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Bound Cytochrome c as Proton Donor and Acceptor During Enzymic Oxidoreduction

D.E. Green and H. Vande Zande

Institute for Enzyme Research
University of Wisconsin
Madison, WI 53706

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SUMMARY: When ferrocytochrome c reacts with delipidated cytochrome oxidase under conditions which prevent oxidation, one proton is taken up per molecule of ferrocytochrome c bound to cytochrome oxidase. When ferricytochrome c reacts with delipidated Complex III, one proton is released per molecule of ferricytochrome c bound to Complex III. From these data it can be concluded that the oxidation of ferrocytochrome c by cytochrome oxidase leads to the release of a proton and an electron, whereas the reduction of ferricytochrome c by Complex III leads to the uptake of a proton and an electron. Thus ferrocytochrome c like QH_2 and NADH is both an electron and proton donor, and ferricytochrome c like Q and O_2 is both an electron and proton acceptor. The pattern for the three mitochondrial electron transfer sequences NADH → Q, QH_2 → ferricytochrome c and ferrocytochrome c → O_2 involves separation of an electron and proton on the side of the membrane where electron transfer is initiated and recombination of an electron and a proton in the terminal acceptor on the side of the membrane where electron transfer terminates.

INTRODUCTION: When ferrocytochrome c is oxidized by Ca^{2+}-state mitochondria, protons are released on the exterior (I side) of the inner membrane; and when the same reaction sequence is studied in electron transfer particles, protons are taken up on the interior side (M side) of the membrane (1). Furthermore, the H^+/e ratio is unity for both the release in mitochondria and the uptake in the electron transfer particles. The simplest conclusion to be drawn from these observations would be that oxidation of ferrocytochrome c leads to the separation of an electron and a proton on one side of the inner membrane (the electron moving into the membrane and the proton being extruded), whereas the reduction of oxygen involves electron-proton recombination on the other side of the membrane (the electrons brought in via the transmembrane chain and the protons originating from the aqueous space on the M side of the membrane). However, there is a strong impression in the literature that cytochrome c is exclusively an electron donor or acceptor (2); and thus ferrocytochrome c would not be expected to be the source of a proton when oxidized. While it is indeed correct that the oxidoreduction of free cytochrome c at pH 7.0 does not involve protonic change, there is no evidence to

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exclude such involvement in the oxidoreduction of bound cytochrome c. Rodkey and Ball (3) have shown that at pH 7.8 and higher the oxidation-reduction potential of cytochrome c is indeed pH dependent--a token of the involvement of a proton in the oxidoreduction. Thus the capability of free cytochrome c to act as a proton donor and acceptor is demonstrable at alkaline pH. Binding of cytochrome c to Complexes III and IV (the complexes in which it is reduced and oxidized, respectively) may lower the pH at which protonic changes accompany oxidoreduction. The present communication is addressed to the testing of this possibility. Since bound ferrocytochrome c releases a proton on oxidation, we would predict that the binding of ferrocytochrome c to cytochrome oxidase would lead to proton uptake. Similarly since bound ferricytochrome c takes up a proton during reduction, we would expect the binding of ferricytochrome c to Complex III to lead to proton release. In other words, there should be a group in bound cytochrome c that dissociates a proton in the ferro to ferri transition and takes up a proton in the ferri to ferro transition. Were such the case, the negatively charged ionizable group in the protein would serve as the charge partner for the positive charge generated by oxidation of ferrocytochrome c. In the reduction of ferricytochrome c this ionized group would become protonated.

The question of the origin of the proton released during energy coupling is crucial to an understanding of the mechanism of energy coupling and once resolved the uncertainty with respect to two issues (the theoretical value for the H^+/e ratio and the role of the proton in energy coupling) can also be resolved.

