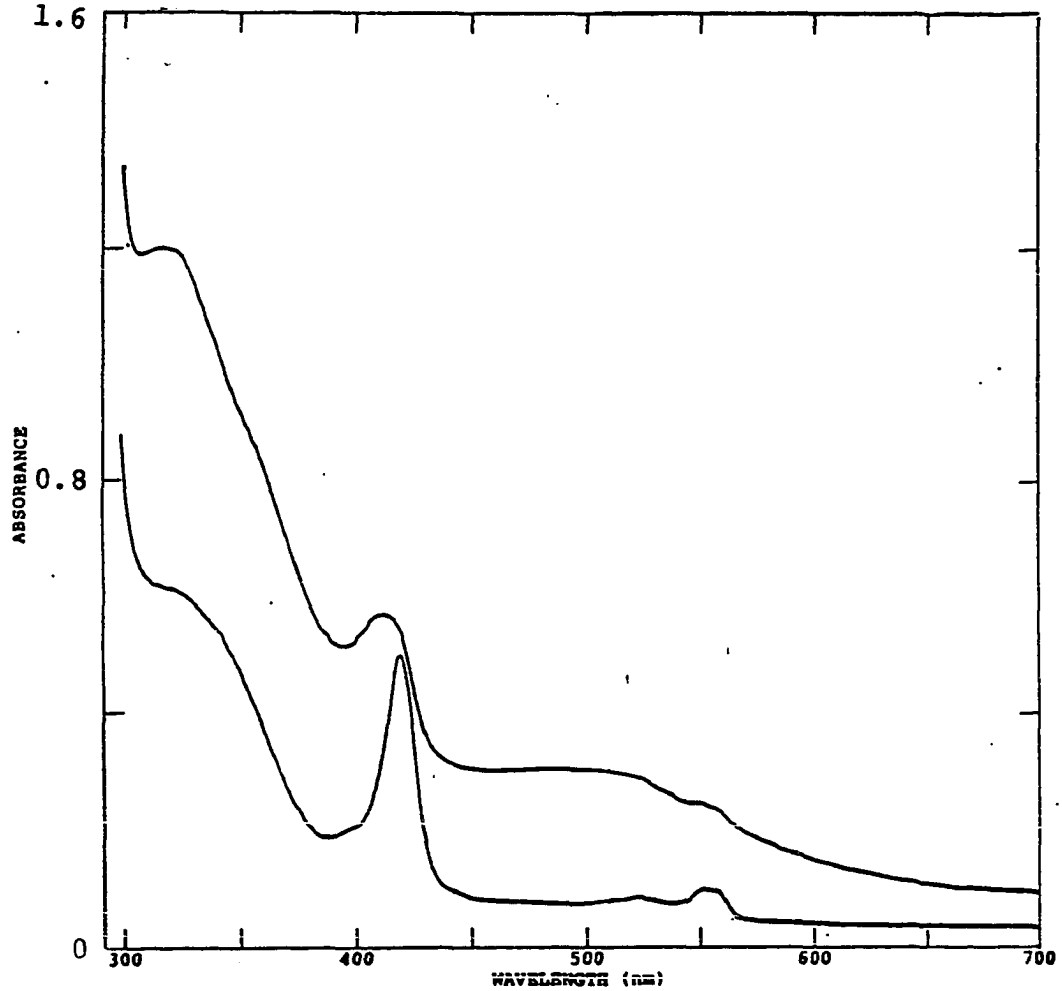
0.7 μM cytochrome b_5 in 10 mM phosphate buffer pH 7.5, 25 °C, 100 μM NADH, 100 μM metHr, 3 U/ml cytochrome b_5 reductase, one scan every 30 minutes starting from highest A_{340}. Last scan obtained at 420 minutes.

Figure III-27. Absorbance decreases during reduction of metHr by reductase system: low cytochrome b_5 concentration



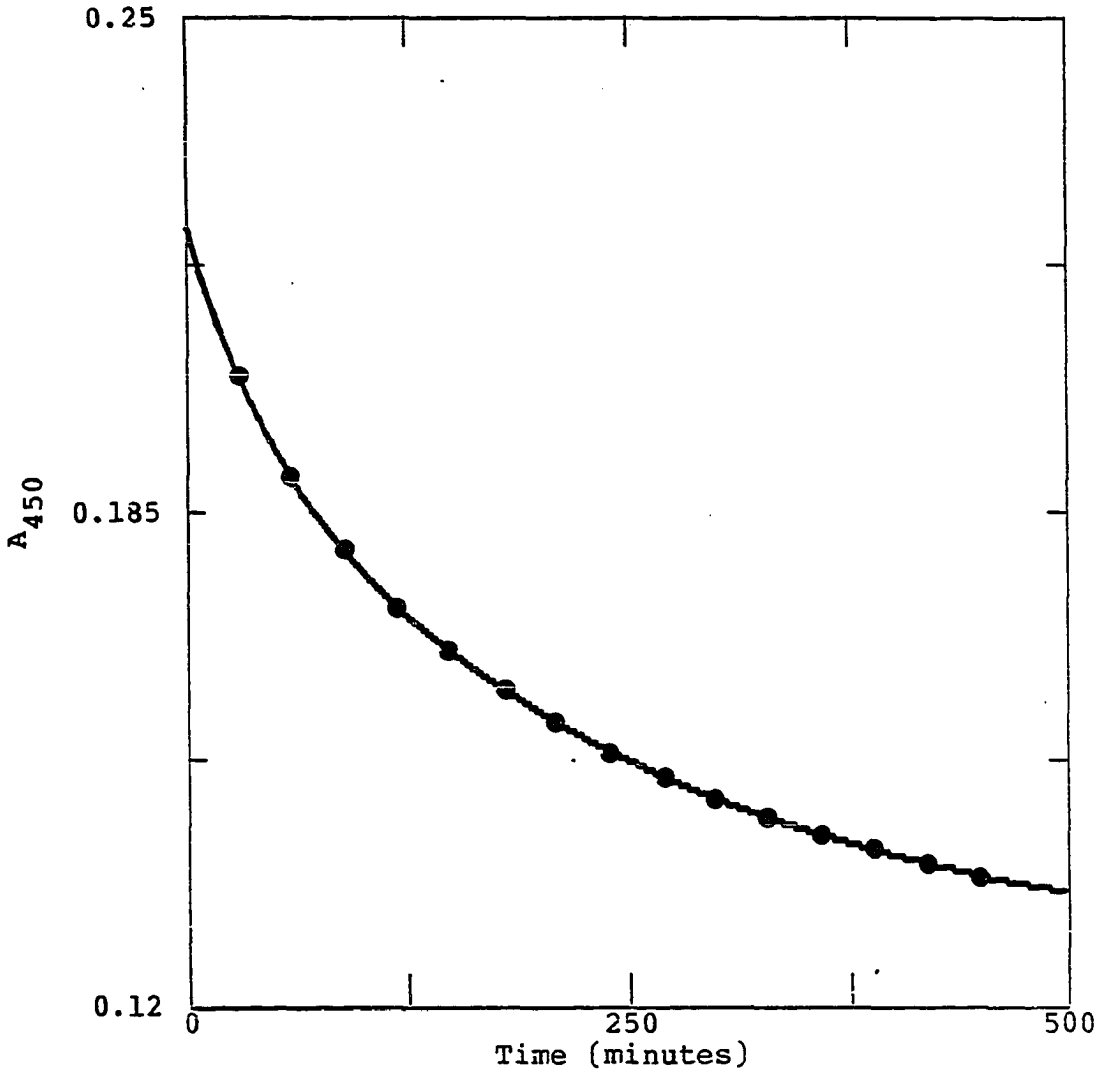
Three μM cytochrome b_5 in 10 mM phosphate buffer pH 7.5, 25 °C, 100 μM NADH, 100 μM metHr, 3 U/ml cytochrome b_5 reductase, one scan every 30 minutes starting from highest A_{340}. Last scan obtained at 630 minutes.

