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ENERGIZED CATION TRANSPORT BY COMPLEX III (UBIQUINONE-CYTOCHROME c REDUCTASE)

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SUMMARY

Energized cyclical and net transport of cations and anions has been demonstrated in liposomes containing Complex III using a protamine-aggregation technique. Energization with reduced ubiquinone induces a rapid ion uptake followed by a more gradual efflux upon exhaustion of the available reductant. Both monovalent and divalent cations are transported, but at relatively high concentrations divalent cations are transported in preference to monovalent cations. Results are consistent with a cation:electron ratio of unity.

INTRODUCTION

The protamine-aggregation technique of Rosier et al. (1) for measuring radiolabeled solute uptake by liposome preparations is an effective alternative method to the use of ion-selective electrodes. Using this technique, Rosier and Gunter (2) have demonstrated the energized accumulation of calcium by cytochrome oxidase-liposome preparations. We have also shown that cytochrome oxidase can mediate the transport of a wide variety of cationic species including both monovalent and divalent cations as well as amino acids (3). An important feature of these experiments was that the cytochrome oxidase-mediated energized transport of cations did not require the addition of ionophore-uncoupler combinations. Ionophore-uncoupler combinations are frequently added to such preparations to release respiratory control, supposedly by collapsing the pH-membrane potential according to the chemiosmotic theory of Mitchell (4). Green et al. (5) have demonstrated another important aspect of electron-transfer mediated cation transport, namely that cation transport can be a cyclical process. Thus initiation of electron transfer is paralleled by a rapid unidirectional movement of cations; upon exhaustion of the available reductant or oxidant, cation flux occurs under nonenergized conditions and of opposite directionality to the original energized flux. Quantitation of this process has shown the cation:electron ratio to be invariably equal to unity (5).

With the above studies in mind (2, 3, 5), we have set about characterizing the energy-dependent ion-transport properties of liposomes containing

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Complex III (ubiquinone-cytochrome c reductase) of the mitochondrial respiratory chain. The results of the present study suggest a basic similarity between the ion-transport properties of purified Complex III and those measured in cytochrome oxidase (3) or mitochondrial preparations (5).

MATERIALS AND METHODS

Complex III was prepared from beef-heart mitochondrial paste as previously described (6) essentially according to Rieske (7). Preparations of Complex III contained per gm of protein an average of 6.6 μmoles cytochrome b, 3.4 μmoles cytochrome c_1, and 6.4 μmoles nonheme iron. Asolectin (95% pure soybean phospholipid) was obtained from Associated Concentrates, Woodside, NY. Protamine sulfate (from herring, Grade III) was a product of Sigma Chemical Company. DB (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone), a CoQ_2 derivative (8), was kindly provided by Dr. B.L. Trumpower, Department of Biochemistry, Dartmouth Medical School, Hanover, NH. Reduced DB (DBH) was prepared according to Trumpower and Edwards (9).

The term "buffer" as used below will refer to 50 mM Tris-HCl, pH 7.4, containing where stated the appropriate concentration of other monovalent or divalent cations. Liposomes containing Complex III were routinely prepared as follows. Asolectin (2.5 gm) was added to 30 ml of buffer, 0.5% (v/v) in cholate, and sonicated for 5 min at 50 W power (Branson Sonifier), the vessel contents being kept cold by immersion of the vessel in an ice-water mixture. Complex III, suspended in buffer containing 0.1% (v/v) cholate, was added to the asolectin mixture (phospholipid:protein wt. ratio 10:1) together with 2 μmoles of cytochrome c. The volume was made up to 50 ml with buffer and briefly homogenized before dialyzing against 4 l of buffer for 12 hr at 4°C. After dialysis, the liposome mixture was gassed with nitrogen for 15 min, an appropriate radiolabeled cation or anion added to give approximately 0.1 μCi/ml, and the mixture left to stand under a nitrogen atmosphere at 0-4°C for a further 12 hr to allow for the complete equilibration of the radiolabel.

