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Differential Exposure of Components of Cytochrome b–c_1 Region in Beef Heart Mitochondria and Electron Transport Particles[1]

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Abstract

The reduction of cyctochromes $c + c_1$ by durohydroquinone and ferrocyanide in electron transport particles (ETP) and intact cytochrome c-depleted beef heart mitochondria has been studied. At least 94% of the ETP are in an inverted orientation. Durohydroquinone reduces 80% of $c + c_1$ in ETP but less than 20% in mitochondria; sonication of mitochondria allows reduction of cytochromes $c + c_1$ (80%). Addition of ferrocyanide (effective redox potential +245 mV) to electron transport particles results in 30% reduction of cytochromes $c + c_1$. Addition of ferrocyanide to intact cytochrome c-depleted mitochondria does not reduce cytochrome c_1; treatment with N,N,N',N'-tetramethylphenylenediamine, Triton X-100, or sonic oscillation results in 30% reduction of cytochromes $c + c_1$. The K_m value of ferrocyanide oxidase for K-ferrocyanide is pH-dependent in ETP only, increasing with increasing pH. The extent of reduction of cytochrome c_1 is also pH-dependent in ETP only, the extent of reduction increasing with decreasing pH. On the basis of these data cytochrome c_1 is exposed to the matrix face and cytochrome c is exposed to the cytoplasmic face. No redox center other than cytochrome c in the segment between the antimycin site and cytochrome c is exposed on the C-side.

Key Words: Mitochondria; electron-transport particles; cytochrome c_1; topography; respiration; ferrocyanide; durohydroquinome; Q-cycle; complex III.

Introduction

The ubiquinol-cytochrome c reductase segment of the mitochondrial electron transport system is associated with the second side of oxidative phosphoryla-

[1]Abbreviations Used: MES, 2(N-morpholino)-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TMPD, N,N,N',N'-tetramethylphenylenediamine; ETP, electron transport particles; NAD-NADH, nicotinamide adenine dinucleotide; PMS, phenazine methosulfate.

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tion. The segment, called Complex III (Rieske, 1967), is composed of at least two distinct *b*-cytochromes, cytochrome c_1, cytochrome *c*, the Fe–S center ($g = 1.90$) as described by Rieske and co-workers (Rieske *et al.*, 1964; Baum *et al.*, 1972; Rieske, 1976), and possibly two other paramagentic centers (Eisenbach and Gutman, 1974; Lee and Slater, 1974), although the existence and identification of multiple Fe–S centers in Complex III has been seriously challenged (Rieske, 1976; Yu *et al.*, 1974; King *et al.*, 1975; Orme-Johnson *et al.*, 1974). Central to the understanding of the mechanisms of electron transfer and energy-linked functions at site II is the determination of the locations of the electron carriers of Complex III and the path of electron flow in or across the membrane (Chance, 1977).

Previous studies (Harmon and Crane, 1973; Harmon *et al.*, 1974; DePierre and Ernster, 1977) have suggested a transmembranous arrangement of Complex III electron carriers found between the site of antimycin A inhibition and cytochrome *c*. In these studies antimycin-sensitive succinate-ferricyanide reductase was observed in both mitochondria and electron transport particles of opposite orientation. Since cytochrome *c* is sequestered within the membrane vesicle of electron transport particles (ETP) (see Harmon *et al.*, 1974, and DePierre and Ernster, 1977, for reviews), the ferricyanide-reactive site exposed on the matrix face of ETP was suggested to be cytochrome c_1 or an Fe–S center. The *b*-cytochromes are on the substrate side of the antimycin block and cannot be reactive species. Schneider and Racker (1971) found that antibodies against cytochrome c_1 are reactive with mitochondria, but not with ETP, suggesting the location of cytochrome c_1 on the cytoplasmic or C-side of the membrane. Case and Leigh (1976) suggest that *all* Complex III electron carriers are located on the cytoplasmic face of the membrane on the basis of the alteration of the component EPR signals by Ni^{2+} or GD^{2+} in pigeon heart mitochondria but not submitochondrial particles. Bell *et al.* (1978) report the interaction of specific anti-cytochrome c_1 antibodies only when added to intact mitochondria, suggesting the location of cytochrome c_1 on the cytoplasmic face.

The midpotentials of the Complex III components are as follows (Dutton and Wilson, 1974): cytochrome b_{566}, -30 mV; cytochrome b_{562}, $+30$ mV; cytochrome c_1, $+228$ mV; Fe–S (Rieske), $+280$ mV; and cytochrome *c*, $+225$ mV when bound to the membrane. If ferrocyanide is to reduce any of these components, ferrocyanide solutions must have a potential less than $+280$ mV. Kolthoff and Tomsicek (1935) and O'Reilly (1973) indicate that the midpotential of the ferri-/ferrocyanide couple is approximately $+400$ mV. Our data, however, demonstrates that ferrocyanide is an effective impermeant reductant of complex III.

Durohydroquinone (DQH_2) is oxidized at different rates in ETP and mitochondria (Harmon and Crane, 1976; Ruzicka and Crane, 1971; Crane,

1977). This fact, the inhibition of DQH_2 oxidation by poly-L-lysine (Ruzicka and Crane, 1971; Crane, 1977), and the stimulation of DQH_2 oxidation by bathophenanthroline sulfonate in ETP (only) (Harmon and Crane, 1976) indicate that DQH_2 is impermeant. While duroquinol-cytochrome *c* reductase is antimycin-sensitive, DQH_2 can also reduce cytochrome *c* directly. The antimycin-insensitive duroquinol oxidase activity in ETP and mitochondria is also CN^--sensitive, indicating that a DQH_2-reactive site(s) is found on the oxygen side of the antimycin block (Boveris *et al.,* 1971). Data will be presented to indicate that DQH_2 ($E_m = +5$ mV; see Dutton and Wilson, 1974) is an impermeant electron donor useful in the elucidation of Complex III topography.

In this communication we provide evidence that cytochrome c_1 or a nearby respiratory component is located on the matrix face of the inner membrane and that the extent of reduction of cytochrome c_1 by exogenous impermeant reductants is dependent on the pH of the medium at the matrix face.

Methods and Materials

Intact cytochrome *c*-depleted mitochondria were isolated from fresh bovine heart by the method of Harmon and Crane (1973). Electron-transport particles of inverted orientation were isolated from beef heart mitochondria by the alkaline treatment described by Crane *et al.* (1956).

Ferrocyanide oxidase activity was measured using a Clark electrode. Membranes was suspended in 20 mM MES-Tris buffer at the desired pH value at 37°C or in medium containing 83 mM sodium phosphate–83 μM EDTA at the desired pH values. Initial rates of oxygen uptake were measured and plotted as a Lineweaver–Burke plot to determine the Michaelis constant and maximum velocity at infinite ferrocyanide concentration.