Figure III-28. Absorbance decreases during reduction of metHr by reductase system: high cytochrome b_5 concentration



End product, (last spectrum), of system reaction in Figure III-28 (lower line), and after the addition of air (upper line).

Figure III-29. Changes in absorbance upon exposure to air of end product of metHr-reductase system reaction at pH 7.5



1.7 μM cytochrome b_5, 100 μM NADH, 100 μM metHr, 3 U/ml cytochrome b_5 reductase in 10 mM phosphate buffer pH 7.5, 150 mM Na_2SO_4, 25 °C.

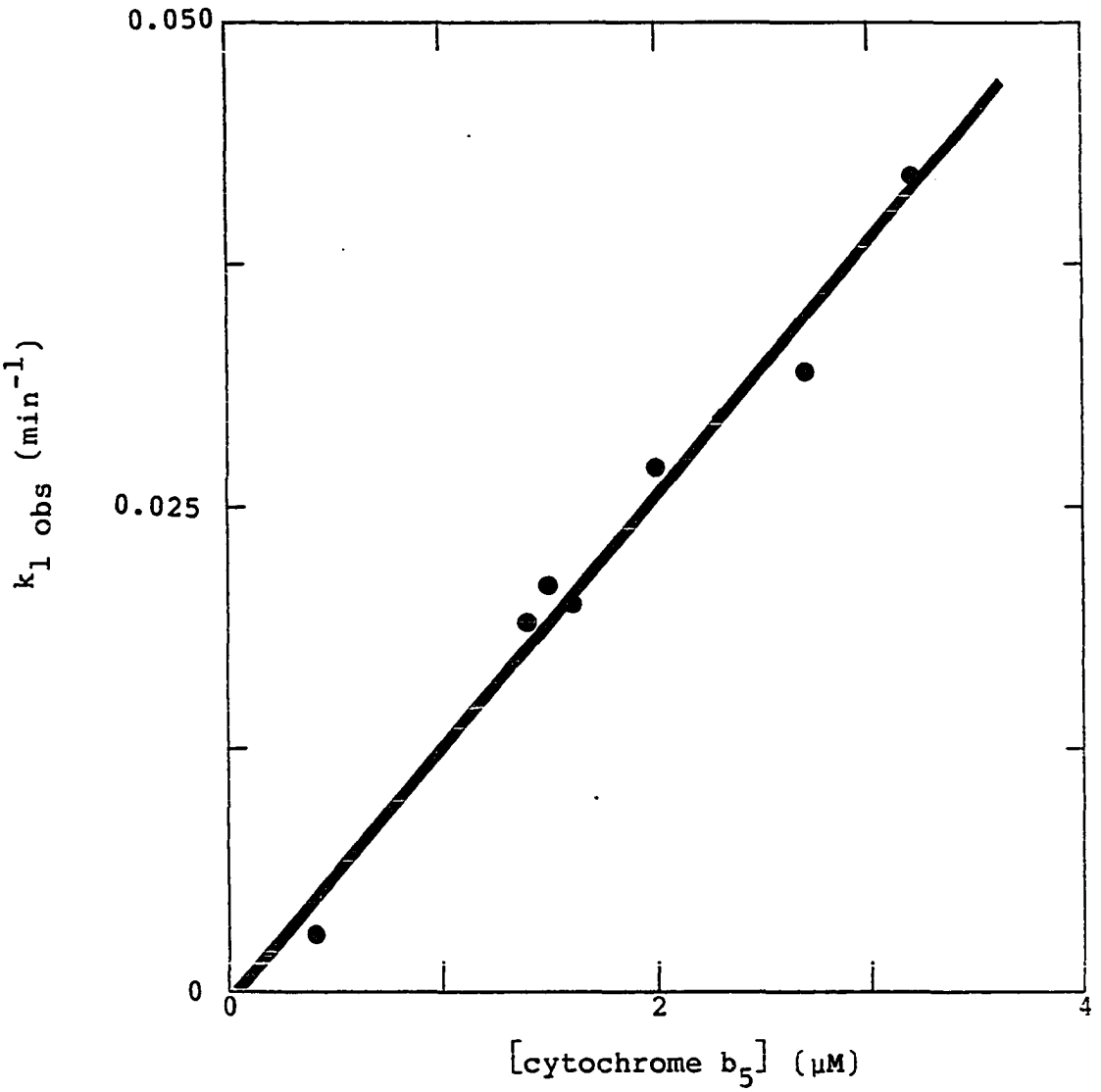
Figure III-30. Two-exponential fit of ΔA_{450} versus time for reduction of metHr by reductase system

Figure III-31. The second order rate constant for this reaction is 200 $M^{-1}s^{-1}$, close to the value obtained for the reduction of metHr by cytochrome b_5 under the same conditions (160 $M^{-1}s^{-1}$). The second phase of the reduction is not dependent on cytochrome b_5 concentration, and has a rate constant of $5 \pm 2 \times 10^{-5}$ s^{-1}. Table III-14 summarizes these data.

Each phase of the reduction of metHr has a characteristic absorbance change associated with it. Figure III-32 is the spectrum of metHr at pH 7.5, and the spectrum of $(semi\text{-}met)_R Hr$ produced by the addition of two equivalent of dithionite to the metHr. Below 400 nm, the absorbance changes are quite dramatic, but at 450 nm the loss of absorbance on reduction of metHr to $(semi\text{-}met)_R Hr$ is 20% of the total absorbance change in the metHr to deoxyHr reduction. In the reductase system reductions of metHr, the loss in absorbance in the first phase is 18% ± 4% of the total absorbance change (Table III-14). This result agrees very well with the conversion of metHr to $(semi\text{-}met)_R Hr$ showing that the first stage of the reductase system reaction is the reduction of metHr to $(semi\text{-}met)_R Hr$.

