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PROTONIC AND CATIONIC CHANGES DURING CYCLICAL CATION TRANSPORT DRIVEN BY ELECTRON TRANSFER

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SUMMARY: Complexes I and III in mitochondrial particles mediate the coupling of electron transfer (NADH → Q; QH_2 → ferricytochrome c) to either cyclical or net transport of K^+. Cyclical transport was characterized by a U-shaped profile for the protonic and cationic changes observed during an oxygen pulse. The K^+/e and H^+/e ratios were found to be unity for the coupled reactions mediated by Complexes I and III. Protons were released on the side of the inner membrane where oxidation was initiated (I side) and taken up on the side where oxidation was terminated (M side). The pattern now shown to be general for the three electron transfer complexes that collectively carry out the NADH → O_2 sequence includes dehydrogenation of the primary reductants on the I side, hydrogenation of the terminal acceptors on the M side, and nonenergized transfer of a proton from the I to the M side of the membrane by cation/proton exchange. From these data it has been concluded that the movements of the electron and cation are coupled and synchronized, whereas the proton plays no direct role in energy coupling mediated by electron transfer complexes.

INTRODUCTION: In the study of cyclical cation transport mediated by cytochrome oxidase in both Ca^{2+}-state mitochondria and lysolecithin dispersions thereof (1-3), a characteristic coupling pattern could be recognized; and equally important the structural basis for translating this pattern could be specified (4-6). Coupling of electron transfer to ion transport in cytochrome oxidase had its structural counterpart in two chains in close proximity attached to the same transmembrane subunit--a chain for transfer of electrons and a cardiolipin-containing chain for transport of cations. Events in these two chains were paired and synchronous. In the electron transfer chain, coupling was initiated by the oxidation of ferrocytochrome c on the I side of the membrane, leading to the release of an electron and a proton--the electron moving into its chain in the membrane and the proton being extruded into the aqueous phase on the I side. In the ion transport chain, coupling was also initiated on the I side of the membrane by separation of a salt into a cation and an anion--the cation moving into its chain in the membrane and the anion being extruded into the aqueous phase on the I side. Coupling was terminated in both chains on the M side of the membrane--in the electron transfer chain by uptake of an electron by the terminal acceptor concomitant with the uptake of a proton from the aqueous phase on the

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M side, and in the ion transport chain by release of the cation and combination with a hydroxyl ion in the aqueous phase. The question posed in the present communication is whether the coupling pattern established for cytochrome oxidase is general for the two other electron transfer complexes that mediate coupled reactions. Several experimental observations that we have made indicate that such might be the case. Isolated Complex III inlaid in liposomes has been shown to couple electron transfer to net transport of monovalent or divalent cations (7). Cardiolipin has been implicated as a prosthetic group of all three coupled electron transfer complexes (8).

By studying cyclical cation transport in three overlapping electron transfer sequences (NADH → O_2; QH_2 → O_2; and ferrocytochrome c → O_2) it was possible to estimate the K^+/e and H^+/e ratios for the electron transfer sequences mediated by the individual complexes. These ratios were determined by the two slope method for measuring either ΔK^+ or ΔH^+ (1). By an independent method that depends upon the use of ferricyanide as oxidant, the H^+/e ratio either for the reaction NADH → Q mediated by Complex I or for the reaction QH_2 → ferricytochrome c mediated by Complex III was evaluated. This is a titration method with a sharp end point--a token that energized cation uptake is not complicated by nonenergized cation efflux leading to proton influx by the exchange reaction. The rationale of this titration method will be considered in the Experimental Methods section.