$DQH_2 \rightarrow O_2$ activity was determined at 25°C in a medium containing 83 mM sodium phosphate and 83 μM EDTA (pH 7.4). Durohydroquinone (0.25 mg) from K & K Chemicals was used as substrate. Where indicated, antimycin A, 200 μg cytochrome *c* (type VI, Sigma), 25 μg superoxide dismutase, or 150 μM NaCN (final concentration) were added to the reaction mixture. Protein (0.1–0.12 mg) was used in a 1.9-ml chamber.

The extent of reduction of cytochromes $c + c_1$ was measured with a Cary 14 spectrophotometer equipped with a 0.1 A slidewire or a Johnson Foundation DBS-3 double-beam spectrophotometer. Electron transport particles (ETP) and mitochondria were suspended in 0.25 M sucrose containing 50 mM sodium phosphate buffer or 0.25 M sucrose containing 50 mM MES-Tris at the desired pH values. The protein concentration (noted in the figure

legends) was between 1.25 and 1.5 mg/ml. Ferrocyanide was added to a final concentration of 9 mM in the presence of antimycin and NaCN. The total cytochrome $c + c_1$ content was determined following the addition of excess solid sodium dithionite.

Oxidation-reduction potentials were measured in a Dutton-type cuvette at room temperature using a platinum–calomel electrode pair. Spectra were recorded with the Cary 14 instrument. The cuvette contents were continually flushed with N_2 gas. Fe^{++}–EDTA (5 μM) and *N,N,N′,N′*-tetramethylphenylenediamine dihydrochloride (TMPD) (0.58 mM) were used as mediators. The potential was adjusted by the addition of freshly made dithionite and ferricyanide solutions. An oxidized sample was placed in the reference cuvette at all times. Cytochrome *c* reduction was determined using the difference in absorbance at 550 and 535 mm.

Results

Orientation of Membrane Preparations

Previously published studies have demonstrated that intact beef heart mitochondria and electron transport particles are routinely of 95% or greater desired orientation (Harmon *et al.*, 1974; Harmon and Crane, 1976). The isolation of mitochondria involves 2 washes in medium containing 0.15 M KC1. As a result, 90% or more of the endogenous cytochrome *c* is removed (Harmon *et al.*, 1974; Harmon and Crane, 1976; Lee *et al.*, 1971; Table I); to measure maximal respiratory activity, cytochrome *c* must be added to the reaction medium. Intact mitochondria show a 10-fold (minimum) increase in

Table I. Effect of Exogenous Cytochrome *c* on Respiration in Mitochondria and Electron Transport Particles[a]

	Oxygen uptake (μmol O_2/min/mg protein)	
Substrate	Mitchondria	Electron transport particles
NADH (0.5 mM)	0.073	4.00
+ cytochrome c^{+}	0.79	3.74
Succinate (27.8 mM)	0.08	1.94
+ cytochrome c^{+}	1.02	1.83
+ 200 μg cytochrome *c*, Type III Sigma		

[a]Medium contained 83 mM sodium phosphate and 83 μM EDTA at pH 7.4. 100 μg mitochondrial protein was used for each determination.

the rate of oxygen reduction upon addition of cytochrome *c* using either succinate, ferrocyanide, ascorbate *plus* TMPD (or phenazine methosulfate, PMS), NADH, duroquinol, or glutamate *plus* malate as reductants (cf. Tables I and II). Intact mitochondria oxidize exogenous NADH extremely slowly; the observed rate is routinely 1/20 to 1/30 that of NADH oxidation by ETP. As shown previously (Harmon *et al.*, 1974; Harmon and Crane, 1976), low NADH oxidase activity is indicative of mitochondrial intactness since the NADH-reactive site is located on the matrix face of the cristae. Freshly isolated mitochondria have a specific activity of 75 nmol O_2/mg protein/min (cytochrome *c* present) using malate *plus* glutamate as substrates; this activity is not observed after freezing–thawing, although the other criteria of "intactness" are unaltered by the freezing procedure (see Harmon *et al.*, 1974, for criteria).

Electron transport particles, conversely, have extremely high NADH oxidase activity with 6% or less increase in activity upon addition of cytochrome *c* (cf. Table I). This indicates that the cytochrome-*c* reactive site is sequestered inside the vesicles and that ETP are intact inverted vesicles with NADH dehydrogenase exposed to the medium (Harmon *et al.*, 1974).

This facility routinely isolates ETP preparations that are 94% or more inverted. ETP of such quality exhibit high NADH oxidase activity (Harmon *et al.*, Harmon and Crane, 1976), high ATPase activity (Harmon *et al.*, 1974) high inhibition by the addition of (isolated) endogenous ATPase inhibitor protein (Harmon *et al.*, 1974) and low stimulation/inhibition of respiration by addition of cytochrome *c*/protamine (Table I; see also Harmon *et al.*, 1974).

Reduction of Cytochromes by Durohydroquinone

Addition of durohydroquinone to electron transport particles results in the reduction of 76% of cytochromes $c + c_1$ and 50% of the cytochromes *b*. The extensive reduction of the *c*-type cytochromes and 50% reduction of cytochrome *b* is consistent with the value of +5 mV for the midpotential of duroquinol; spectra of dithionite-reduced *minus* duroquinol-reduced samples indicate that b_{562} (+30 mV) is the species reduced by the quinol (data now shown), as was also shown by Boveris *et al.* (1971).

Addition of DQH_2 to intact 90%-depleted mitochondria, however, results in 50% reduction of cytochromes *b* but less than 20% reduction of cytochrome c_1 and the remaining cytochrome *c* (10% of endogenous amount), as shown in Fig. 1B, lower panel. Addition of duroquinol to mitochondria (90% cytochrome *c*-depleted) subjected to brief sonication (30 sec) results in 80% reduction of the total cytochromes $c + c_1$ but still only 50% reduction of total cytochrome *b* (Fig. 1B, upper panel, curve 2).

Since 10% of the endogenous cytochrome *c* remains on the membrane in the mitochondria, 10% of the cytochrome c_1 will be reduced since the two cytochromes are in equilibrium. Of the total $c + c_1$ content, DQH_2 can reduce 2/11 of $c + c_1$ (the remaining endogenous 10% cytochrome *c* and an equal amount of 100% of the endogenous cytochrome c_1). This is based on the evidence that *c* and c_1 are reduced in equimolar amounts and possess similar extinction coefficients (Chiang and King, 1975). Thus, a minimum of 18% reduction will be observed by reduction via cytochrome *c*; this is in excellent agreement with the data.