EXPERIMENTAL METHODS:

Protonic change during interaction of ferricytochrome c with Complex III. Complex III, prepared by the method of Rieske (4), was delipidated by the method of Fry and Green (5, 6). Triton X-100 (the detergent used in the delipidation procedure) was removed from Complex III by exchange with potassium cholate. This was achieved by passing a solution of Complex III, solubilized with Triton X-100, through a LH-20 column equilibrated with 50 mM Tris chloride, 1% in potassium cholate. After elution from the column, all samples were reduced in volume by dialysis against carbowax and then dialyzed against 100 vol of a solution, 2 mM in Tris chloride (pH 7.6) and 50 mM in choline chloride for 72 hr at 5° with two changes of buffer. H^+ release was measured at 25° in a thermostatted cell containing in 5 ml total volume the delipidated complex (10-30 mg) and ferricytochrome c in excess. The diluent used to bring the volume to 5 ml was the external dialysis mixture of Tris and choline chloride which had been equilibrated with the solution of Complex III. The samples of Complex III were brought to the pH of the solution of ferricytochrome c (ca 25 mg prot/ml) by addition of 5 µmol of Tris base and then back titrated to pH 7.5 - 7.75 (the range of pH values to which Complex III and ferricytochrome c were adjusted before mixing). After the pH of the two solutions were adjusted to the same value and the recording electrode was stable, aliquots of ferricytochrome c were added until no further change in pH was observed. Standard HCl was then added to calibrate the observed changes in pH. A Corning combination electrode fed into a Beckman expandomatic pH meter (SS-2)

Table 1. Proton release during binding of ferricytochrome c ($Fe^{3+}c$) to Complex III at pH 7.5 - 7.75

Complex III added (mg) (3.64 nmol/mg)	Residual Triton X-100 in Complex III	H^+ released on addition of excess $Fe^{3+}c$	% Theory for uptake of $1H^+$/molecule of $Fe^{3+}c$ bound
11.2	0	35	86
14.7	0	51	95
16.6	0	56	93
13.5	+	44	88
19	+	43	64
28	+	51	50

See Experimental Methods for description of the procedures used to delipidate Complex III and for other details.

was used for measuring pH. The electrode measurements were recorded on a Soltec Rikadenki Recorder (Mark II). The titration was conducted under N_2.

Protonic change during interaction of ferrocytochrome c with Complex IV. Complex IV prepared by the method of Fry et al. (7) was depleted of bulk lipid (one pass through a LH-20 column) by the method of Fry and Green (5, 6) A 5.5 mM solution of ferricytochrome c was reduced with a slight excess of dithionite and the solution was aerated to oxidize excess reductant. The pH was adjusted to 6.9 with dilute HCl and the solution was diluted to 3.3 mM with water. 40 µl of this solution was mixed with 3 ml of the following mixture: 1.8 ml of 0.25 M sucrose, 1 ml of 1.95% cytochrome oxidase (12 nmol a heme/mg prot), 0.1 ml of 5% lysolecithin and 100 µl of 0.5 M sodium azide (to prevent enzymic oxidation). The pH of the mixture was brought to the same pH as that of the 3.3 mM solution of ferrocytochrome c before mixing of the two solutions and recording the pH change with a glass electrode. The observed pH change was standardized by addition of a known amount of 0.1 N HCl to the reaction mixture after the reaction had taken place.

The H^+/e ratio for the oxidation of bound NADH. The electron transfer sequence, NADH → Q, mediated by Complex I can be selectively studied in heavy beef heart mitochondria in presence of pyruvate + malate as substrates for electron transfer, ferricyanide as oxidant and antimycin or cyanide as inhibitor to suppress oxidation between QH_2 and oxygen. Ferricyanide makes it possible to carry out a precise titration with a sharp end point. Bound NADH is generated by enzymic reduction of endogenous NAD^+ by pyruvate + malate. The extent of reduction of ferricyanide is a precise measure of the electron flux originating from bound NADH. Hence the H^+/e ratio can be evaluated by measuring simultaneously the electron flux via reduction of ferricyanide and the release of protons electrometrically. This ratio can also be measured in presence of exogenous NAD^+ or in absence of pyruvate + malate and in presence of exogenous NADH. The concentrations of reagents used in these studies are specified in the legend for Table 3. Heavy beef heart mitochondria were prepared by the method of Hatefi and Lester (8).