EXPERIMENTAL METHODS: Cyclical cation transport energized by the NADH → Q and QH_2 → ferricytochrome c oxidative sequences

Ca^{2+}-state mitochondria prepared by the method of Haworth and Hunter (9) mediate the antimycin-sensitive oxidation of durohydroquinone by O_2 coupled to the cyclical transport of a cation. This oxidation is a composite of two sequences--QH_2 → ferricytochrome c and ferrocytochrome c → O_2, mediated by Complexes III and IV, respectively. Heavy beef heart mitochondria prepared by the method of Hatefi and Lester (10) after twice freezing and thawing or after incubation at 38° for several min. in presence of 0.2 mM $CaCl_2$, mediate the oxidation of pyruvate and malate by O_2 coupled to the cyclical transport of a cation. This oxidation is a composite of three sequences--NADH → Q and the two subsequent sequences listed above, mediated by Complexes I, III and IV, respectively. If the stoichiometry for cyclical cation transport in Complexes I, III and IV is identical (K^+/e and H^+/e = 1), then the value of these ratios will be the same regardless whether the measured oxidative reaction involves 1, 2 or 3 complexes in series. The K^+/e and H^+/e ratios were determined in oxygen pulse experiments by evaluating the total flux of K^+ or by the two slope method described by Green et al. (1) and then relating either of these fluxes to the electron flux measured by the slope of the O_2 uptake curve. The K^+ flux is the sum of the energized influx rate (a) and the nonenergized efflux rate (b). The slope of the descending limb of the profile for ΔK^+ is a measure of a-b, and the slope of the ascending limb is a measure of b. The sum of the values for these two slopes (a-b + b) is then numerically equal to the energized influx rate. ΔH^+ during an oxygen pulse includes three processes: energized proton release in the oxidation of ferrocytochrome c (a); nonenergized proton uptake (b); and nonenergized proton release in the reduction of ferricytochrome c by durohydroquinone (c).

The slope of the ascending limb of the profile for ΔH^+ is a measure of a-b + c, and the slope of the descending limb is a measure of b-c. The sum of the values for these two slopes (a-b + c + b-c) is then numerically equal to the rate of energized proton release or the energized proton release flux (a). In evaluating the ratio of the energized proton or cation flux to the electron flux, there is a discrepancy in time in the measurements of the respective fluxes--the proton or cation flux being measured in the approach to equilibrium and the electron flow being measured at equilibrium. But since the rate of O_2 uptake is constant, whether at the equilibrium phase or in the approach to the equilibrium plateau phase, there is no need to measure the slope of the oxygen uptake curve over the same short time interval as the slope of the curve for either ΔK^+ or ΔH^+. The constancy of the oxygen uptake curve throughout the pulse period was determined in a special experiment carried out below 10° in which H_2O_2 was pretreated with catalase before introduction into the reaction cell, thereby eliminating the time required for catalatic decomposition of H_2O_2; and under these conditions the rate of O_2 uptake could be measured before and after the equilibrium phase set in.

The assay conditions relevant to the measurement of ΔH^+, ΔK^+ and ΔO_2 are specified in the legends for Figs. 1 and 2. Green et al. (1) have already described the details of the electrode measurements.

To demonstrate that protons are taken up on the M side during an oxygen pulse, electron transfer particles prepared by the method of Linnane and Ziegler (11) were used. These particles expose the M side of the inner membrane to the external phase (12). The so-called uncoupled respiration of these particles can be identified as energized cyclical cation transport as evidenced by the U-shaped profile for ΔH^+--a profile diagnostic for cyclical cation transport. Since K^+ is the only cation present in the assay medium, it is reasonable to assume that K^+ is the dominant cationic species being transported.

Ferricyanide titration method

Alexandre and Lehninger (12) have introduced the ferricyanide method to evaluate the H^+/e ratio for the oxidative sequences mediated by Complexes I and III, respectively. In presence of ferricyanide and K^+ salts it can be shown that the NADH → Q and QH_2 → ferricytochrome c oxidative sequences are coupled to the net transport of K^+. There is no evidence for proton cycling. In determining the H^+/e ratio, the concentration of protons released is compared to the concentration of ferricyanide that has undergone reduction (one electron taken up per molecule of ferricyanide reduced). The H^+/e ratio can be expressed as a ratio either of concentrations or of rates since the two are equivalent when the reactants measured reflect the same enzymic reaction. We have used heavy beef heart mitochondria (frozen and thawed 2 x) to measure the NADH → Q and QH_2 → ferricytochrome c reactions in absence of valinomycin and K^+, and rat liver mitochondria prepared by the method of Lardy et al. (13) to measure the same two oxidative sequences in presence of added valinomycin + K^+. The H^+/e ratio is the same whether valinomycin is added or not. The necessity for valinomycin depends upon the extent of respiratory control.