Antimycin (10 μg/mg protein) was added to ETP and mitochondria to

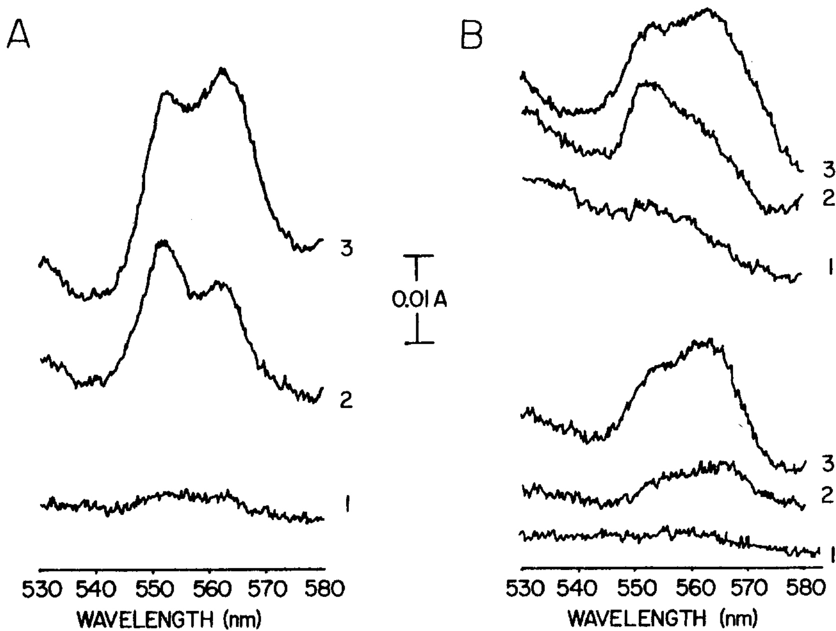

Fig. 1. (A) Difference spectra (reduced *minus* oxidized) of the cytochromes of electron transport particles using durohydroquinone as reductant. ETP are suspended in 0.25 M sucrose–50 mM sodium phosphate buffer (pH 7.4) at a concentration of 1.5 mg/ml. 300 μM CN^- and antimycin A (10 μg/mg protein) were present. Additions as follows: curve 1, no additions; curve 2, 0.082 mM duroquinol to sample, ethanol to reference; curve 3, solid dithionite to sample, nothing to reference. (B) Difference spectra of the cytochromes of beef heart mitochondria using durohydroquinone as reductant. Mitochondria (90% cytochrome *c*-depleted) were suspended in 0.25 M sucrose–50 mM sodium phosphate buffer (pH 7.4) at 1.5 mg protein/ml. Upper panel: mitochondria exposed to sonic irradiation for 30 sec. Additions as follows: curve 1, none; curve 2, 0.082 mM duroquinol to sample, 20 μl ethanol to reference cuvette; curve 3, solid dithionite to sample, no addition to reference. Lower panel: nonsonicated control mitochondria. Additions: curve 1, none; curve 2, 0.082 mM duroquinol to sample, ethanol to reference; curve 3, solid dithionite to sample, nothing to reference curvette. Antimycin A present in all cuvettes (10 μg/mg protein). Similar results were observed using 300 μM CN^-.

prevent the transfer of electrons from cytochrome b_{562} to cytochrome c_1. The reduction of the c-type cytochromes is thus via exposed electron-transfer components on the oxygen side of the antimycin block and *not* due to a "leak" past the inhibition site. Were such a leak existent, the extents of reduction of cytochrome c_1 in ETP and mitochondria would be far more similar. The extent of reduction of cytochromes $c + c_1$ in ETP is similar to that of mitochondria only *after* sonication (and more than three times greater than in intact mitochondria), indicating that mitochondria possess a permeability barrier to duroquinol that is destroyed by sonication. The data in Fig. 1 indicates (1) that duroquinol is not a permeant reductant and (2) that cytochrome c_1 or an electron-transfer component associated with cytochrome c_1 is exposed to the matrix face. In short, at least one component of Complex III on the oxygen side of the antimycin site is on the M-side of the membrane in opposition to the findings of Case and Leigh (1976).

Duroquinol Oxidase Activity in ETP and Mitochondria

Addition of antimycin to ETP results in 85% inhibition of duroquinol oxidase activity; CN^- inhibits 90% of the activity (cf. Table II). That the activity is inhibited 96% by antimycin and 98% by CN^- in the presence of superoxide dismutase (SOD) indicates that most of the antimycin-insensitive activity is due to O_2^- generation on the M-side.

In cytochrome c-replenished mitochondria, however, addition of antimycin inhibits only 86% of the duroquinol oxidase activity, indicating that DQH_2 can reduce cytochrome c directly. Addition of antimycin to cytochrome c-depleted mitochondria in the presence of SOD inhibits only 16% of the duroquinol oxidase activity compared to 26% in the absence of SOD. This

Table II. Duroquinol Oxidase Activities in Mitochondria and Electron Transport Particles[a]

Assay system	Oxygen consumption (μmol O_2/min/mg protein) −25 μg SOD	+25 μg SOD
Electron transport particles	1312	1250
+ AA	192	47.2
+ NaCN	137.6	29.5
Mitochondria + cytochrome (200 μg)	1166	1270
+ AA	193.7	179.8
+ NaCN	83.5	44.1
Mitochondria (cyt c-depleted)	60.3	41.5
+ AA	44.1	34.8
+ NaCN	24.9	17.4
Purified cytochrome oxidase	—	23
+ Na CN	—	12.8

[a]SOD, superoxide dismutase; AA, antimycin A; cyt c, cytochrome c (Type VI); NaCN added to 150 μM final concentration.

suggests that respiration is not due to O_2^- generation. That CN^--sensitive O_2 consumption of isolated cytochrome oxidase (in the absence of cytochrome *c*) is observed in the presence of SOD suggests that O_2 uptake of cytochrome *c*-depleted mitochondria is catalyzed, in part at least, by cytochrome oxidase.

In the absence of SOD, it is possible that reduction of $c + c_1$ in ETP could occur via O_2^-. While O_2^- generation at the ubiquinone site is increased by addition of antimycin, it is also inhibited by CN^- (Turrens and Boveris, 1980). Even though the reduction of $c + c_1$ in ETP occurs in the presence of SOD (in N_2-purged and flushed cuvettes), apparently considerable (but not all) O_2 consumption is due to O_2^- generation on the M-face. SOD is not expected to cross the cristae membrane and can thus catalyze the dismutation of superoxide on the M-face. It is possible that superoxide generated within the ETP vesicle on the C-face results in $c + c_1$ reduction. Since cytochrome *c* is present within the ETP vesicle and data in Table II indicate that DQH_2 (or $DQH^\cdot$ or O_2^-) can react with cytochrome *c* directly, respiration in ETP may proceed via (C-side-generated) reduction of sequestered cytochrome *c*, although the presence of CN^- should inhibit superoxide-driven reduction of $c + c_1$ in Fig. 1A.

If superoxide is the reductant in cytochrome *c*-depleted mitochondria, it apparently is unable to cross the membrane to reduce cytochrome c_1 (cf. Fig. 1). Addition of DQH_2 to cytochrome *c*-loaded liposomes in the presence of O_2 and the absence of SOD results in the reduction of cytochrome *c* with a half-time of 200 sec; O_2^-, if generated, is unable to cross the membrane rapidly. Superoxide generated on one membrane face is unable to cross the membrane and directly reduce a component on the opposite face.