2. Effect of cytochrome b_5 reductase concentration

The reduction of metHr by the reductase system was studied with varying concentrations of cytochrome b_5 reductase. The results are shown in Figure III-33 and



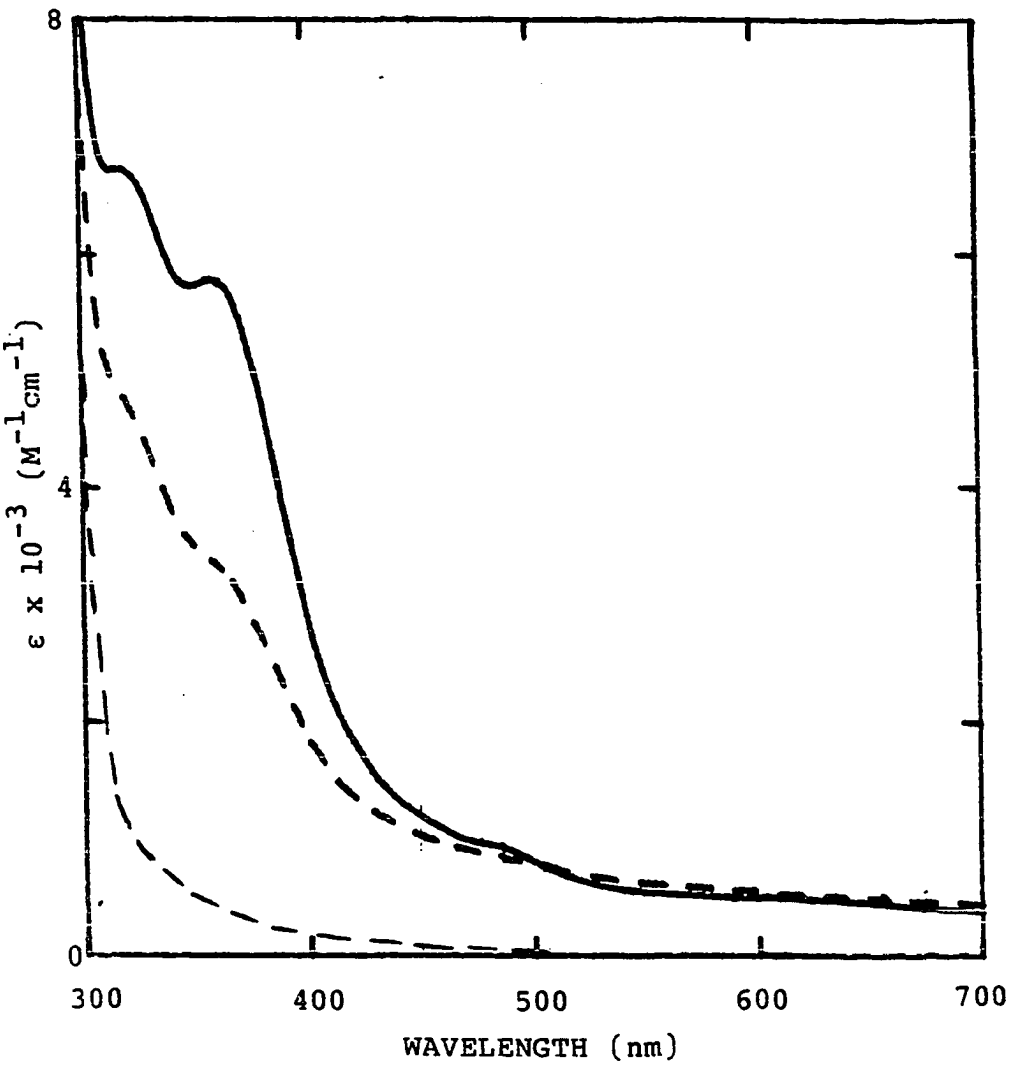
Ten mM phosphate buffer pH 7.5 with 150 mM Na_2SO_4, 100 μM metHr, 100 μM NADH, 3 U/ml cytochrome b_5 reductase.

Figure III-31. Dependence of first phase of reduction of metHr by the reductase system on P. gouldii cytochrome b_5 concentration

Table III-14. Kinetic data for reduction of metHr by the reductase system[a]

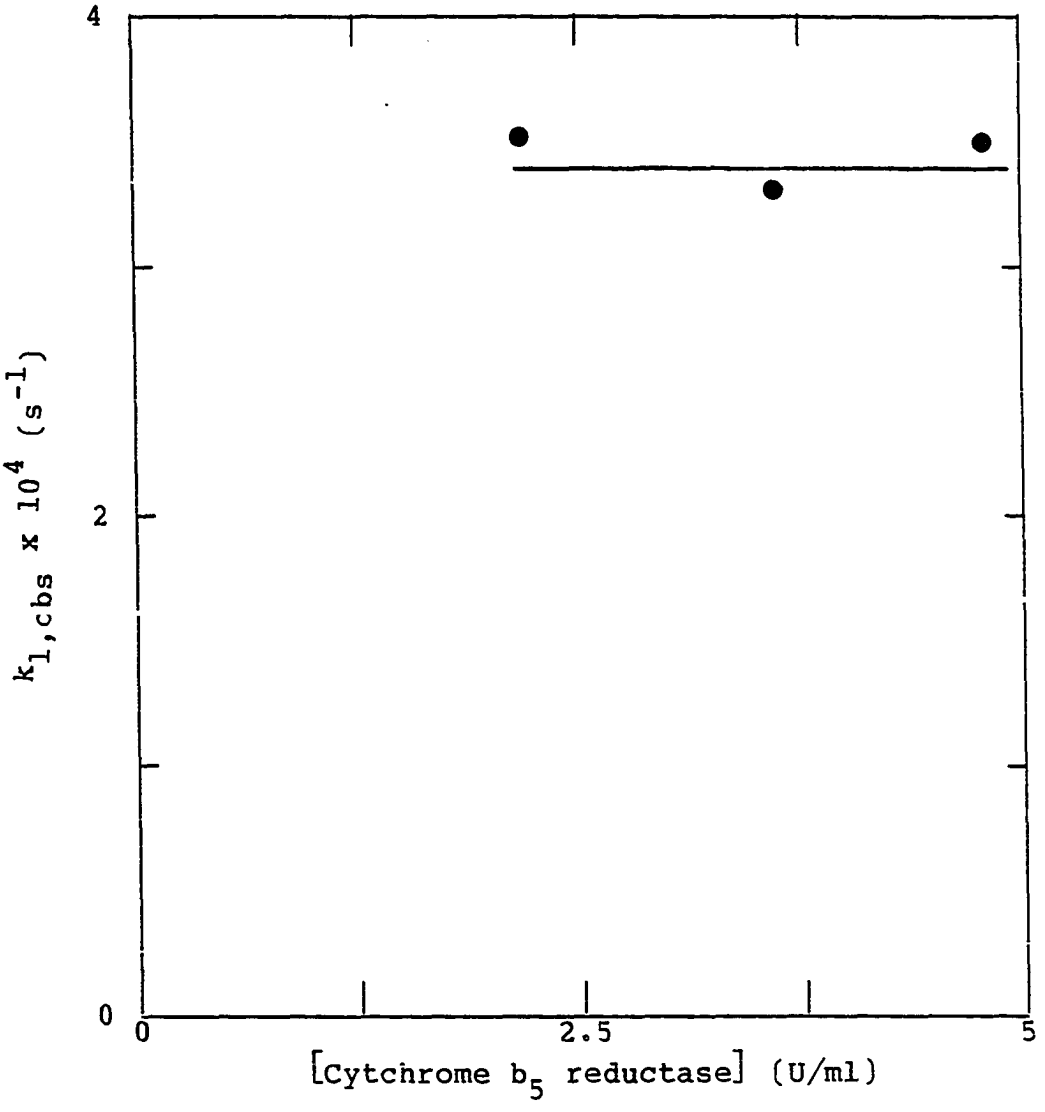
[cytochrome b_5] (μM)	$k_{1,obs}$ ($10^{-4}s^{-1}$)	$k_{2,obs}$ ($10^{-5}s^{-1}$)	% absorbance change in first phase
0.66	1.0	----	----
1.4	3.17	3.83	14
1.5	3.50	5.00	12
1.6	3.33	4.17	21
2.0	4.50	3.33	20
2.7	5.33	3.33	19
3.2	7.00	6.33	24

[a]25 °C, 10 mM phosphate buffer pH 7.5 with 150 mM Na_2SO_4, 3 U/ml cytochrome b_5 reductase, 100 μM metHr, 100 μM NADH.



