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On reagents that convert cytochrome oxidase from an inactive to an active coupling state

(respiratory control/release of control/activity-inducing reagents/reversibility of induction/control of coupling)

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ABSTRACT **Cytochrome oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) of beef heart mitochondria, prepared by a standard method and brought to the highest purity level, is essentially inactive when tested in the aerobic assay involving oxidation of reduced cytochrome *c* by molecular oxygen. Three reagents (lysolecithin, Tween 20, and exogenous phospholipids) can convert cytochrome oxidase from an inactive to an active coupling state. These conversions are reversible: i.e., removal of the inducing agent leads to loss of activity. The evidence for the intrinsic coupling capability is that cytochrome oxidase in the active state invariably generates a proton gradient during respiration, and such gradient formation is demonstrable even when cytochrome oxidase is not inserted into a liposome.**

Cytochrome oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) is an intrinsically coupled system in the sense that electron transfer in the unsupplemented complex is coupled to some process that is intrinsic to cytochrome oxidase. The demonstration of this intrinsic coupling capability does not require supplementation with other coupling systems such as factors F_0–F_1 or coupling devices such as valinomycin–K^+, nor does it require introduction of cytochrome oxidase into a liposome. An essential prerequisite for studying intrinsic coupling in cytochrome oxidase has been the recognition that cytochrome oxidase prepared by standard methods is inactive and that it can be converted to an active coupling state by a limited set of inducers. In the active state electron transfer in cytochrome oxidase invariably generates a proton gradient that is the hallmark of both a membrane and a coupled reaction. Elsewhere we shall be presenting evidence that intrinsic coupling in cytochrome oxidase can be identified as cyclical ion transport. The present communication deals with reagents that induce the active state with the formation of a characteristic proton gradient by the activated oxidase.

There is an extensive literature dealing with the phenomena covered in the present communication (1–4), but up to now the distinction between intrinsic and extrinsic coupling has been neither made nor recognized. This distinction is the element of novelty in the presentation that follows and by virtue of the experiments based on this distinction it has been possible to shed new light on coupling in cytochrome oxidase.

MATERIALS AND METHODS

Materials. Phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, cardiolipin, phosphatidylglycerol, lysolecithin (lysophosphatidylcholine), cytochrome *c*, and tetramethylphenylenediamine were all products of Sigma. Asolectin was obtained from Associated Concentrates (Woodside, NY); lysophosphatidylethanolamine from P-L Biochemicals; Tween 20 and Tween 80 (polyoxyethylene sorbitol esters) from Nutritional Biochemicals; and Triton X-100 (polyoxyethylene *p*-*t*-octylphenol) from Rohm and Haas (Philadelphia, PA).

Preparation of Cytochrome Oxidase. The standard preparation of cytochrome oxidase previously described by Fry *et al.* (5) contained about 6–8% phospholipid by weight. The high-lipid preparation (20% phospholipid by weight) was obtained by omitting the last two exposures of the enzyme to cholate and ammonium sulfate as described in the standard preparation. The low-lipid preparation (1% phospholipid by weight) was obtained by exposing a standard cytochrome oxidase preparation to repeated precipitations with ammonium sulfate in the presence of cholate according to the method of Tzagoloff and MacLennan (6).

Preparation of Phospholipid Complexes of Cytochrome Oxidase. The procedure of Hunter and Capaldi (7) based on the procedure of Hinkle *et al.* (8) and Hinkle (9) was used to prepare phospholipid complexes of cytochrome oxidase in weight ratios varying from 1:1 to 1:20 (cytochrome oxidase to phospholipid).

Assay of Cytochrome Oxidase Activity. Activity was measured at 36°C with an oxygen electrode according to the method of Wharton and Griffiths (10) except for the omission of asolectin. The assay mixture (7 ml) was 50 mM in potassium phosphate (pH 7) and was saturated with air. It contained 0.1 ml of 5.5 mM cytochrome *c* and 0.1 ml of 2 M Tris/ascorbate. The reaction was usually initiated by the addition of cytochrome oxidase (about 50 μg of protein). Because the aliquot used for the assay was taken from a solution of cytochrome oxidase containing 10–50 mg of protein per ml, the aliquot introduced into the assay mixture was necessarily small and the dilution correspondingly high. The activities reported in this study were calculated from the linear part of the oxygen uptake curve, corrected for the relatively low activity of the assay mixture in the absence of cytochrome oxidase.