These data indicate that the reduction of $c + c_1$ in ETP occurs via a component on the matrix face. Reduction of c_1 in cytochrome *c*-depleted mitochondria is apparently limited by the permeability of reducing equivalents of DQH_2 to reach the component on the M-side. If the $c + c_1$ reduction is dependent on diffusion to a component on the C-side, then 80% reduction of $c + c_1$ in ETP would require greater than 200 sec; the reduction of $c + c_1$ in ETP occurs in less than 30 sec, however. If the reduction of $c + c_1$ (on the C-side) were due to C-side superoxide generated by a C-side component, then rapid and extensive reduction of c_1, would be observed in mitochondria; this is not observed, suggesting that c_1 is not located on the C-side. The presence of 300 μM CN^- (cf. Fig. 1) would inhibit superoxide generation at the ubiquinone site (Turrens and Boveris, 1980), further indicating that O_2^- is not likely to be the reductant in ETP or mitochondria.

To determine the probable identify of the component reduced by DQH_2 in ETP (above) and to determine the topography of Complex III components on the oxygen side of the antimycin site, ferrocyanide was used as a reductant of ETP and 96% cytochrome *c*-depleted intact mitochondria. To reduce the

cytochrome c + c_1 components (+228 mV) or even the Rieske FE–S center (+280 mV), ferrocyanide must possess a midpotential range overlapping that of these components.

Determination of the Effective Potential of Ferrocyanide Solutions

The midpotential of ferri-/ferrocyanide (or the reducing potential of ferrocyanide) is frequently stated as +420 mV (Kolthoff and Tomsicek, 1935; O'Reilly, 1973) Ferrocyanide has been used by numerous investigators to reduce cytochrome c (Harmon *et al.,* 1974; Jacobs and Sanadi, 1960; Zabinski *et al.,* 1974; Stellwagen and Cass, 1975) and act as a source of electrons for the cytochrome c oxidase reaction (Mitchell and Moyle, 1967; Wikström, 1977). To determine the effective potential of ferrocyanide solutions, the extent of reduction of isolated soluble cytochrome c (E_m = +275 mV; Dutton and Wilson, 1974) by ferrocyanide was measured. As indicated

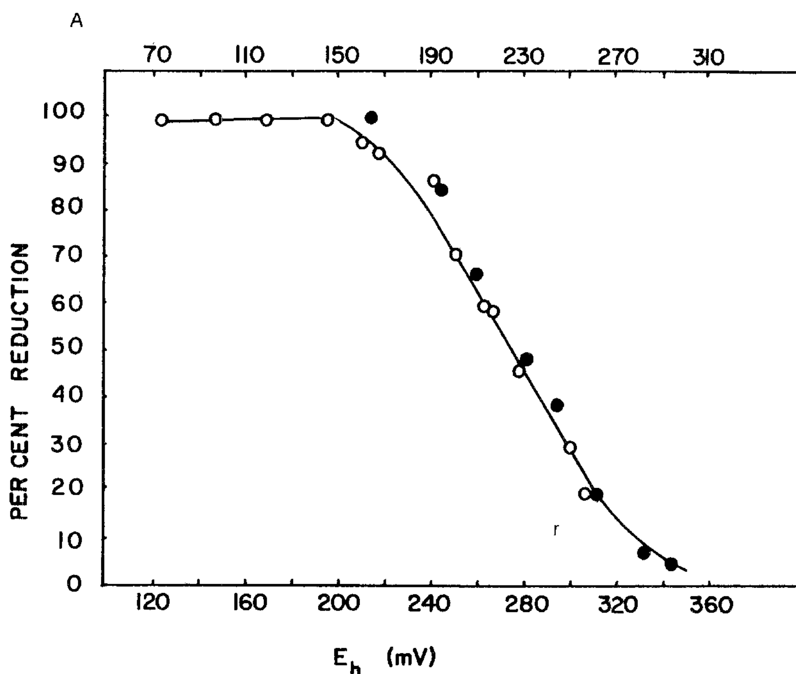

Fig. 2A. Oxidation-reduction potential dependence of soluble cytochrome c. 3.2 μM soluble cytochrome c was suspended in 0.25 M sucrose–50 mM sodium phosphate buffer at pH 7.4. Potentials measured as described in methods section. Absorbance changes determined by measuring the difference in absorbance at 550 *minus* 535 nm. Open circles, reductive titration; closed circles, oxidative titration. Lower potential scale for soluble cytochrome c. Upper potential scale for membrane-bound cytochrome c with 50% reduction at +225 mV (Dutton and Wilson, 1974)

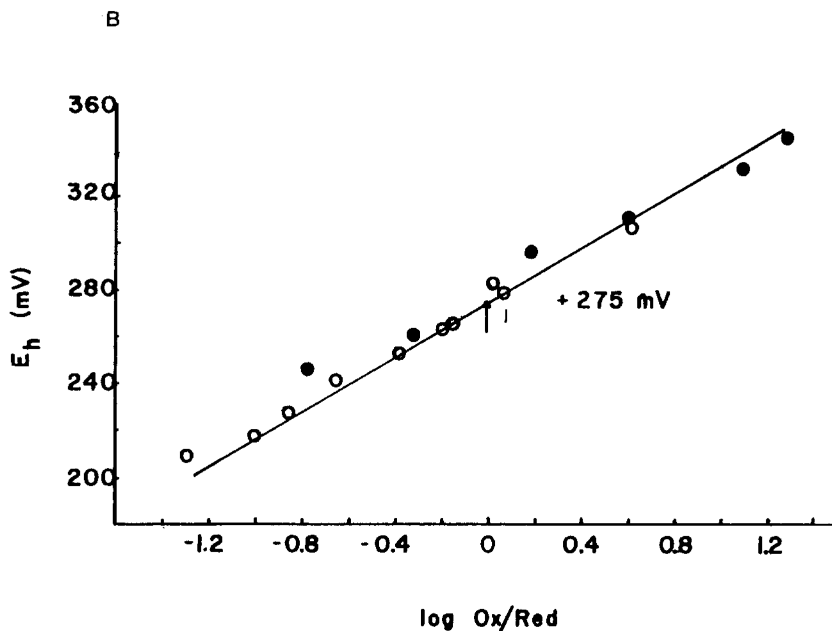

Fig. 2B. Determination of oxidation-reduction potential of soluble cytochrome *c*. Data from Fig. 2A. The straight line denotes theoretical $n = 1$ component.

in Figs. 2A and B, the midpotential of cytochrome *c* used in these experiments is indeed +275 mV; cytochrome *c* is suspended in 0.25 M sucrose containing 50 mM sodium phosphate buffer, pH 7.4 (or 5.5), used to suspend ETP and mitochondria in these experiments. Addition of 9 mM ferrocyanide (final concentration) results in 75% reduction of soluble cytochrome *c* at both pH 5.5 and 7.4 as shown in Fig. 3A and 3B. A 75% reduction is observed at +245 mV (see Fig. 2A, lower scale). This indicates that the effective reduction potential of 9 mM ferrocyanide solution is at least 150 mV less positive than the ferri-/ferrocyanide midpotential. Ferrocyanide solutions are capable of reducing (at least partially) a component with a midpotential of +225 mV or more. As seen on the upper scale of Fig. 2A (midpotential of membrane-bound cytochrome *c* of c_1 at +225 mV), 30% reduction is observed at +245 mV potential. This is in agreement with the ability of ferrocyanide to reduce cytochromes $c + c_1$ as reported by Hinkle and Mitchell (1970).