MetHr in 10 mM phosphate buffer pH 7.5 with 150 mM Na_2SO_4 (solid line), $(semi\text{-}met)_R Hr$ produced by the addition of two equivalents of dithionite (upper dashed line), deoxyHr (lower dashed line).

Figure III-32. UV-visible spectra of metHr, $(semi\text{-}met)_R Hr$ and deoxyHr



1.5 μM cytochrome b_5, 54 μM metHr, 10 mM phosphate buffer pH 7.5, 150 mM Na_2SO_4, 25 °C.

Figure III-33. Dependence of $k_{1,obs}$ for the reduction of metHr by the reductase system on P. gouldii cytochrome b_5 reductase concentration

tabulated in Table III-15. When NADH, cytochrome b_5 reductase and metHr are incubated, there is no decrease in A_{450}, indicating no metHr reduction.

These results indicate that the reductase does not directly reduce metHr, as the increase in reductase concentration does not increase the rate of reduction. These results also show that increasing the concentration of cytochrome b_5 reductase does not increase the concentration of reduced cytochrome b_5. Therefore, under these conditions the amount of reduced cytochrome b_5 can be treated as a constant equal to the total amount of cytochrome b_5 present.

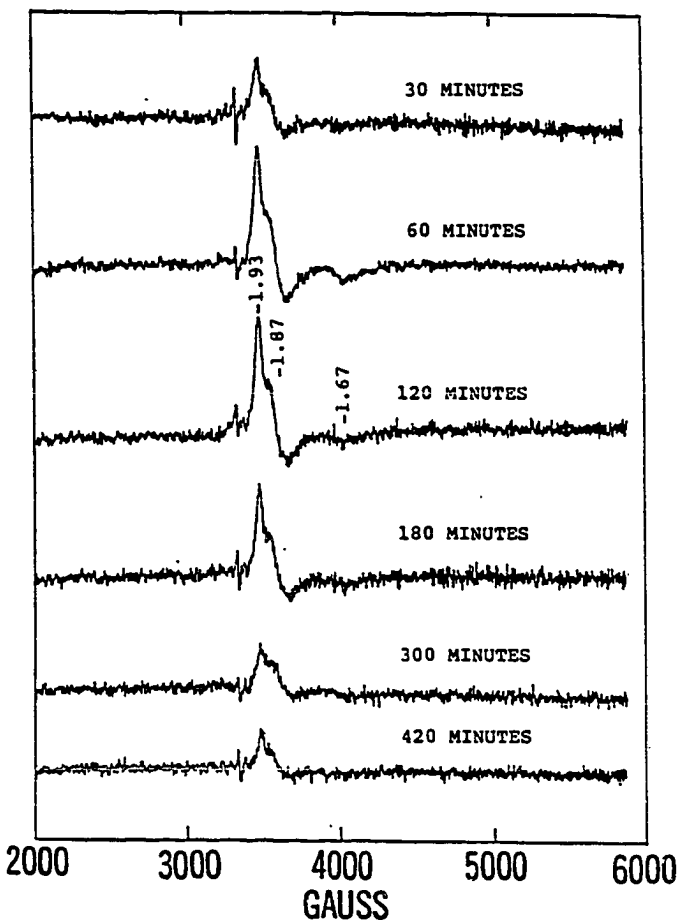
3. EPR of metHr reduction by reductase system

The reductase system reaction was followed by EPR. Three milliliters of an anaerobic sample of of 1.2 μM P. gouldii cytochrome b_5, 100 μM NADH, 100 μM metHr and 3 U/ml reductase was prepared and incubated at 25 °C. Tenth milliliter samples were removed and frozen in liquid nitrogen for EPR at various times. Figure III-34 shows the $(semi\text{-}met)_R Hr$ signals formed during the reaction time course. The g-values seen in the EPR are 1.93, 1.87, and 1.67. The $(semi\text{-}met)_R Hr$ signal intensity increased with time at the beginning of the reaction, and then decreased as expected for a reaction intermediate. All EPR signals in Figure III-34 have had a cavity signal near g=2 subtracted from them. Because the cavity signal was as

Table III-15. Effect of reductase concentration on the kinetics of reduction of metHr by the reductase system[a]

[reductase] (U/ml)	$k_1 \times 10^4$ (s^{-1})	$k_2 \times 10^5$ (s^{-1})
2.2	3.5	3.67
3.6	3.3	3.81
4.8	3.5	4.22

[a]25 °C, 1.5 μM P. gouldii cytochrome b_5, 10 mM phosphate buffer pH 7.5, 150 mM Na_2SO_4, 100 μM NADH, 100 μM metHr.



1.2 μM cytochrome b_5, 100 μM NADH, 100 μM metHr, 3 U/ml reductase in 10 mM phosphate buffer pH 7.5 with 150 mM Na_2SO_4, 25 °C. EPR conditions: Temperature 4 K; frequency 9.42 GHz; power 201 μW; gain 5.0 x 10^5; modulation 16 G at 100 kHz; time constant 0.10 sec; 5 scans.

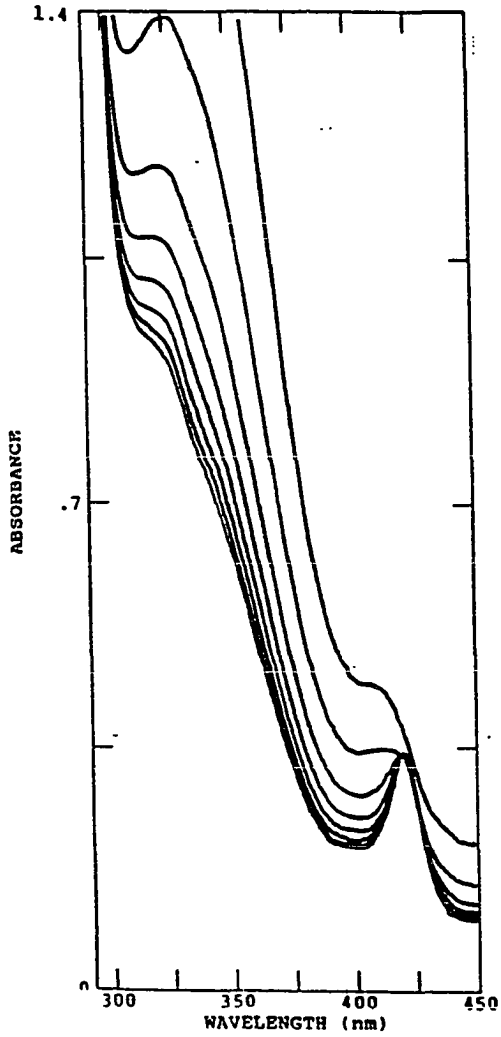
Figure III-34. EPR time course of reduction of metHr by the metHr reductase system

intense as the $(semi\text{-}met)_R Hr$ signal, signals were not integrated to give the concentrations of $(semi\text{-}met)_R Hr$ at any time. However, these results are fully consistent with the reduction of metHr by the reductase system occurring in two sequential one-electron processes.