RESULTS

Induction of cytochrome oxidase activity by lysolecithin

When the standard preparation of cytochrome oxidase (6–8% phospholipid) was assayed for activity under conditions specified in *Materials and Methods*, the result was either zero or close to zero, but activity could be induced by addition of an appropriate amount of lysolecithin (Table 1). It should be mentioned that the activity of the standard preparation of cytochrome oxidase, even when the enzyme is stored frozen at −20°C, deteriorates with time and thus the maximal activity achievable by addition of lysolecithin can vary over wide limits (15–50 μmol of atomic oxygen per min per mg of protein) de-

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Abbreviation: cyt. oxidase, cytochrome oxidase.

Table 1. Response of standard cytochrome oxidase (6–8% phospholipid) to the inducing action of lysolecithin

Exp.	Additions	Activity, μmol O/min per mg protein
1	Cyt. oxidase	1
	Cyt. oxidase + lysolecithin	28
2	Cyt. oxidase	0
	Cyt. oxidase + lysolecithin	21
3	Cyt. oxidase	2
	Cyt. oxidase + lysolecithin	20
	Cyt. oxidase + lysolecithin + cholate	35
	Cyt. oxidase + cholate	0

The final concentration of lysolecithin in experiments 1–3 was 0.14 mg/ml and the final concentration of cholate in experiment 3 was 1%. These two reagents were used from concentrated aqueous solutions and added directly to the assay mixture. Cyt. oxidase, cytochrome oxidase.

pending upon the period of storage of the enzyme as well as other factors such as the concentration of bile acids, etc. No incubation was necessary for the induction of activity by lysolecithin, and the effect was demonstrable as soon as the addition to the assay mixture had been made. The induction could not be duplicated by cholate, deoxycholate, uncouplers, or Triton X-100, each tested at various concentrations, but cholate could often enhance the inductive effect of lysolecithin even though inactive in its own right. Lysophosphatidylethanolamine acted in a similar fashion to lysolecithin. The same inductive effect of lysolecithin was demonstrable with cytochrome oxidase preparations containing 1% and 20% bound phospholipid (Table 2). This result suggested that the inductive effect of lysolecithin was independent of the concentration of endogenous phospholipid in preparations of cytochrome oxidase. Admittedly the maximal attainable and inducible activity was lowest in a low-lipid preparation and highest in a high-lipid preparation, but the possibility of induction was independent of the variable of the endogenous lipid concentration in any preparation of cytochrome oxidase. The inducible activity of cytochrome oxidase with 1% lipid was surprisingly high.

Fig. 1 (curve a) shows the activity of cytochrome oxidase as a function of the concentration of lysolecithin. When lysolecithin was added to a concentrated suspension of cytochrome oxidase (20–30 mg/ml) and then a small aliquot (containing 50 μg of enzyme) was withdrawn for introduction into the assay mixture, it was found that the concentration of lysolecithin required to achieve a particular enzyme activity was about 1/20th the concentration of lysolecithin required if added directly to the assay mixture containing cytochrome oxidase

Table 2. Lysolecithin effect on enzyme activity of low- (1%) and high- (20%) lipid preparations of cytochrome oxidase

Additions	Activity, μmol O/min per mg protein
Low-lipid cyt. oxidase	1
Low-lipid cyt. oxidase + lysolecithin	34
High-lipid cyt. oxidase	3
High-lipid cyt. oxidase + lysolecithin	72

The final concentration of lysolecithin was 0.14 mg/ml in the assay mixture.