Ferrocyanide Oxidation by ETP and Mitochondria

Addition of ferrocyanide to aerobic ETP results in CN^--sensitive but antimycin-insensitive oxygen reduction. Because of the homogeneously oriented particles used, the rate of oxygen uptake is not altered more than 6%

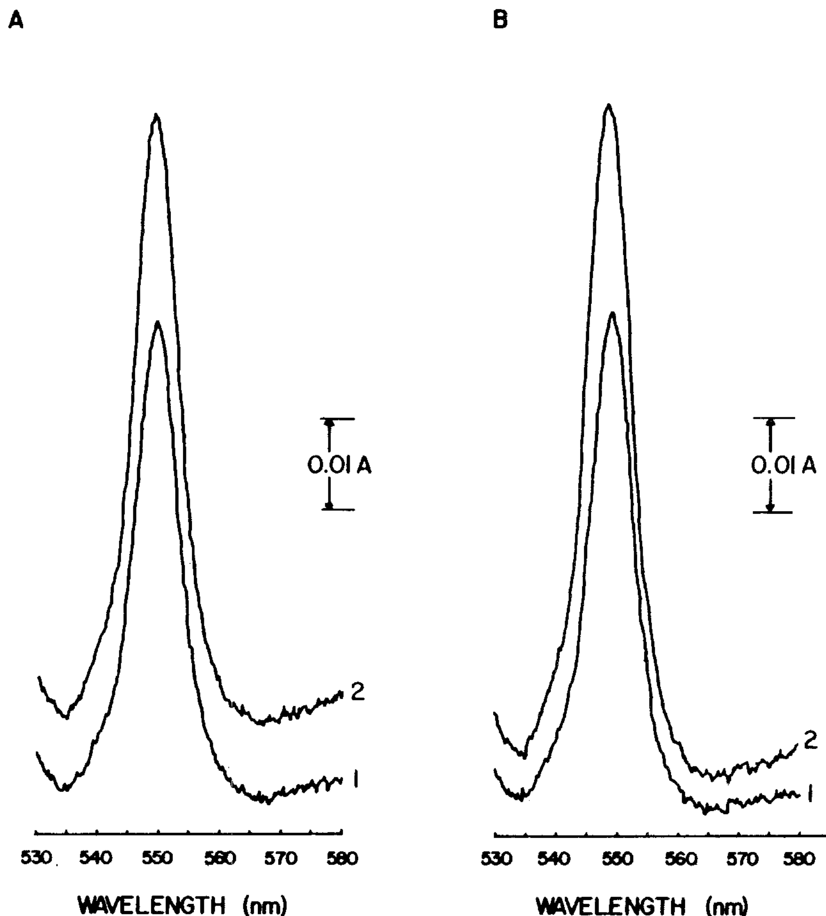

Fig. 3. Reduction of soluble cytochrome c by ferrocyanide. 3.2 μM cytochrome c was suspended in 0.25 M sucrose–50 mM sodium phosphate buffer at pH 5.5 (A) and pH 7.4 (B). 9 mM ferrocyanide was added to the sample cuvette (curve 1). Full reduction was obtained by addition of solid dithionite to sample cuvette (curve 2).

by the addition/removal of cytochrome c or the addition of protamine sulfate or poly-L-lysine which inhibits respiration by blocking the cytochrome c-binding site (cf. Harmon *et al.*, 1974). In contrast, the rate of oxygen uptake by mitochondria is dependent on the presence of cytochrome c and is inhibited by the addition of protamine or poly-lysine. As in ETP, ferrocyanide oxidase in mitochondria is antimycin-insensitive and CN^--sensitive.

The observed rates of oxygen uptake were plotted against the substrate concentration in a Lineweaver–Burke plot to determine the value of the Michaelis constant (K_m). At pH 5.5 and 7.4, the K_m for ferrocyanide is

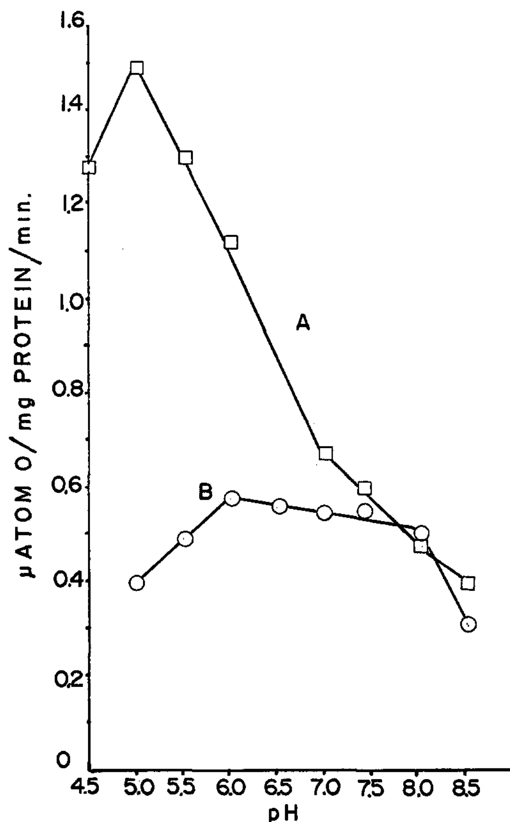

Fig. 4. Effect of pH of suspending medium on ferrocyanide oxidase rate in electron transport particles (A) and intact mitochondria (B). Oxygen uptake measured as described in Methods section.

calculated to be 0.5 mM. The K_m of ferrocyanide in ETP, however, varies linearly with the pH of the medium as shown in Fig. 5. The value of K_m varies from 3.2 mM at pH 5.5 to 12.4 mM at pH 8.0. As shown in Fig. 4, ferrocyanide oxidation in mitochondria (plus cytochrome c) is constant across a wide pH range, while the respiratory rate in ETP is higher at acidic pH values. The data in Fig. 3 indicate that the midpotential of ferrocyanide is constant at these pH values. Thus, it is likely that different ferrocyanide-reactive sites are present on each membrane face.

Reduction of Electron-Transport Components by Ferrocyanide

Addition of 9 mM ferrocyanide (final concentration) to ETP results in the partial reduction of cytochromes $c + c_1$ and cytochrome $a + a_3$ as seen in

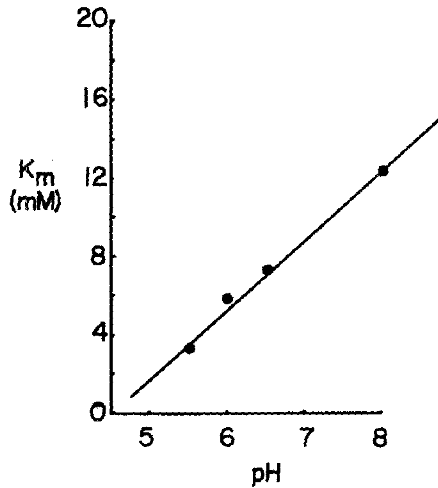

Fig. 5. Plot of K_m of ferrocyanide of ferrocyanide oxidase in ETP vs. pH. The K_m values were determined from linear Lineweaver–Burke plots of ferrocyanide oxidase activity in ETP.