Energized cation uptake was measured using the protamine-aggregation technique of Rosier et al. (1) as follows. Samples of Complex III-liposomes (1 ml) were added to 1.5 ml plastic centrifuge tubes. Typically, 20 μl of 0.1 M $K_3Fe(CN)_6$ was added to each tube and mixed, and the enzymic reaction started by addition of 500 nmoles of DBH (no more than 5 μl of a concentrated ethanolic solution of DBH). After addition of DBH, tubes were immediately stoppered, the contents mixed by inversion, and the reaction effectively stopped at the required time by the addition and mixing of 100 μl of protamine sulfate in buffer (25mg/ml). The aggregated liposomes were immediately sedimented by centrifugation for 2 min in a Brinkmann 3200 microcentrifuge, the pellet rinsed once with buffer, and resuspended in 1 ml of buffer for addition to 10 ml Aquasol scintillation fluid for counting. The amount of transported cation was corrected for by subtraction of zero time readings (with addition of ethanol in place of DBH or addition of protamine sulfate before adding DBH) and by allowance for variations in the pellet entrapped label. Pellet entrapped label was estimated under the various conditions used (e.g., ± DBH, ± $K_3Fe(CN)_6$) by employing [^{14}C] glucose that was not transported under these conditions during the duration of such experiments. Energized transport of ions was measured at a temperature of between 3-6°C with the liposome mixtures maintained on ice; at this temperature the enzymic activity of Complex III-liposome preparations averaged about 163 nmoles cytochrome c reduced/sec/mg protein.

RESULTS

The addition of 500 nmoles of DBH to a Complex III-liposome mixture, containing 2 μmoles of $K_3Fe(CN)_6$, induced a reduction-oxidation cycle of cy-

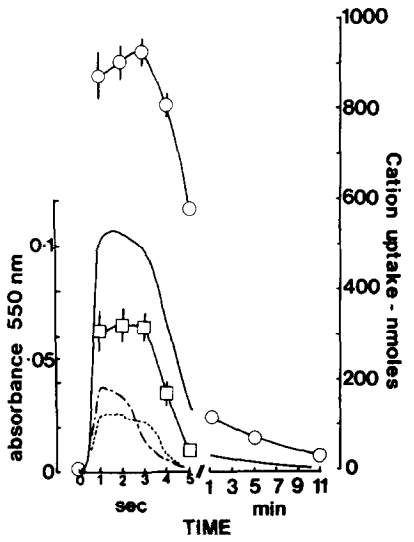

Fig. 1. Profiles of cytochrome c reduction-oxidation and cation uptake--efflux cycles. Reduction-oxidation of cytochrome c was followed spectrophotometrically with time. Antimycin and uncoupler (FCCP-carbonylcyanide-p-trifluoromethoxy-phenylhydrazone) were preincubated with Complex III-liposomes from the time of equilibration with radiolabel (see Methods) at concentrations of 7.3 µg/mg protein and $1x10^{-3}$M, respectively. The liposome mixture (1 ml containing 50 mg asolectin and 5 mg Complex III) contained 50 mM Tris-HCl, pH 7.4 (for $Tris^+$ transport), or 50 mM Tris-HCl and 5 mM $CaCl_2$ (for Ca^{2+} transport). Solid line is absorbance change at 550 nm due to reduction-oxidation of cytochrome c following addition of 500 nmoles of DBH. O-O = $Tris^+$ uptake, □-□ = Ca^{2+} uptake, -.-.- = $Tris^+$ uptake in the presence of antimycin, ----- = $Tris^+$ uptake in the presence of FCCP.

tochrome c (and cytochrome c_1) that could be followed spectrophotometrically at 550 nm (Fig. 1). Concomitant with reduction-oxidation of cytochrome c there was a rapid uptake of cations, followed by a more gradual efflux of cations upon exhaustion of available substrate. In Fig. 1 is shown the energized uptake and release of $Tris^+$ and Ca^{2+} by a Complex III-liposome preparation. The rapidity of the enzymic reaction following addition of DBH was too great to allow a meaningful estimation of the rate of cation uptake, and readings taken over the first 1-3 sec of the reaction were difficult to accurately quantitate (shown by the bars in Fig. 1). Nevertheless, that the measured cation uptake over this period remained relatively constant was taken to mean that this plateau phase did represent a reliable estimate of the maximum cation uptake for a given amount of DBH. Maximum cation uptakes tabulated in this report are therefore based on estimates over the first 3 sec of reaction, i.e., during the plateau phase of cation uptake. In the experiments shown in Fig. 1, the addition of 500 nmoles of DBH (equivalent to 1000 reducing equivalents) to a liposome mixture 50 mM in Tris-HCl induced a maxi-