4. Effect of pH on reduction of metHr by the reductase system

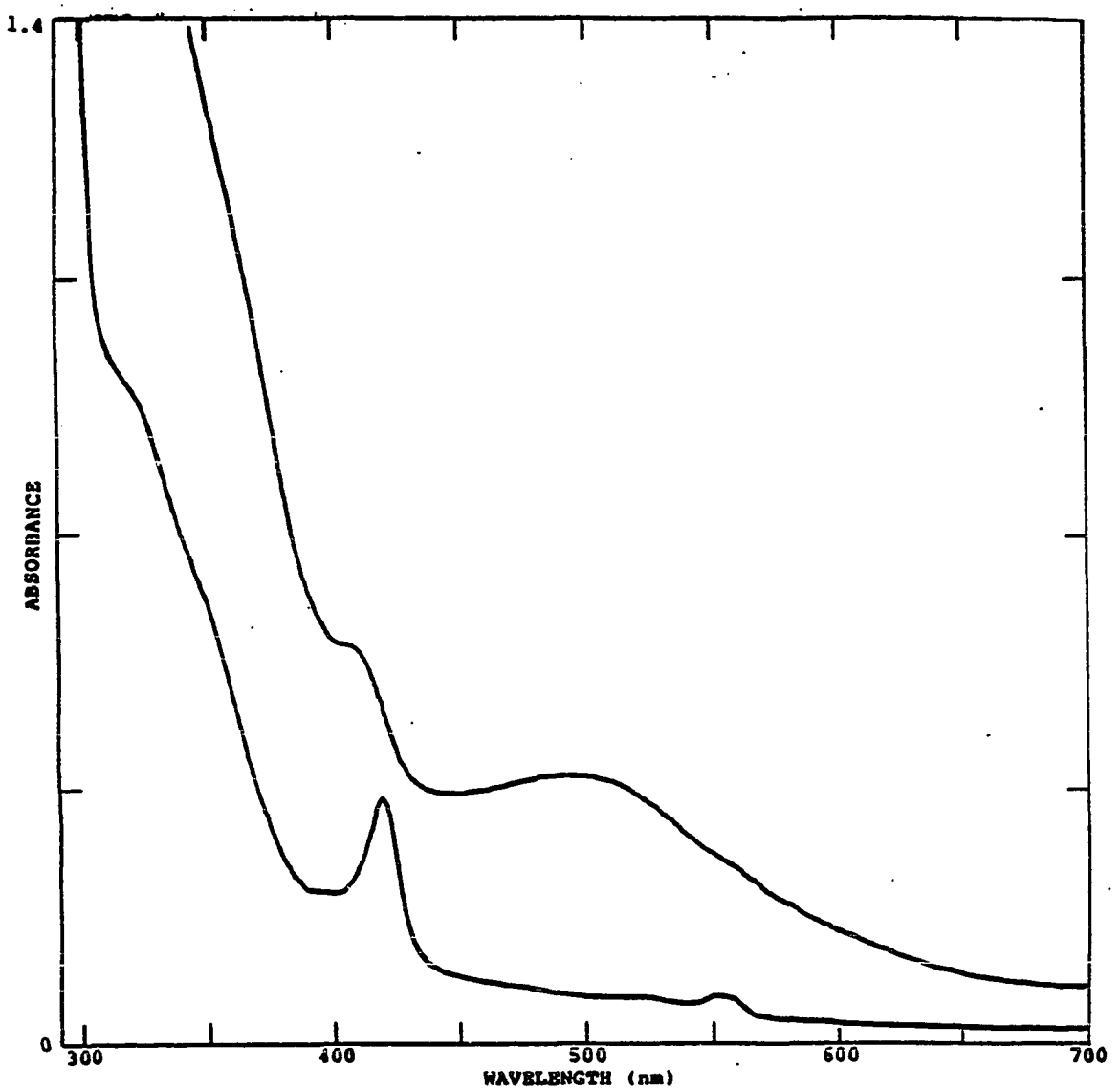
The reduction of metHr by the reductase system was studied at pH 6.5 and 7.0 in addition to pH 7.5, which was discussed above. The reaction mixtures consisted of 1.5 μM cytochrome b_5, 0.05 mM metHr, 0.12 mM NADH and 3 U/ml cytochrome b_5 reductase. The cytochrome b_5 was not totally reduced throughout during the early stages of the reaction. The oxidation state of the cytchrome b_5 could be determined by the position of the Soret peak. The metHr was converted to deoxyHr. The reaction was complete after four hours, much faster than at pH 7.5, where the reaction is complete in approximately eight hours (Figures III-27, III-28). The time course of this reaction at pH 6.5 is shown in Figure III-35. The end product of this reaction is shown in Figure III-36.

At pH 6.5 or 7.0 the reaction mixture is not as well-behaved as at pH 7.5. The reaction mixture is less soluble and over the time course of the reaction precipitation becomes a problem. The cytochrome b_5 reductase reduction of cytochrome b_5 is apparently slower at pH 6.5 than at pH 7.5,



1.5 μM cytochrome b_5 in 10 mM phophate buffer pH 6.5, 25 °C, 100 μM NADH, 100 μM metHr, 3 U/ml cytochrome b_5 reductase, one scan every 30 minutes, starting from highest A_{340}. Last scan obtained at 210 minutes.

Figure III-35. Absorbance decreases during reduction of metHr by reductase system at pH 6.5



End product, (last spectrum), of the system reaction in Figure III-35 (lower line), and after the addition of air (upper line).

Figure III-36. Absorption spectra of end product of the reductase system reaction at pH 6.5 before and after exposure to air

and does not allow for the full reduction of the cytochrome b_5 during the reaction time course under these conditions. The rate limiting step becomes electron transfer from cytochrome b_5 reductase to cytochrome b_5, rather than from cytochrome b_5 to metHr. The production of deoxyHr in a shorter amount of time at pH 6.5, than at pH 7.5 is apparently due to the faster reduction of $(semi\text{-}met)_R Hr$ at pH 6.5. A similar pH dependence is seen for reduction of metHr by inorganic reagents (20).

5. Effect of ionic strength on the reductase system

The effect of ionic strength on the reduction of metHr by the reductase system was not possible to study due to experimental difficulties. Precipitation was a problem when the reaction was run at concentrations of Na_2SO_4 lower than 100 mM. The resulting turbidity of the solution prevented determination of rate constants by absorbance changes. DeoxyHr was the end product of the reaction in 10 mM phosphate buffer pH 7.5, 25 °C, 100 lM NADH, 100 μM metHr, 3 U/ml cytochrome b_5 reductase, 25 °C, 420 minute reaction time as evidenced by the increase in A_{500} and developement of a red color upon oxygenation.