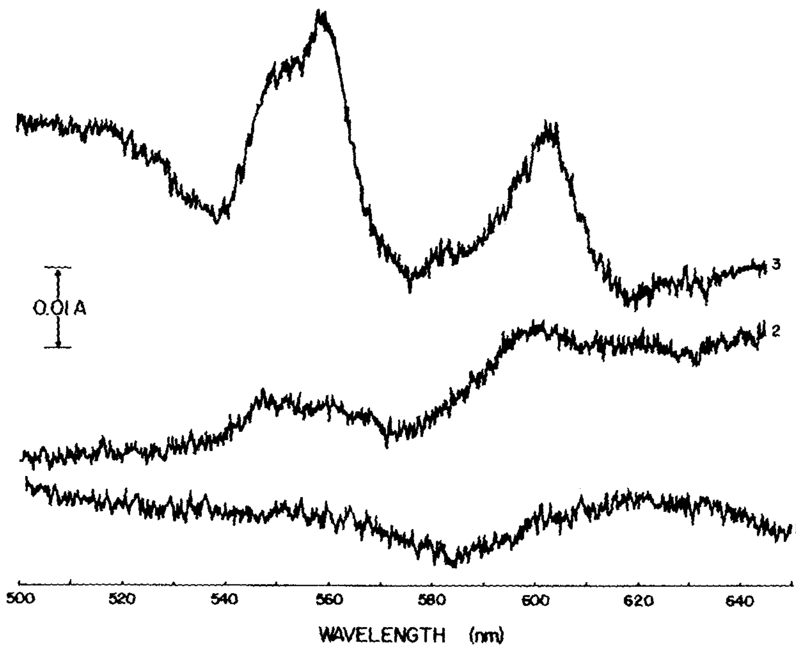

Fig. 6. Difference spectra (reduced *minus* oxidized) of electron transport particles using ferrocyanide as reductant. ETP are suspended at 1.5 mg/ml in 0.25 M sucrose–50 mM sodium phosphate buffer at pH 7.4. Additions: curve 1, 300 μM CN^- and antimycin to each; curve 2, 9 mM ferrocyanide to sample, 9 mM KCl to reference cuvette; curve 3, solid dithionite to sample, nothing to reference cuvette.

Fig. 6 by the increase in absorbance at 552 and 608 nm. Comparison of ferrocyanide-reduced ETP with dithionite-reduced ETP indicates that approximately 30% of the cytochromes $c + c_1$ are reduced. Membrane-bound cytochrome c is reported to have a midpotential of +225 mV while cytochrome c_1 has a midpotential of +228 mV (Dutton and Wilson, 1974). The calculated extent of reduction of components with a +225 mV midpotential by a +245 mV reductant is 30% as seen in Fig. 2A (upper scale); this is in excellent agreement with the observed data. Further, since 30% of the total $c + c_1$ content is reduced, cytochromes c and c_1 are in electronic equilibrium. Were only one of the two cytochromes reduced, the observed extent of reduction would only have been 15% (30% of 50% of the 552–540 nm absorbance).

Addition of ferrocyanide to intact mitochondria does not reduce cytochrome c_1 ($+c$), as seen in Fig. 7A, curve 2. (Mitochondrial respiration was stimulated 24-fold in these mitochondria.) Addition of TMPD *plus* ferrocyanide (curve 3) results in an increase in 552 nm absorbance to a value approximately 40–45% of that reduced by dithionite (curve 5). If Triton X-100 is added to solubilize the membranes (curve 4), an increase in absorbance to approximately 30% of the maximum value is also observed.

The increase in reduction of $c + c_1$ in the presence of TMPD (compared to that in the presence of Triton X-100) is due to the greater interaction of reduced TMPD with c_1 than of ferrocyanide alone. Addition of TMPD in the presence of ferrocyanide to sonicated mitochondria also results in 40–45% reduction of cytochrome c_1. As shown below, increased interaction of ferrocyanide with c_1 (by increasing the ferrocyanide concentration) results in increased reduction of $c + c_1$.

Figure 7B illustrates that addition of ferrocyanide alone to sonicated (30 sec) mitochondria also results in a significant increase in 552 nm absorbance to about 30% reduction (compare curve 2 of Fig. 7A to curve 1 of Fig. 7B). This data indicates that addition of TMPD or detergent does not cause an increase in 552 nm absorbance by altering the midpotential of cytochrome c_1, but that a permeability barrier is breached or destroyed by treatment with TMPD, Triton, or sonication, allowing ferrocyanide (or electrons from) access to the matrix surface of the mitochondria.

It is of interest to determine if the extent of reduction of cytochromes $c + c_1$ in ETP increases linearly with increasing pH as does the K_m value for ferrocyanide. In the presence of 10 μg antimycin A and 300 μM NaCN, the extents of reduction of $c + c_1$ in buffers of different pH and in the presence of 9 mM ferrocyanide were observed, as illustrated in Fig. 8. At alkaline pH values (high K_m) the extent of reduction is small (less than 10%), increasing in a linear manner to approximately 45% at pH 5.3 (low K_m). This seems inconsistent with the data presented in Fig. 2, namely that the expected