RESULTS: Table 1 shows that Complex III delipidated by the procedure described in Experimental Methods releases a proton on interaction with ferri-

Table 2. Uptake of H^+ accompanying the binding of ferrocytochrome c ($Fe^{2+}c$) to cytochrome oxidase (turnover suppressed by sodium azide)

Cytochrome oxidase added (nmol)	$Fe^{2+}c$ added (nmol)	H^+ taken up (nmol)	Theory for uptake of $1H^+$/molecule of $Fe^{2+}c$ bound (nmol)
116	132	122	116
116	132	100	116

See Experimental Methods for description of the procedures used to delipidate Complex IV and for other details.

cytochrome c. The binding of ferricytochrome c to Complex III as measured by the release of a proton is not stoichiometric. The maximal release of protons is achieved at a lower molar ratio of ferricytochrome c to Complex III when Complex III is essentially free of Triton X-100 (ratio about 1.5). When significant residual amounts of Triton X-100 are present in Complex III, this ratio increases markedly, particularly at high concentrations of Complex III. The values for the ratio of H^+ released per molecule of cytochrome c bound were sufficiently close to 1.0 (0.86, 0.95, 0.93) that we may safely assume that the theoretical ratio is indeed unity.

Table 2 shows that the interaction of ferrocytochrome c with delipidated cytochrome oxidase leads to the uptake of a proton. The same experiment was repeated five times with similar results. This interaction is essentially stoichiometric as judged by the fact that proton uptake per molecule of ferrocytochrome c is maximal when the concentrations of the two reactants are exactly equal. The molar ratio of H^+ taken up to cytochrome c bound is close to unity (observed 1.1 and 0.86). To prevent oxidation of ferrocytochrome c when bound to cytochrome oxidase, 170 mM sodium azide was added to the system. At this high concentration of inhibitor the enzymic reaction was completely suppressed.

Green et al. (1) unwittingly carried out basically the same experiment as shown in Table 2. They found that in the cytochrome oxidase reaction mediated by Ca^{2+}-state mitochondria, protons were released concomitant with energized influx of K^+, whereas in the same reaction mediated by a lysolecithin dispersion of cytochrome oxidase protons were taken up concomitant with energized influx of K^+. In the Ca^{2+}-state mitochondrial system no addition of cytochrome c was made since the concentration of the bound form of cyto-

chrome c is sufficient for the reaction to proceed maximally. But addition of exogenous cytochrome c was mandatory for lysolecithin dispersions of cytochrome oxidase since these do not contain bound cytochrome c. In this system the binding of ferrocytochrome c to cytochrome oxidase leads to the uptake of a proton and the oxidation of bound ferrocytochrome c leads to the release of a proton. Thus the oxidative release of a proton is matched exactly by the binding-dependent uptake. The net result is that the profile for ΔH^+ of the lysolecithin dispersion of cytochrome oxidase appears to be out of phase with the profile for ΔK^+. When exogenous cytochrome c is added to Ca^{2+}-state mitochondria carrying out the coupled cytochrome oxidase reaction, the same anomaly was observed, namely the apparent uptake of protons accompanying the energized influx of K^+. These observations confirm that binding of ferrocytochrome c to cytochrome oxidase leads to proton uptake under energy coupling conditions.