The effect of chloride on the reductase system is of obvious importance in an organism that lives in the sea and has a high concentration of chloride in the erythrocyte. Solubilized P. gouldii cytochrome b_5 reductase is inhibited by

500 mM NaCl but is still capable of reducing cytochrome b_5. The rate of reduction of metHr by cytochrome b_5 is slower in NaCl than is expected if the only function of the NaCl is to adjust the ionic strength.

The reductase system reaction was run in 10 mM phosphate buffer pH 7.5 with 500 mM NaCl present. During the course of the reaction, turbidity developed that prevented the calculation of a rate constant. The end product of the reaction after 420 minutes was deoxyHr as evidenced by the increase in A_{500} and development of a red color upon exposure to air.

J. Concentration of Reduced Glutathione in P. gouldii Erythrocytes

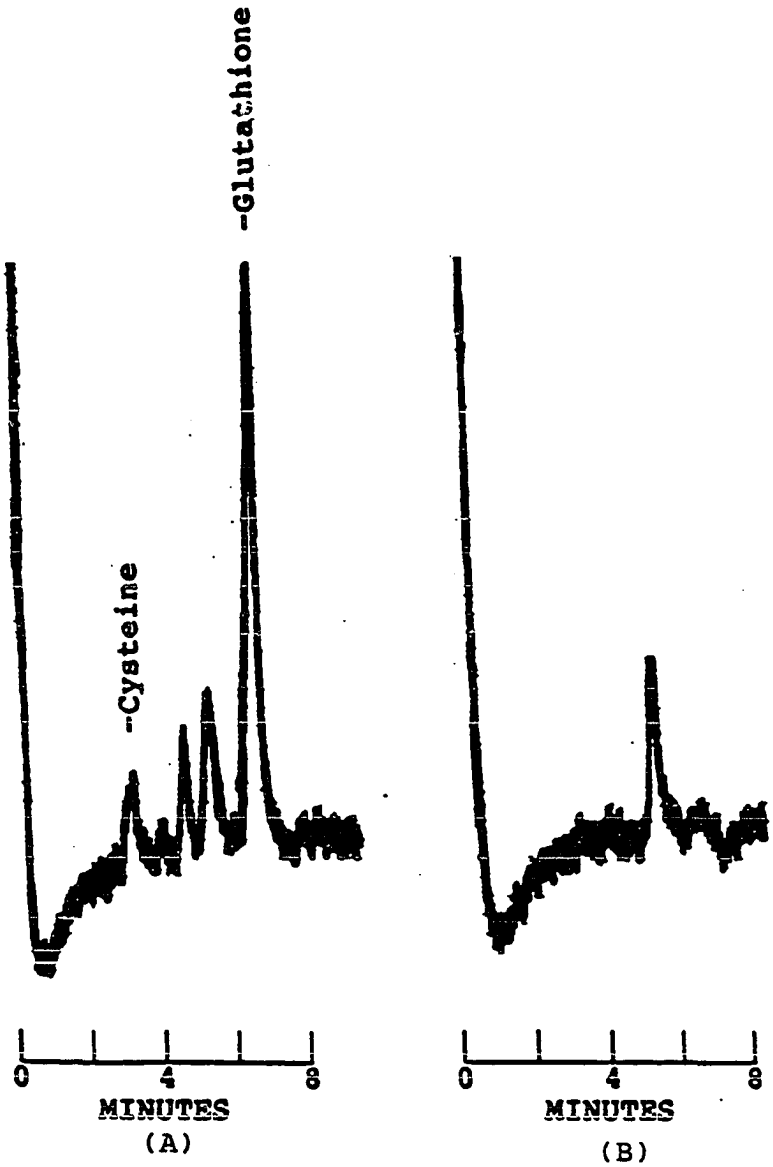
Four different thiols were detected in the erythrocytes of P. gouldii by HPLC and postcolumn reaction with 6,6'-dithiodinicotinic acid. Comparison of their retention times to those of pure thiols without postcolumn reaction showed one thiol to be reduced glutathione and another to be cysteine. Comparison of the peak heights to those of a standard curve gave a concentration of 0.97 mM glutathione and 0.10 mM cysteine. When the worms were deoxygenated in sealed plastic jars at 16 °C for 24 hours prior to conducting the assay, no reduced glutathione or cysteine was detected. The

HPLC traces for both the oxygenated and deoxygenated erythrocytes are shown in Figure III-37.

K. Reduction of MetHr by Reduced Glutathione

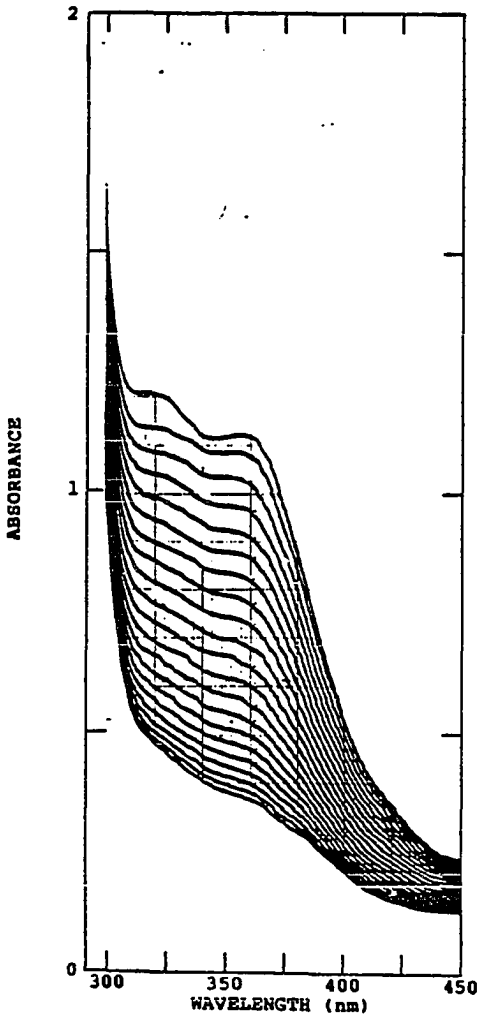
MetHr can be reduced by reduced glutathione (GSH). The reaction is very slow and difficult to follow due to precipitation of the protein over the long time course. Figure III-38 shows the absorbance time course of the reduction of metHr by GSH. EPR shows that $(semi\text{-}met)_R Hr$ is formed as an intermediate (Figure III-39). The end product of the reaction is deoxyHr, and is readily oxygenated upon exposure to oxygen. The observed rate constant for the reduction of metHr (0.22 mM) by GSH (18 mM) is 8×10^{-6} s^{-1}.