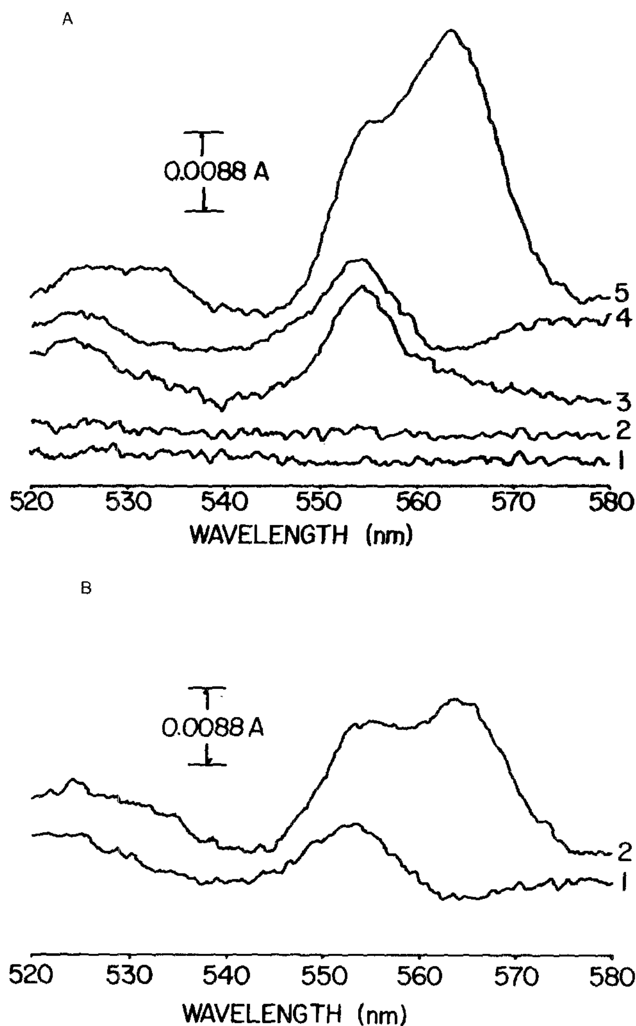

Fig. 7. Difference spectra (reduced *minus* oxidized) of cytochromes of intact beef heart mitochondria using ferrocyanide as reductant. 96% cytochrome *c*-depleted mitochondria were suspended in 0.25 M sucrose–50 mM sodium phosphate buffer at pH 7.4. 10 μg antimycin/mg protein and 300 μM NaCN present in each cuvette. 500 nm wavelength used as reference in dual-beam (DBS-3) spectrophotometer. (A) Baseline, oxidized mitochondria. Additions: curve 1, solid ferricyanide (separate assay mixture); curve 2, 9 mM ferrocyanide; curve 3, 9 mM ferrocyanide plus 0.24 mg *N,N,N′,N′*-tetramethylphenylenediamine; curve 4, 9 mM ferrocyanide to mitochondria treated with 0.13% (final concentration) Triton X-100; curve 5, solid dithionite. Protein concentration is 2 mg/ml. (B) Baseline, oxidized sonicated mitochondria. Additions: curve 1, 9 mM ferrocyanide; curve 2, solid dithionite. Protein concentration is 1.5 mg/ml.

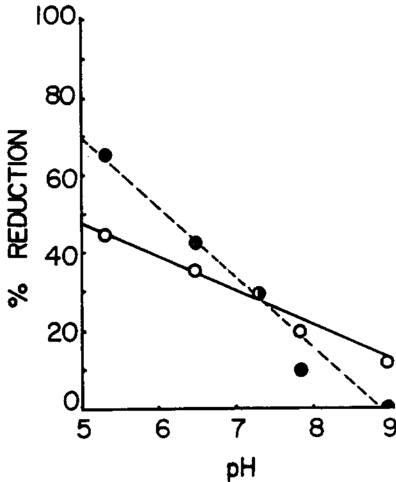

Fig. 8. Plot of reduction of cytochromes c_1 and $c + c_1$ vs. pH. Data obtained from difference spectra of ETP in the presence of 300 μM NaCN and 10 μg antimycin/mg protein; 9.0 mM ferrocyanide was used as a reductant (final concentration). 100% reduction value was obtained from spectra in which solid excess dithionite was used as reductant. Open circles, $c + c_1$; closed circles, calculated extent of reduction of cytochrome c_1 (Eisenbach and Gutman, 1974).

extent of reduction of membrane-bound cytochrome c (+225 mV) or cytochrome c_1 is 30%. The observed deviation from this value may be explained simply by the sequestering of cytochrome c within the ETP vesicle and the exposure of cytochrome c_1 to the medium; cytochrome c will always be reduced 30% while c_1 reduction is different at different pH values. Since both c-cytochromes contribute essentially equally to the 552 nm absorbance, an observed 15% of total $c + c_1$ reduction can be explained if cytochrome c is reduced 30% (due to its +225 mV midpotential and the 245 mV potential of ferrocyanide solution) and cytochrome c_1 is not reduced. Similarly at 45–50% reduction of total $c + c_1$ at pH 5.3 can be explained if one assumes that 15% of the 552 nm absorbance due to $c + c_1$ is due to 30% reduction of cytochrome c and a 60–70% reduction of cytochrome c_1 (assuming the two cytochromes are present in equal amounts and have similar extinction coefficients).

By measuring the extent of reduction of soluble cytochrome c in the presence of 56 mM ferrocyanide, we determined that the potential of 56 mM ferrocyanide, like that of 9 mM ferrocyanide, is approximately +245 mV. If the extent of reduction of cytochrome c_1 is dictated by pH-induced changes in the midpotential of c_1, then the extent of reduction of $c + c_1$ should remain the same in the presence of 56 mM reductant. At pH 9, 40% reduction of $c + c_1$ is observed in ETP in the presence of 56 mM ferrocyanide (compared to 10% reduction in the presence of 9 mM ferrocyanide), however, indicating that the extent of reduction is due to K_m of ferrocyanide oxidase. At pH 5.3 the S/K_m ratio is approximately 4 and 40–50% $c + c_1$ reduction is observed (9 mM ferrocyanide present). At pH 9.0 in the presence of 56 mM ferrocyanide, the S/K_m ratio is also approximately 4; the extent of $c + c_1$ reduction is also approximately 40%. When the value of S/K_m decreases, the extent of

reduction of $c + c_1$ decreases. Because the extent of $c + c_1$ reduction increases with increasing reductant concentration (at the same potential), the differences in c_1 reduction are not due to a pH-dependent increase in cytochrome c_1 midpotential. Indeed, changes in E_m of cytochrome c_1 at various pH values has *not* been observed (Dutton and Wilson, 1974).

Discussion

Ferrocyanide has been shown previously to be an impermeant electron donor (Mitchell and Moyle, 1961; Klingenberg and Buchholz, 1970) and is expected to donate electrons to those respiratory components that are exposed. Respiration is antimycin-insensitive, indicating that the electrons do not enter the respiratory chain via ubiquinone or a cytochrome *b*. Electrons are donated in mitochondria at or on the reducing side of cytochrome *c*; electrons are donated on the reducing (low potential) side of cytochrome *c* in ETP (but not *at* cyt *c*).

That separate and different ferrocyanide oxidation sites are involved is shown by the fact that the K_m values for ferrocyanide oxidation are greater in ETP that in mitochondria. Further, the respiration and extent of c_1 reduction are not affected by pH in mitochondria. These facts indicate that ferrocyanide is impermeant and reacting with different sites on each exposed surface.

It is possible that a single transmembranous component could have a site on each membrane surface possessing the characteristics described above. We would expect to observe similar extents of reduction of cytochrome $c + c_1$ in ETP and mitochondria, assuming that the portions exposed to each face are in electronic equilibrium. This is not observed, however.

The difference in extent of reduction of cytochrome c_1 in mitochondria or ETP is not due to a decreased interaction of ferrocyanide with a C-side carrier since the extent of reduction does not increase even after one hour incubation in the presence of ferrocyanide or in the presence of high concentrations of ferrocyanide (mitochondria only). To explain the data, the interaction of ferrocyanide with the C-side would have to be orders of magnitude less than that with the M-side.