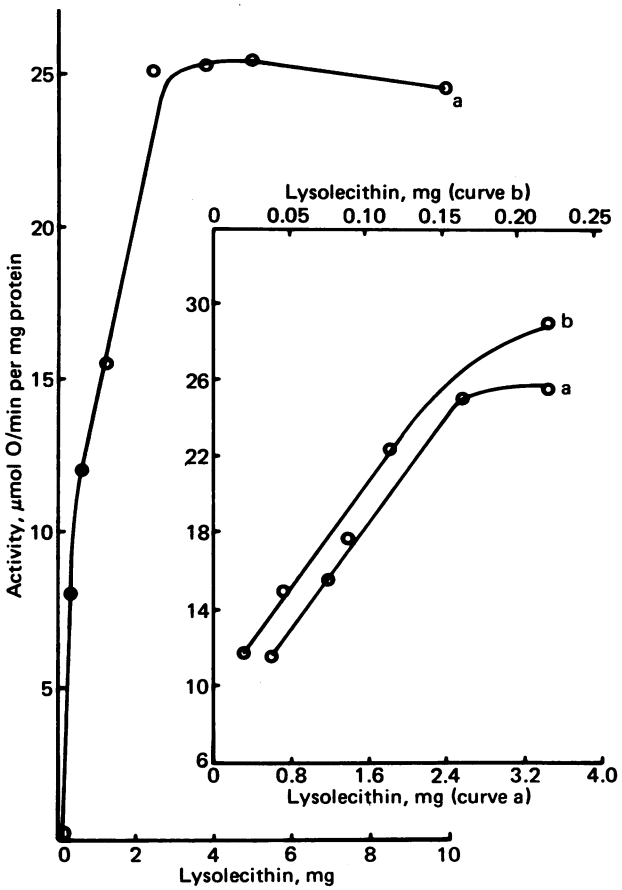

FIG. 1. Cytochrome oxidase activity as a function of the concentration of lysolecithin. Curve a, no preincubation of lysolecithin and cytochrome oxidase before addition of lysolecithin to the assay mixture; curve b, preincubation of lysolecithin and cytochrome oxidase before addition of the two components to the assay mixture. In the preincubation experiments, the concentration of the cytochrome oxidase suspension was between 20 and 40 mg/ml.

(curve b of Fig. 1). We take this result to mean that the interaction of lysolecithin with cytochrome oxidase is some 20-fold more efficient when the reactants are combined in concentrated solution, compared to interaction when the reactants are several hundredfold more dilute. Note that the abscissa scale for the concentration of lysolecithin in curve a is 16 times the corresponding scale for curve b. In the *Inset* of Fig. 1 showing the relationship of curves a and b, we have selected the experimental values in the middle range of each curve. The concentration of cytochrome *c* required to achieve a particular activity was correspondingly lower when cytochrome oxidase and lysolecithin were combined in concentrated solution in the presence of cytochrome *c* (data not shown). Apparently the affinity of the enzyme for cytochrome *c* increases when lysolecithin and cytochrome oxidase combine.

The inductive effect of lysolecithin can be eliminated by exhaustive washing of lysolecithin-activated suspensions of cytochrome oxidase. This result shows that the induction is not permanent and depends upon the continued presence of the inducer.

Induction of cytochrome oxidase activity by Tween 20

Under the conditions described above for demonstration of lysolecithin induction, the activity of a standard cytochrome oxidase preparation can be induced by addition of Tween 20 in an appropriate amount. The activity of cytochrome oxidase as a function of the concentration of Tween 20 is shown in Fig.

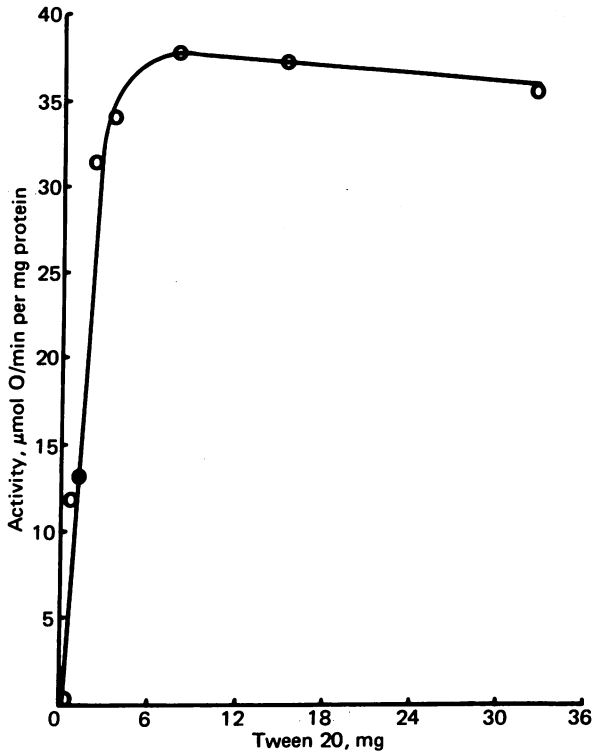

FIG. 2. Cytochrome oxidase activity as a function of the concentration of Tween 20.