When ferricyanide is used as terminal oxidant, cyclical cation transport is replaced by net cation transport. The alkalinization of the matrix space is the event that triggers nonenergized efflux since this efflux generates protons for the matrix space. This alkalinization is avoided by the use of ferricyanide since the uptake of protons on the M side during reduction is balanced by the release of protons during oxidation by ferricyanide. Without alkalinization of the matrix space, the capacity for cation uptake is increased by an order of magnitude particularly as ferrocyanide formed by reduction of ferricyanide provides the anion needed for retention of accumulated cation.

RESULTS: Figs. 1 and 2 show the U-shaped profiles for ΔK^+ and ΔH^+, respectively, during an oxygen pulse of a mitochondrial suspension. Fig. 3

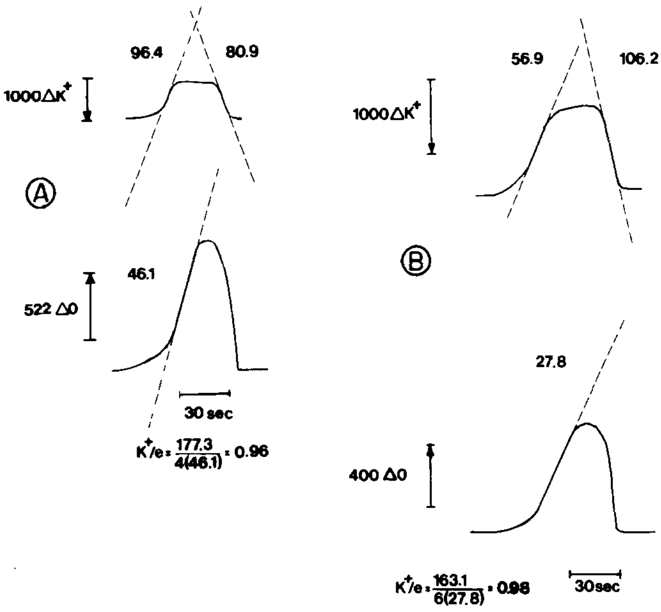

Figure 1. U-shaped profiles for ΔK^+ in mitochondria carrying out the $QH_2 \rightarrow O_2$ reaction (A) and the NADH $\rightarrow O_2$ reaction (B)

The numbers adjacent to the dotted lines indicate the rate of K^+ or oxygen (O) change in nmol/sec.

A - The reaction mixture (10 ml) was 0.25 M in sucrose, 50 mM in choline chloride, 1 mM in KCl and 2 mM in K^+-Hepes buffer (pH 7.4). It contained in addition 300 μl of a saturated solution of durohydroquinone in ethanol, 20 μl of 5% catalase and 500 μl of a suspension of Ca^{2+}-state mitochondria (25 mg/ml). The reaction at 30° was initiated by the addition of H_2O_2 (10 μl of 0.5% H_2O_2).