The existence of a redox center on the C-side between the antimycin block and cytochrome *c* (other than cytochrome *c*) can be ruled out. If the center were in equilibrium with cytochrome *c* and c_1, then 30% reduction of cytochrome c_1 would be observed in either ETP or mitochondria in the presence of ferrocyanide. Since this is not observed, either a C-side redox center does not exist or it interacts with oxidant/reductant very poorly. This is in agreement with the observed lack of succinate-ferricyanide reductase activity in cytochrome *c*-depleted mitochondria (Harmon and Crane, 1973;

Harmon *et al.,* 1974). This conclusion is in agreement with the findings of Ohnishi *et al.,* (Ohnishi, Blum, and Salerno, in press; Ohnishi, Blum, Harmon, and Hompo, in press), indicating that the Rieske center is not on the C-face but likely to be situated in the middle of the membrane.

Addition of durohydroquinone to ETP results in substantial reduction of the *c*-type cytochromes. Addition of DQH_2 to 90% cyt *c*-depleted mitochondria results in less than 25% reduction. This alone suggests that at least one component of Complex III, presumably cytochrome c_1, is exposed on the M-side. These data also indicate that DQH_2 is impermeant and that cytochrome c_1 cannot be reduced via the now vacant cytochrome *c* site of cyt *c*-depleted mitochondria.

Finally, we can rule out the possibility that electrons from ferrocyanide are reducing cytochrome c_1 via another redox complex. Since antimycin is present at all times, electrons from components in NADH or succinate dehydrogenase cannot readily reduce cytochrome c_1. Components of cytochrome oxidase are at a higher potential than ferrocyanide (Dutton and Wilson, 1974) and, thus, could possibly accept electrons from ferrocyanide; since the midpotential of cytochrome *a* and its associated copper atom are similar to that of cytochrome $c + c_1$, electrons theoretically could flow from the oxidase component to the *c*-cytochromes. In the absence of cytochrome *c*, ferrocyanide cannot reduce cytochromes *a* and a_3 either in mitochondrially bound or detergent-solubilized oxidase (Harmon and Crane, 1973; DePierre and Ernster, 1977; Krab and Slater, 1979). Further, in the absence of cytochrome *c*, oxygen uptake is severely limited in mitochondria; if ferrocyanide could reduce cytochrome oxidase components, an appreciable uptake of O_2 would be observed. Thus, reduction of cytochrome $c + c_1$ proceeds via Complex III components only.

Cadenas *et al.*, (1977) demonstrated that DQH_2 can generate superoxide, a powerful reductant. Our evidence indicates that superoxide dismutase decreases the rate of oxygen consumption in both mitochondria and ETP. It is possible that the $c + c_1$ reductions observed are due to O_2^- instead of direct interaction with duroquinol. Because the reducing equivalents derived from duroquinol do not readily cross liposome membranes, we expect duroquinol (or superoxide generated by it) to react with electron-transport components located on the membrane face exposed to the medium. The reduction of $c + c_1$ is observed under anaerobic conditions in the presence of SOD as well as under aerobic conditions. If O_2^- is involved, it is membrane impermeant. The involvement of superoxide thus does not alter the interpretation of the results.

That superoxide generated by $QH^\cdot$ (Cadenas *et al.*, 1977; Boveris *et al.*, 1976; Boveris *et al.*, 1971) within the ETP or mitochondria may be the reductant of $c + c_1$ can be discounted since CN^- (present during spectropho-

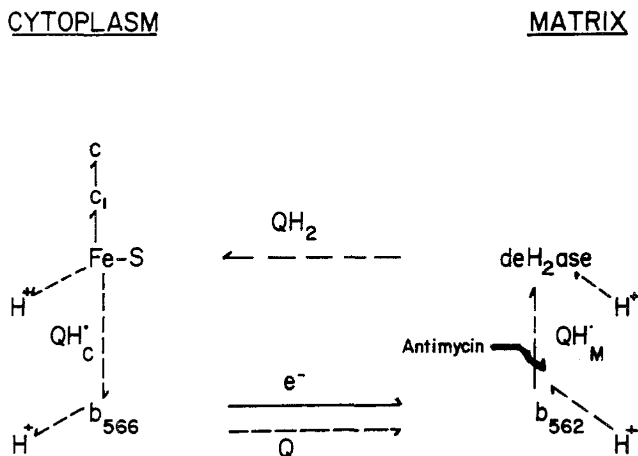

Fig. 9. Diagrammatic Q-cycle as described by Bowyer and Trumpower (1981) $QH_M^{\cdot}$ and $QH_C^{\cdot}$ located on the matrix and cytoplasmic faces respectively are capable of lateral diffusion only. QH_2 and Q can diffuse across the membrane. Solid arrows denote direct electron transport. As described by Bowyer and Trampower (1981) and Mitchell (1975, 1976), cytochrome c_1 and the Rieske Fe–S center are located on the C-side, in contrast to the evidence presented in this publication.

tometric assays) inhibits superoxide generation via $QH^{\cdot}$ (Turrens and Boveris, 1980). As explained previously, the experimental results expected would be opposite to those observed.

The preceding discussion has considered *direct* interaction of impermeant reductants with the electron-transport components as if a linear sequence existed. If the UQH_2-cytochrome c reductase portion is considered a "Q-cycle," a different interpretation of results appears possible. As shown in Fig. 9, a hypothetical model based on data of Mitchell (1975, 1976) and Bowyer and Trumpower (1981) places the Fe–S center and c_1 on the C-side. From our data, it is evident that direct reduction of these components is not observed, even though the midpotential of DQH_2 is below that of these components.

With the "Q-cycle" arrangement, however, reduction of a component from the M-side need not indicate its exposure to that surface. Addition of electrons from DQH_2 at the matrix face would reduce $QH_M^{\cdot}$ to QH_2 which would then reduce Fe–S and c_1 regardless of their location. Mitchell (1976) suggests that the E_m of the $QH^{\cdot}/QH_2$ couple is high (but this has not been directly measured); ferrocyanide could thus reduce $QM_M^{\cdot}$ resulting in reduction of c_1 and Fe–S. Indeed, the reaction would be expected to be faster at lower pH, ideally increasing 10-fold per pH unit change. However, V_{max} is not

observed to increase linearly with decreasing pH nor is it clear why the K_m value for ferrocyanide would be pH-dependent in the reduction of $QH_M^{\cdot}$.

Because the ETP used are inhibited by antimycin and CN^-, an increase in rate of $QH^{\cdot}$ reduction by ferrocyanide would not be expected to cause an increase in reduction of c_1. If steady-state oxidation of cytochrome c_1 were to occur at a slower rate than reduction, then increased $QH_M^{\cdot}$ reduction would result in an increase in steady-state reduction of cytochrome c_1. However, the presence of antimycin and CN^- does not allow for such an oxidation pathway. But the reduction of $c + c_1$ is not different in the presence or absence of antimycin, nor is cytochrome *b* reduction observed in the presence/absence of antimycin. The data do not support the idea that cytochrome c_1 is not exposed to either surface and that ferrocyanide reduces $QH^{\cdot}$ on the M-side (which reduces c_1 eventually). The data are consistent with the location of c_1 on the matrix face.

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