2. When Tween 20 interacted with cytochrome oxidase in concentrated solution, the interaction was about 4–5 times more efficient than the interaction in dilute solution (data not shown). The induction was reversible, because exhaustively washed preparations of cytochrome oxidase previously activated with Tween 20 lost their activity and required reactivation with this detergent. The activation of cytochrome oxidase by Tween 20 was independent of the endogenous lipid content of the enzyme (tested in preparations of cytochrome oxidase varying from 1 to 20% in phospholipid content). Tween 80 was also active as an inducer, but much higher concentrations were required to achieve the same activity induced by lower concentrations of Tween 20.

Induction of cytochrome oxidase activity by exogenous phospholipids

We have found that the interaction of a preparation of standard cytochrome oxidase with any of the usual set of phospholipids induces the inactive-to-active transition (Table 3). All the phospholipids tested were allowed to interact in a 20-fold excess by weight compared to the weight of cytochrome oxidase. The interaction took place in concentrated solution and then an aliquot containing 50 μg of cytochrome oxidase and 1 mg of phospholipid was introduced into the assay bowl. Interaction of enzyme and phospholipid required the presence of cholate; the phospholipid solution was first sonicated in the presence of 2% cholate to yield either a solution or a fine suspension and then added to the concentrated enzyme. The final concentration of cholate was 2% in the interaction mixture *prior to* removal of the appropriate aliquot. The interaction was carried out at 0°C and no incubation period was found necessary.

The data of Table 4 show that the weight ratio of phospholipid to cytochrome oxidase need not be 20:1 to achieve maximal activity. For some phospholipids a 1:1 ratio was found to be sufficient, though in other cases maximal activity required relatively high ratios.

Table 3. Enzyme activities of different phospholipid complexes of cytochrome oxidase

Phospholipid	Activity, μmol O/min per mg protein
None	0
Phosphatidylserine	60.6
Phosphatidylcholine	39.4
Asolectin	81
Phosphatidic acid	158.4
Phosphatidylinositol	93.6
Cardiolipin	60.0
Mitochondrial phospholipid	38.8
Phosphatidylserine/phosphatidylethanolamine/ phosphatidylcholine 1:1:1 by wt	12
Phosphatidylserine/phosphatidylethanolamine/ phosphatidylcholine 1:2:4 by wt	27
Cardiolipin/phosphatidylethanolamine/ phosphatidylcholine 1:1:1 by wt	23

The phospholipid to cytochrome oxidase weight ratio was 20:1 in all cases. The enzyme was preincubated with the phospholipid in presence of 2% cholate before an aliquot was introduced into the assay mixture.

Lysolecithin could augment the activity of certain phospholipid complexes of cytochrome oxidase but had little effect on other such complexes (Table 5). There would seem to be no simple rule to explain this synergistic effect of lysolecithin because phospholipid complexes such as that between cytochrome oxidase and mitochondrial phospholipid, which show relatively high activity alone, nonetheless showed augmented activity when lysolecithin was present in the assay mixture.

Removal of cholate by dialysis or precipitation led to the loss of activity of relatively few cytochrome oxidase–phospholipid complexes (notably asolectin, mitochondrial phospholipid, and phosphatidylserine). The rest were unaffected by the presence or absence of cholate once the interaction had taken place. The initial interaction, however, *always* required the presence of cholate.

When the phospholipid complex of cytochrome oxidase was resolved into its component elements by fractionation with

Table 4. Induction of respiratory activity by exposure of inactive cytochrome oxidase to phosphatidylinositol, phosphatidylethanolamine, and phosphatidic acid in various weight ratios in presence of 2% cholate

Phospholipid	Weight ratio of cyt. oxidase to phospholipid	Activity, μmol O/min per mg protein
Phosphatidylinositol	1:1	25
	1:5	27
	1:10	44
	1:20	54
Phosphatidyl- ethanolamine	1:1	24
	1:5	35
	1:20	124
Phosphatidic acid	1:1	44
	1:5	42
	1:10	46
	1:20	51

Cytochrome oxidase (*ca.* 25 mg/ml) was mixed at 0°C with phospholipid in the specified weight ratios. The phospholipid solutions were made by sonicating the phospholipid in 2% cholate to a final concentration of 50 mg/ml before addition to the enzyme.