Table I. Energized uptake of cations by Complex III-liposomes

nmoles of	Maximum[a] Cation Uptake (nmoles)								
DBH added	$Tris^+$	$Tris^+ + Ca^{2+}$		$Tris^+ + Sr^{2+}$		$Tris^+ + Rb^+$		$Tris^+ + Na^+$	
50	88	58	27	61	29	78	9	77	18
100	186	128	66	125	64	176	19	157	42
300	581	379	198	382	196	531	52	484	97
500	974	652	330	655	331	882	86	783	192

Cation uptake was measured at approximately 4°C as described under Methods. The concentration of Tris-HCl was 50 mM in all cases. Other cations were added as their chloride salts at concentrations of Ca^{2+} = 5 mM, Sr^{2+} = 5 mM, Rb^+ = 5 mM, Na^+ = 10 mM and their energized uptake measured by inclusion of the appropriate radioisotope. $Tris^+$ uptake was measured in different cation media by inclusion of [^{14}C] Tris only.

[a]Maximum cation uptake was determined by averaging nine readings, three each at 1, 2 and 3 sec following addition of DBH (see Fig. 1 and text).

mum measurable $Tris^+$ uptake of about 930 nmoles within the first 3 sec of the reaction; this was immediately followed by an efflux of $Tris^+$ and by 1 min the accumulated cation had decreased to some 14% of its level of maximum accumulation. A further 10 min were required for efflux to restore the internal and external $Tris^+$ concentrations to equilibrium. In a medium 50 mM in Tris-HCl and 5 mM in $CaCl_2$, the energized uptake and nonenergized efflux of Ca^{2+} was qualitatively similar to that observed for $Tris^+$ (Fig. 1). Complex III-liposomes preincubated with antimycin showed little uptake of $Tris^+$ upon addition of DBH; similarly, addition of uncoupler significantly diminished the extent of cation uptake (Fig. 1).

Experiments with Complex III of the type shown in Fig. 1 clearly demonstrate the cyclical nature of energized cation transport. The rate of rapid energized cation uptake (that is consistent with the enzymic activity of such preparations) probably exceeds several fold the rate of cation efflux. Thus a maximum cation uptake can be attained and held for a short duration until substrate is exhausted and cation efflux down its concentration gradient can proceed.

The extent of energized cation uptake correlated closely with the amount of DBH added to Complex III-liposomes (Table I). For all concentrations of DBH added, a stoichiometry almost equal to or slightly less than unity was obtained for the ratio, amount of reducing equivalents added:maximum cation uptake. This stoichiometry held in media containing only monovalent cations ($Tris^+$) or a mixture of monovalent and divalent cations (Table I).

In media containing mixtures of different monovalent cations (e.g., $Tris^+ \pm Na^+, \pm Rb^+$) no preference was found for transport between these cations. In

Table II. Relation of the extent of energized cation uptake to its concentration in the medium

Concentration of cation tested (mM)	nmoles of maximum cation uptake	
Ca^{2+}	Ca^{2+}	$Tris^+$
0	0	972
0.5	10	962
1	55	921
2	142	829
5	328	654
5[a]	521	445
Na^+	Na^+	$Tris^+$
0	0	976
1	19	954
5	89	884
10	162	816
10[a]	328	644

Maximum cation uptake was measured according to Methods and legend to Table I. $Tris^+$ uptakes given are those measured at the corresponding Ca^{2+} or Na^+ concentrations. Concentration of Tris-HCl was 50 mM. DBH was added at 500 nmoles.

[a]Concentration of Tris-HCl = 20 mM.

other words, the uptake of any particular monovalent cation reflected the proportion of that cation of the total monovalent cation concentration in the medium. However, this straightforward relationship between different monovalent cation uptake did not necessarily hold in media containing divalent cations. In Table II is given the maximal cation uptakes measured in media containing $Tris^+$ and Ca^{2+} or Na^+. For a given amount of DBH (500 nmoles) it can be seen that at low concentrations of Ca^{2+} (<1 mM) the combined uptake of $Tris^+$ and Ca^{2+} is approximately consistent with their relative concentrations. Thus in a medium containing 50 mM Tris-HCl and 0.5 mM $CaCl_2$, the addition of 1000 reducing equivalents produced a maximal cation uptake of ∿972 nmoles; this was comprised of ∿962 nmoles $Tris^+$ and ∿10 nmoles Ca^{2+}, a ratio of 96:1 ([Tris]:[Ca] = 100:1). However, as the divalent cation concentration of the medium was increased, the ratio of monovalent to divalent cation transported was decreased well below that expected from their relative concentrations. Thus in a medium containing 20 mM Tris-HCl and 5 mM $CaCl_2$, the amount of Ca^{2+}