B - The reaction mixture (8 ml) was 0.2 M in sucrose, 5 mM in K-Hepes buffer (pH 7.3), 40 mM in choline chloride, 2.5 mM in KCl, 0.5 mM in EDTA, 1.25 mM in choline glutamate and 1.25 mM in choline malate. It contained in addition 25 mg of heavy beef heart mitochondria and 20 μl of 5% catalase solution. The reaction was initiated by addition of 0.5% H_2O_2. The reaction mixture was incubated at 30° for 2-3 min before addition of glutamate and malate. All acids were neutralized to pH 7.3 with choline base. The mitochondrial suspension was frozen and thawed twice before use in the experiment.

shows the profile for ΔH^+ during an oxygen pulse of a suspension of electron transfer particles. These profiles describe the changes observed in the coupling of electron transfer (NADH $\rightarrow O_2$, $QH_2 \rightarrow O_2$) to cyclical transport of K^+. A value close to unity was consistently found for both ratios and for the two kinds of particles. Table 1 shows that variation in the values of the H^+/e ratios obtained by repetition of the same experiment is sufficiently small as to eliminate the possibility of the ratio being significantly greater than unity. With the data now available for the values of the K^+/e and H^+/e ratios obtained for the three oxidative sequences (NADH $\rightarrow O_2$, $QH_2 \rightarrow O_2$ and ferrocytochrome *c* $\rightarrow O_2$), we can conclude that these ratios have a value

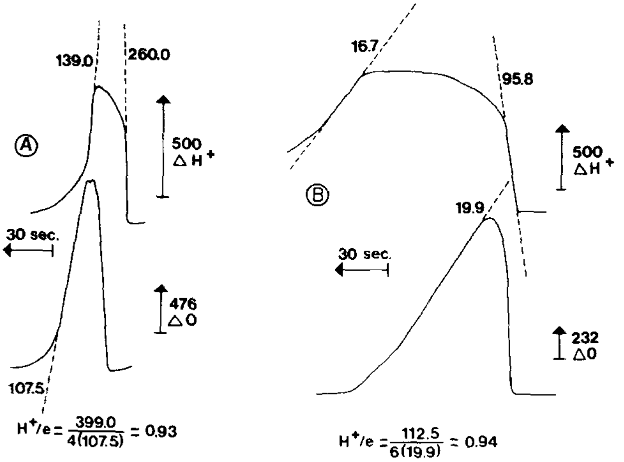

Figure 2. U-shaped profiles for ΔH^+ in beef heart mitochondria carrying out the $QH_2 \rightarrow O_2$ reaction (A) and the NADH → O_2 reaction (B)
The rates of H^+ or oxygen (O) change are expressed in nmol/sec. The assay medium (10 ml) was 0.25 M in sucrose and 50 mM in KCl. It contained in addition 500 µl of mitochondrial suspension (50 mg/ml) and 20 µl of 5% catalase. To A was added 300 µl of a saturated ethanolic solution of durohydroquinone. To B were added 20 µg of oligomycin, 50 µl of 1 M pyruvate, 100 µl of 0.2 M malate and 2 µmol of $CaCl_2$. The reaction at 38° was initiated by addition of 10 µl of 0.5% H_2O_2.

of unity for the oxidative sequences mediated by Complexes I and III, respectively. It can also be concluded that protons are released oxidatively in mitochondria (I side external) and taken up in electron transfer particles