Table 5. Augmentation of activity by the cytochrome oxidase–phospholipid complex when supplemented with lysolecithin

Exp.	Phospholipid	Weight ratio of cyt. oxidase to phospholipid	Activity, μmol O/min per mg protein: No addition	Activity, μmol O/min per mg protein: With lysolecithin
1	Phosphatidylcholine	1:20	25	46
	Asolectin	1:20	28	25
2	Phosphatidylcholine	1:5	27	24
3	Mitochondrial phospholipid	1:5	19	50
4	Phosphatidylinositol	1:5	48	54
	Phosphatidylserine	1:5	43	56
	Phosphatidylglycerol	1:5	40	45
	Phosphatidic acid	1:5	63	82
	Mitochondrial phospholipid	1:5	51	93

The final concentration of lysolecithin in the assay mixture was 0.14 mg/ml and of Tween 20, 2% by volume. The phospholipid–cytochrome oxidase complexes were formed by interaction of the two components in the designated weight ratios in the presence of 2% cholate prior to assay.

cholate and ammonium sulfate, then the cytochrome oxidase so derived was inactive. It should be remarked that the phospholipid-induced augmentation of cytochrome oxidase activity can usually exceed that induced by lysolecithin or Tween. This high augmentation applies to only a few phospholipids (see Table 5). Such variability in the maximal achievable activity by the set of inducers considered thus far would suggest that there is a continuum of active states of cytochrome oxidase, each with its own characteristic maximal activity.

Proton gradient formation by cytochrome oxidase activated by lysolecithin or Tween 20

Fig. 3 shows the characteristic proton gradient formed during respiration of cytochrome oxidase activated by lysolecithin or Tween 20. What is notable about these gradients is that the rate of proton uptake is constant for the duration of the respiration. The gradient appears as soon as respiration begins and disappears when oxygen in the reaction mixture is exhausted. The orientation of cytochrome oxidase under the assay conditions must be that of submitochondrial particles because an alkaline gradient is found.

DISCUSSION

The conclusion to be drawn from the experiments described in this report is that cytochrome oxidase is a coupling system that can fluctuate between an active and an inactive state, and that this fluctuation is inducible by specific reagents. Moreover, coupling in cytochrome oxidase does not depend upon its insertion into a phospholipid liposome. In fact, the liposome is merely another inducer of the active state. That means that the vagaries of introducing cytochrome oxidase into a phospholipid liposome, and there are many such, are irrelevant to the question of what determines coupling in cytochrome oxidase. The data we have presented establish that coupling activity can be induced in cytochrome oxidase even when the phospholipid content has been reduced to 1% and that the requirement for inducer is an inherent property of cytochrome oxidase regardless of the lipid content of the enzyme. There is a misconception that cytochrome oxidase exists in the membrane state