The EPR g-values seen in the $(semi\text{-}met)_R Hr$ formed by the reduction of metHr with reduced glutathione are the same as those normally seen with inorganic reducing agents (1.96, 1.86 and 1.65). The reaction of metHr by GSH is very slow, and could not reduce the metHr produced in the erythrocytes as fast as the cytochrome b_5 reductase system showing that GSH is probably not the primary pathway for metHr reduction. At pH 7.5 with 500 mM Cl^-, the maximium k_{obs} for the reduction of metHr in the erythrocyte with 0.3 μM cytochrome b_5 would be 3.6×10^{-5} s^{-1}. The maximium k_{obs} for 1 mM GSH would be 4.4×10^{-7} s^{-1}. Thus, the cytochrome b_5 metHr reduction pathway



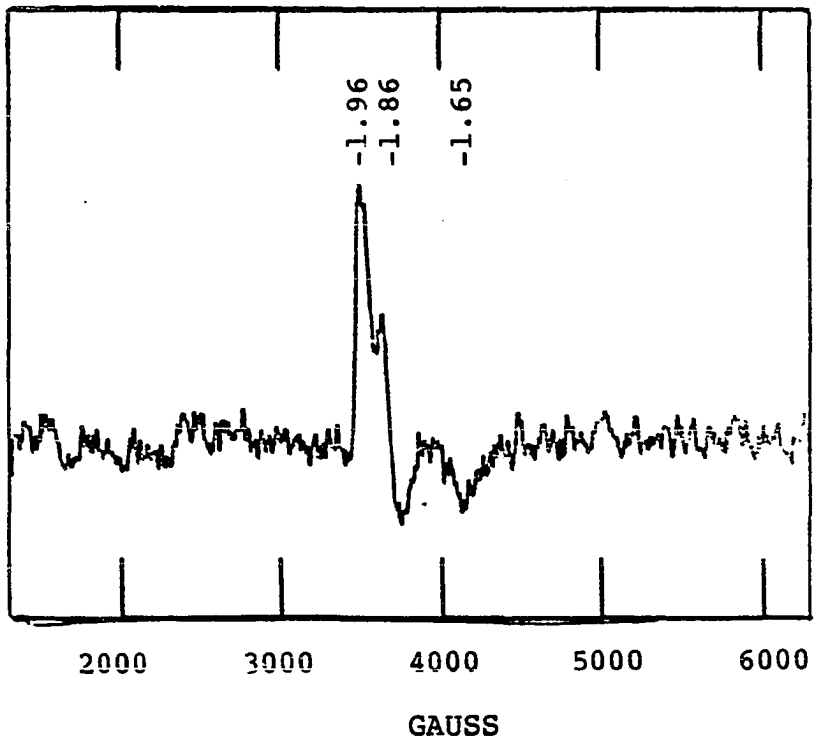
(A) Oxygenated worms; (B) Deoxygenated worms; A_{360} after postcolumn reaction with 6,6'-dithiodinicotinic acid.

Figure III-37. HPLC traces of cellular thiols



Addition of 18 mM GSH to 0.22 mM metHr in 50 mM Tris-$SO_4^=$ with 150 mM Na_2SO_4 at 25 °C, trace every two hours.

Figure III-38. Absorbance changes during reduction of metHr by GSH



0.44 mM metHr and 20 mM GSH incubated anaerobically for 2.5 hours in 80 mM phosphate buffer pH 7.0.
EPR conditions: Temperature 4 K; frequency 9.42 GHz; power 201 μW; gain 5.0 x 10^5; modulation 16 G at 100 kHz; time constant 0.10 sec; 5 scans.

Figure III-39. EPR spectrum of (semi-met)$_R$Hr formed by reduction of metHr by reduced glutathione

probably accounts for ~ 100 times more reduction of metHr than does the GSH pathway.

IV. CONCLUSION

The use of EPR to probe the redox state of the oxygen carrier Hr in the erythrocytes of sipunculids has shown the presence of semi-metHr. This semi-metHr is presumably formed as a one electron reduction product of metHr by the metHr reduction system. This reduction system has been isolated from the erythrocytes of P. gouldii and consists of cytochrome b_5 reductase and cytochrome b_5 with NADH acting as the electron donor. Electrons flow from NADH through cytochrome b_5 reductase to cytochrome b_5. Reduced cytochrome b_5 then reduces metHr to $(semi\text{-}met)_R Hr$. $(Semi\text{-}met)_R Hr$ is then further reduced to deoxyHr in a manner analogous to the manner seen with inorganic reducing agents (20). Cytochrome b_5 was also isolated from T. zostericola erythrocytes, strongly suggesting that cytochrome b_5 is a common component of Hr containing erythrocytes. The erythrocytes of T. zostericola also display a $(semi\text{-}met)_R Hr$ EPR signal. These similarities indicate that the same redox pathways may be involved in both species and that the results reported in this thesis represent a generalized set of properties of sipunculan erythrocytes. These results set the stage for detailed studies of the mechanisms of electron transfer between each pair of components in the system.

The build-up of $(semi\text{-}met)_R Hr$ in the erythrocytes is seen

after a period of stress due to low oxygen availability. The reason for this build-up may be a build-up of metHr in the erythrocytes during the anaerobic incubation, which allows more production of $(semi\text{-}met)_R Hr$ when the erythrocyte is reoxygenated and normal metabolism is resumed. The coelomic fluid of intact S. nudus incubated anaerobically becomes acidic due to the production of acetate, succinate, and propionate (73). It has also been reported that the pH in P. gouldii erythrocytes decreases on anaerobic incubation (74). The anaerobic incubation of Hr containing erythrocytes under acidic conditions leads to the irreproducible production of metHr (75). This increased production of metHr could explain the $(semi\text{-}met)_R Hr$ signal in the erythrocytes incubated anaerobically.

The properties of P. gouldii cytochrome b_5 are very close to those of human erythrocyte cytochrome b_5. Table IV-1 summarizes the properties of P. gouldii and human erythrocyte cytochrome b_5.

The maximum rate constant for reduction of metHr to $(semi\text{-}met)_R Hr$ by 0.3 μM reduced cytochrome b_5 in the erythrocyte at pH 7.5 with 500 mM Cl^- would be (120 $M^{-1}s^{-1}$) x (0.3 x 10^{-6} M) = 3.6 x 10^{-5} s^{-1}. The reduction of $(semi\text{-}met)_R Hr$ to deoxyHr has a rate constant of 5 x 10^{-5} s^{-1}. These rates are apparently sufficient to keep metHr reduced. If all Hr present in the erythrocyte (10 mM, Table III-1) were

Table IV-1. Comparison of cytochromes b_5 from human and P. gouldii erythrocytes

	Human	P. gouldii
molecular weight	13,700[a]	14,000
pI	4.3[a]	3.7-3.9
g-values	3.03[b]	3.07
	2.21	2.22
	1.39	1.4
Scret band		
oxidized	413 nm[c]	412 nm
reduced	423 nm	422 nm
α-band	556 nm	555 nm
β-band	527 nm	526 nm
midpoint reduction potential	0 mV[a,d]	7 mV[e]
Concentration in the erythrocyte	0.22 μM[f]	0.3 μM

[a]Reference 27.
[b]Reference 44.
[c]Reference 43.
[d]Corrected to μ = 0.5 (69).
[e]500 mM NaCl.
[f]Reference 53.

in the met form and the cytochrome fully reduced, the rate of reduction of metHr would be 1.3 mM/hour. This value is very close to the 1 mM/hour observed for the rate of reduction of metHb when measured directly in the human erythrocyte or when calculated from the reconstituted metHb reduction system (52).

Another possible reduction system for metHr has also been isolated but is less efficient than the cytochrome b_5 system. Glutathione is capable of reducing metHr and one millimolar reduced glutathione is present in the erythrocytes of P. gouldii. The reduction of metHr by GSH is very slow with a rate constant of 8×10^{-6} s^{-1} for reduction of 0.22 μM by metHr by 18 mM GSH or presumably 4.4×10^{-7} s^{-1} (1 mM GSH) in the erythrocyte, a value of about 1% that of the cytochrome b_5 system.