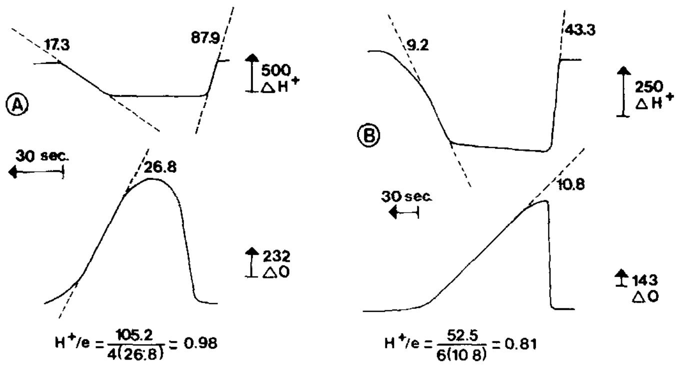

Figure 3. U-shaped profiles for ΔH^+ in electron transfer particles carrying out the $QH_2 \rightarrow O_2$ reaction (A) and the NADH → O_2 reaction (B)
The assay medium (10 ml) was 0.25 M in sucrose and 50 mM in KCl. It contained in addition 500 µl of the particle suspension (15 mg/ml) and 20 µl of 5% catalase. The reaction at 30° was initiated by addition of 10 µl of 0.5% H_2O_2. To A were added 100 µl of 5 mM ferricytochrome c and 100 µl of M succinate. To B were added 100 µl of 5 mM ferricytochrome c, 200 µl of 10 mM NAD^+, 100 µl of a 1% solution of alcohol dehydrogenase, 200 µl of 0.2 M semicarbazide and 100 µl of M ethanol.

Table 1. Range of variation in the determination of the H^+/e ratio for the $QH_2 \rightarrow O_2$ reaction mediated by Ca^{2+}-state mitochondria

Exp.	Temp.	ΔO_2*	Release Rate* of H^+	Uptake Rate* of H^+	Flux* of H^+	H^+/e
1	20	37.3	77.3	56.1	133	0.90
2	20	38.7	84.6	63.0	148	0.95
3	20	47.6	79.7	76.5	156	0.82
4	20	27.7	55.3	43.9	99.2	0.89
5	25	50.5	84.9	76.1	161	0.80
6	30	77.8	141	142	283	0.91

*All rates are n atoms/sec. Average 0.88 ± 0.18

The assay medium (10 ml final volume) was 0.2 M in sucrose, 4 mM in K-Hepes, 50 mM in KCl and contained 10 mg of Ca^{2+}-state mitochondria, 0.5 mg of catalase and 200 µl of a saturated solution of durohydroquinone in ethanol.

The three rates (ΔO_2, Δ Release of H^+ and Δ Uptake of H^+) were determined by the method described in the section on Experimental Methods.

(M side external)--the values of the H^+/e ratios being identical but of opposite sign.

The curves in Fig. 4 describe the release of protons as a function of time on the addition of ferricyanide to a suspension of rat liver mitochondria executing the $QH_2 \rightarrow$ ferricytochrome *c* reaction sequence in presence of valinomycin + K^+. The H^+/e ratio in this experiment was found to be close to unity. In a previous communication (3) data have been presented that addition of ferricyanide to a suspension of beef heart mitochondria executing

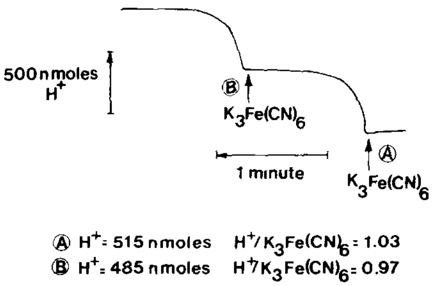

Figure 4. H^+/e ratio for the $QH_2 \rightarrow$ ferricytochrome *c* reaction in rat liver mitochondria

The assay mixture (10 ml) was 2.50 mM in sucrose, 50 mM in KCl, 1 mM in Tris chloride (pH 7.5), 10 mM in succinate and 1 µM in valinomycin. It contained 15 mg of a freshly prepared rat liver mitochondrial suspension. EDTA (0.1 mM) was present throughout the preparation of the mitochondrial suspension. When the recording electrode was stabilized, 500 nmoles of $K_3Fe(CN)_6$ were added and the pH charge recorded. Standard HCl was added to calibrate the chart recording.

the NADH → Q reaction sequence in absence of valinomycin also shows a H^+/e ratio of unity. Thus by an independent method the value of the H^+ ratio for the oxidative sequences mediated by Complexes I and III, respectively, have been shown to be unity.

DISCUSSION: The data presented establish that coupling in Complexes I and III follows the same pattern as that for coupling in cytochrome oxidase. Lehninger and his colleagues have published a series of papers in which values of 2 for the H^+/e and K^+/e ratios for each of the three coupling sites were reported (14-17). We have repeated several of their experiments (Fig. 4) and have consistently found the value of the ratios to be close to unity.

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