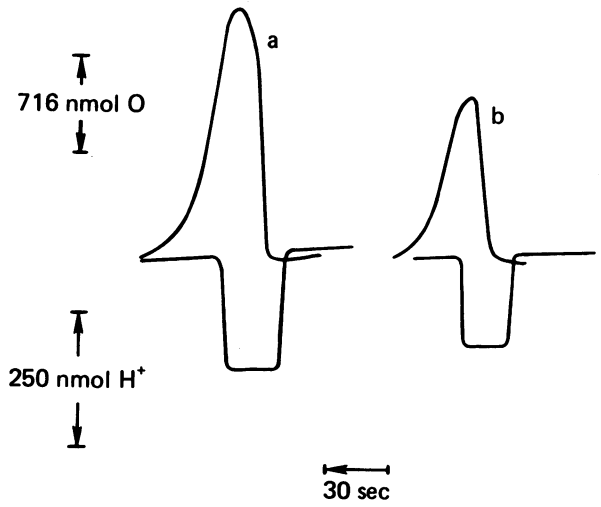

FIG. 3. Generation of a proton gradient by soluble cytochrome oxidase. The reaction mixture contained 1 mM Tris·HCl at pH 7.0, 50 mM KCl, and 250 mM sucrose. To 7 ml of reaction mixture was added 100 μl of 5 mM cytochrome *c* in water, 10 μl of 25 mM tetramethylphenylenediamine in ethanol, 200 μl of a saturated solution of durohydroquinone in ethanol, and 10 μl of catalase (25 mg/ml). After anaerobic conditions had been established, a suitable aliquot of H_2O_2 was added to produce an "oxygen pulse." In experiment a, the assay mixture contained 100 μl of a stock solution of soluble cytochrome oxidase (35 mg of protein per ml, as prepared) and 200 μl of a 10% (wt/vol) solution of Tween 20. In experiment b was added 40 μl of stock cytochrome oxidase and 100 μl of a 50 mg/ml solution of lysolecithin in water. The reaction mixture was maintained at 36°C.

only when inserted into a liposome. But we have shown that the capability for generating proton gradients that is the hallmark of the membrane state is manifested whenever cytochrome oxidase is converted to the active coupling state by lysolecithin or Tween 20. In other words, the membrane state of cytochrome oxidase is inducible even when the phospholipid content is at the catalytic level. There is the possibility that the transition from an inactive to an active state is in fact a transition from a state in which a membrane cannot be formed to a state in which a membrane can be formed. Exogenous phospholipid is not essential for this transition. It is replaceable by lysolecithin or Tween 20. It is curious that the concept of a membrane generated exclusively by a protein system with catalytic amounts of phospholipid should be derivative from a study of the determinants of coupling in cytochrome oxidase.

We have considered three reagents that induce the conversion of cytochrome oxidase from an inactive to an active state. What do these reagents have in common? Either these reagents can interact with the endogenous phospholipid of cytochrome oxidase to form mixed micelles (with lysolecithin or Tween 20) (11) or they can mix with the endogenous phospholipid to form a new mixture of phospholipids (with exogenous phospholipids) (12). In both cases the element in common is the modulation of the phospholipid associated with the coupling unit. This commonality suggests that the phospholipid of the coupling unit is the component that undergoes the transition from the inactive to the active state. It is relevant to point out that lysolecithin and Tween 20 can profoundly affect the transport properties of phospholipid measured in a two-phase system (11).

Cytochrome *c*, the specific reductant for cytochrome oxidase, forms lipid-soluble complexes with phospholipids (13, 14). Is there any connection between lipid–cytochrome *c* complex formation and the transition in cytochrome oxidase? In the inactive state, cytochrome *c* readily forms complexes with

added lipid, and yet this abundance of bound cytochrome *c* cannot be utilized to satisfy the requirement for cytochrome *c* when the oxidase is activated by lysolecithin. It would thus appear that lipid cytochrome *c* cannot be the form of cytochrome *c* that reacts with cytochrome oxidase or at least not the form that is required for the initial reaction in the coupled process.

Perhaps the most fundamental point in this report to be emphasized is that the endogenous phospholipid of cytochrome oxidase is always in an inactive state in the absence of modulation, and this is true even when cytochrome oxidase is incorporated into liposomes of mitochondrial phospholipid. It is the combination of mitochondrial phospholipid and cholate that leads to release of respiratory control. When the cholate is removed by dialysis, respiratory control is reestablished (8). Certain combinations of phospholipids that mimic the composition of mitochondrial phospholipid can also impose respiratory control in cholate-free liposomes of cytochrome oxidase (15–17).

The reversible conversion of cytochrome oxidase from an inactive to an active coupling state should be looked upon as a control phenomenon. There are now many signs that the cellular control of coupling function may very well involve control of the state of the relevant coupling units. We can thus see the extension of the phenomena that underlie the control of coupling in cytochrome oxidase to the general problem of the control of coupling physiologically.

It is not generally appreciated that gradient formation is a token of a coupled reaction carried out in a membrane. A proton gradient can be formed only by separating an electron from a proton and a proton from its associated anion. Such charge separations are the characteristics of coupled systems. The proton gradients formed by cytochrome oxidase provide critical clues as to the mechanism of the intrinsic coupled reaction carried out by the complex.

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