One question that could be asked of the P. gouldii cytochrome b_5-metHr reaction is if there is any specificity of interaction between these two partner proteins. It has been postulated that no specificity occurs in biological electron transport systems (57). DeoxyMb is capable of reducing metHr, but is obviously not a physiological reducing agent. The rates of reduction of metHr by both P. gouldii cytochrome b_5 and deoxyMb may be compared after correction for potential and self-exchange rate. When these corrections are made, the rate of reduction of metHr by these two reagents are the same within an order of magnitude suggesting little or no

specificity of P. gouldii cytochrome b_5 for metHr at least as far as increasing the rate of electron transfer is concerned (32).

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VI. APPENDIX: PURIFICATION OF A COELOMIC HEME PROTEIN

The successful isolation of cytochrome b_5 from both P. gouldii and T. zostericola has prompted a survey for other heme proteins in P. gouldii. The following procedure was developed to isolate a heme protein from the coelomic fluid.

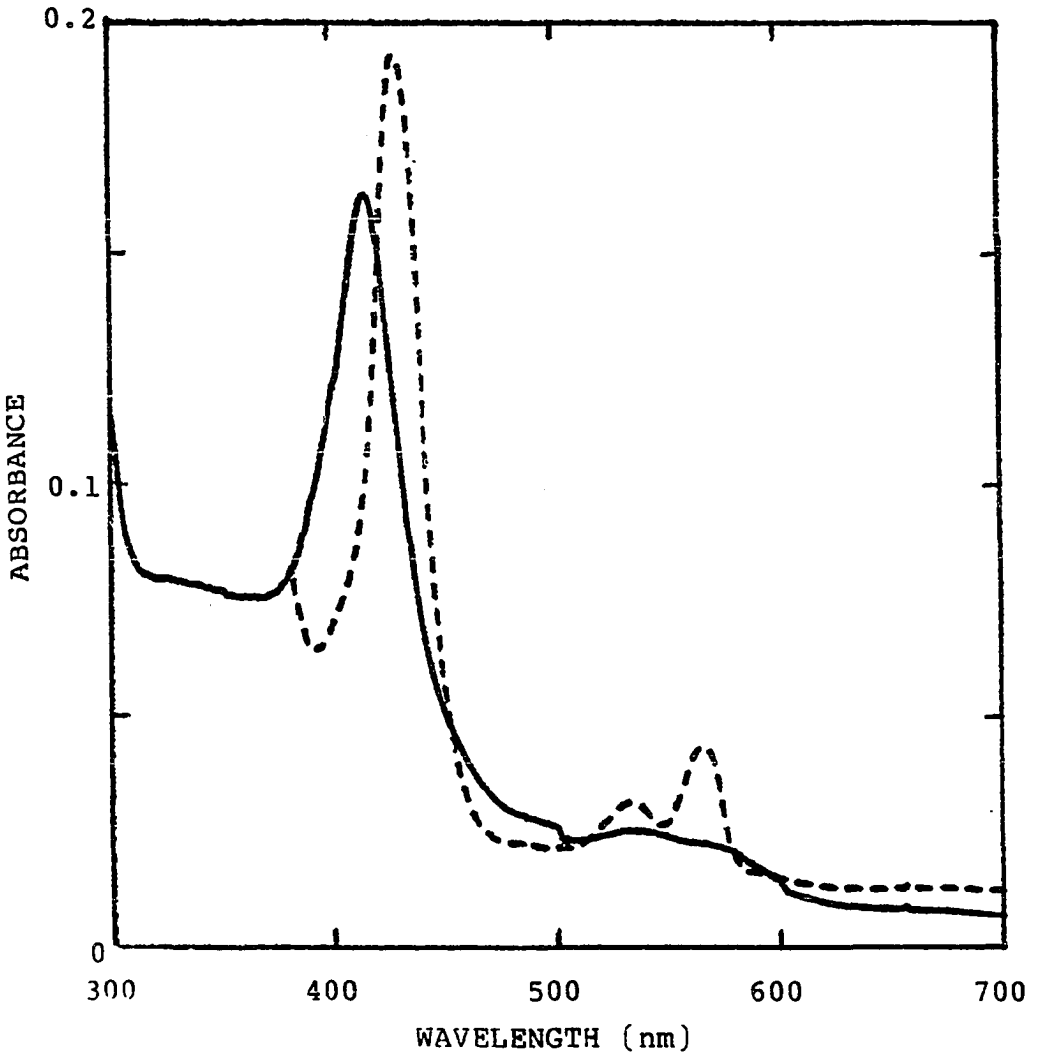
The coelomic fluid is centrifuged at 26,000 g for two hours to remove fatty particles and other debris. The coelomic fluid is dialyzed overnight at 4 °C against 10 mM phosphate buffer pH 7.7 to remove the salt. A white precipitate forms and is removed by centrifugation at 26,000 g for one hour. To obtain a good yield of the coelomic heme protein, the evaporating dish into which the worms are bled must be kept on ice as the blood is drained into it. The centrifugation and dialysis must be carried out at 4 °C and as quickly as possible. The loss of protein with time and at warmer temperatures is presumably due to proteases from the digestive systems of the worms that are damaged during slicing.

The coelomic fluid is passed over a 2 x 10 cm Sephadex A-25 column equilibrated with 10 mM phosphate buffer pH 7.7. The contaminating Hr elutes from the column while a yellow band of protein sticks to the top of the column. The column is flushed with 10 mM phosphate buffer pH 7.7 until the wash is clear. The heme protein is eluted from the A-25 column

with 300 mM NaCl in 10 mM phosphate buffer pH 7.7 and then dialyzed against the eluting buffer. The heme protein is concentrated in an Amicon cell using a PM-30 membrane. Contaminating proteins pass through the membrane, as evidenced by absorbance at 280 nm, but the filtrate contains no Soret absorbance. The concentrated heme protein was passed over a 0.5 x 5 cm Sephadex G-50 column equilibrated in 10 mM phosphate buffer pH 7.7 with 300 mM NaCl. The protein separates into two colored bands of which only the slower eluting band contains a Soret absorbance. The purified protein is then concentrated for use. If 300 mM NaCl is not present at this point, the heme protein will precipitate.

Figure VI-1 shows the spectra of the coelomic fluid heme protein. The dithionite reduced form of the protein has maxima at 566 and 534 nm with a Soret maximum at 429 nm. The oxidized form has a maxima at 535 nm with a shoulder at 570 nm and a Soret maximum at 415 nm.

The coelomic heme protein does not react with CO indicating strong axial ligation of the iron. The type of cytochrome, a, b, or c, may be determined by extraction of the heme in alkaline pyridine solution. Addition of 150 mM NaOH and 25% (v/v) pyridine, and subsquent reduction by sodium dithionite shows an absorbance maximum at 559 nm. This agrees quite well with the value of 556 nm reported for b-type cytochromes (76).



10 mM phosphate buffer pH 7.7 with 300 mM NaCl; oxidized form (solid line), dithionite reduced form (dashed line).

Figure VI-1. Absorption spectra of the coelomic heme protein