In the electron transfer sequence from NADH to O_2 there are three complexes (I, III and IV), three electron transfer substrates (NADH, QH_2 and ferrocytochrome c) and three terminal electron transfer acceptors (Q, ferricytochrome c and O_2). If the pattern of "dehydrogenation" on one side of the membrane and hydrogenation on the other side is general for each complex, then each electron transfer substrate should yield a proton for each electron released ($H^+/e = 1$). This relationship would be correct for the oxidation of QH_2 and ferrocytochrome c but oxidation of NADH is assumed to yield only one proton for each two electrons released. This assumption is, in fact, implicit in the designation NADH. The data of Table 3 show that the H^+/e ratio for oxidation of NADH by Complex I is in fact unity--a token that the oxidation of bound NADH to NAD^+ leads to the release of two protons and two electrons. This 1:1 molar relation of proton to electron is observed only under conditions in which bound or exogenous NAD^+ is reduced to NADH by pyruvate + malate. When NADH is added directly to the mitochondrial system in absence of pyruvate + malate, only one proton per two electrons is released. What this means is that the 1:1 modality is operative when NADH is generated by enzymic reduction of NAD^+ (bound or exogenous), whereas the 0.5:1 modality is operative when enzymic reduction is eliminated. This discrepancy would suggest that bound NADH can exist either in a form compatible with the 1:1 modality or in a form compatible with the 0.5:1 modality. The critical requirement for the 1:1 modality may be the state of ionization of the phosphate residue vicinal to the positively charged nitrogen atom of the pyridine ring. If protonated, the 1:1 modality applies: if ionized, the 0.5:1 modality applies. Enzymic reduction may be a prerequisite for protonation of the cri-

Table 3. Stoichiometry of proton release during mitochondrial oxidation of NADH (bound vs. free) by ferricyanide

State of mitochondria	Additions	H^+ released/$K_3Fe(CN)_6$ added mol/mol
Freshly prepared	P-M*	1.02
Frozen & thawed	P-M	1.04
Frozen & thawed	NAD^+ + P-M	0.93
Frozen & thawed	NADH	0.45

*P-M = Pyruvate + Malate

The assay system (6 ml) was 0.25 M in sucrose, 2 mM in Tris chloride (pH 7.5), 50 mM in KCl and 1.25 mM in KCN. When sodium pyruvate and potassium malate were added, the final concentrations were 3.33 and 1.67 mM, respectively. When NADH was added, the final concentration was 0.33 mM. The concentration of beef heart mitochondria was 2 mg/ml. The assay mixture was incubated at least 2 min at 30° to achieve pH stabilization before addition of 10 µl of 100 mM $K_3Fe(CN)_6$.

tical phosphate residue. In the 1:1 modality, charge neutralization for the second electron is achieved by dissociation of a proton; in the 0.5:1 modality, charge neutralization is achieved by uptake of an anion.

DISCUSSION: The data presented in this communication have established a firm experimental foundation for the thesis that in energy coupled reactions driven by electron transfer the primary substrate for each complex is "dehydrogenated" on one side of the membrane (I side) and the terminal acceptor is "hydrogenated" on the other side (M side). The term dehydrogenated is used loosely to imply that both an electron and a proton in 1:1 molar proportions are released by oxidation. A second important sequella of these data is that theory for the H^+/e ratio is unambiguously unity because the source of the proton is the primary substrate for the electron transfer reaction and the principle of charge neutrality requires equivalence of proton and electron release during oxidation. Given this pattern of dehydrogenation on one side of the membrane and hydrogenation on the other side for the electron transfer process, then we have a complete accounting of the protonic changes during electron transfer coupled to cyclical cation transport (1). Protons are released on the I side by oxidation, taken up on the M side by reduction of the terminal acceptor and transferred across the membrane by cation/proton exchange. Since the net protonic change at the end of an oxygen pulse experiment is zero as deduced from the U-shaped profile, it would follow that there is exact equivalence of the protons released by oxidation and of the protons

taken up in the reductive process. The conclusion we can now draw is that the proton is not directly involved in energy coupling. It is the electron that moves across the membrane together with the cation in the energized phase; the proton is left behind. The proton moves across the membrane only when the cation effluxes under nonenergized conditions. Proton/cation exchange follows in time the coupled transport of the cation.

Takano and Dickerson (9) have recently succeeded in analyzing the three dimensional structure of tuna ferrocytochrome c and ferricytochrome c at 1.5 - 1.8 A° resolution. On the basis of this study it would appear that Tyr-67 is the most likely choice for the group that undergoes the protonation-deprotonation cycle; the buried water molecule hydrogen bonded to Tyr-67 would be the second but less likely choice.

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