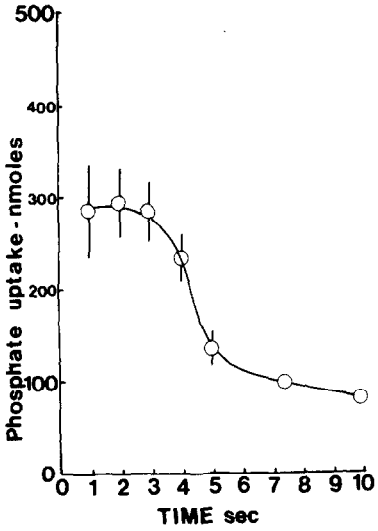

Fig. 2. Energized phosphate uptake by Complex III-liposomes. Phosphate uptake was measured with time upon the addition of 500 nmoles DBH to a medium containing 50 mM Tris-HCl and 10 mM K_2HPO_4, pH 7.4, and equilibrated with ^{32}Pi radioisotope.

transported actually exceeded the amount of $Tris^+$ transported (Table II). The preference for transport of divalent cation was much the same whether the combination was $Tris^+ + Ca^{2+}$ or $Tris^+ + Sr^{2+}$. In contrast, the amount of Na^+ transported compared to $Tris^+$ transported was strictly proportional to the concentration of these two monovalent cations in the medium (Table II).

Concomitant with the energized uptake of cations there was also a parallel uptake of anions. In Fig. 2 are shown the results from an experiment on phosphate uptake, measured in a medium containing Tris-HCl and K_2HPO_4. The energized uptake of phosphate anion was qualitatively similar to that observed for cation uptake, i.e., an initially rapid energized uptake of phosphate followed by a slower efflux upon exhaustion of reductant. No attempt was made to calculate stoichiometries for anion:electron or anion:cation transported because of the difficulty involved in determining the total anion concentration of the medium (in particular, the contribution of the bicarbonate anion dissolved in the medium).

DISCUSSION

The results presented in this study show clearly that under energized conditions, Complex III, incorporated into asolectin liposomes, has the capability to mediate the transport of monovalent and divalent cations as well as anions. Energization results in rapid ion uptake and is followed by non-

energized ion efflux from liposomes upon exhaustion of the available DBH. This ion cycling process mirrors the reduction-oxidation cycle of cytochrome c. Cyclical ion transport observed by "pulsing" Complex III with DBH would, therefore, appear to be a process similar to that observed in the cytochrome oxidase system pulsed with oxygen (5).

The present study and methodology allows for a reasonable quantitation of the uptake of individual cations mediated by Complex III and given amounts of DBH. Therefore, it has been possible to deduce that (a) one cation is transported per reducing equivalent giving a cation:electron stoichiometry of 1, (b) that different monovalent cations are transported with equal efficiency and (c) that at higher concentrations divalent cations are preferentially transported relative to monovalent cations. The first two deductions are in line with similar findings for the cytochrome oxidase system (3, 5). The preference for transport of divalent cations by Complex III may also be similar to that found for cytochrome oxidase. Our studies with cytochrome oxidase (3) indicated a greater net transport of Ca^{2+} relative to monovalent cations, which at the time we attributed to the more extensive cycling of monovalent cations. However, in the case of Complex III it is clear that both monovalent and divalent cations are equally well cycled (Fig. 1). Further experimentation with the cytochrome oxidase system may be required to clarify the basis for the preference shown for divalent cation transport.

It is our position that the present studies and those with cytochrome oxidase (2, 3, 5) are strongly suggestive that each electron-transfer complex has an inherent capability for cation transport. Without such a capability we would have to assume that in the absence of ionophore-uncoupler combinations the very rapid energized ion uptake and relatively rapid ion efflux can occur through the liposomal membrane. These and other experiments on electron-transfer mediated ion transport (2, 3, 5), as well as relevant studies pertaining to cytochrome oxidase (10-12), have led to the proposal of the two chain-direct coupling principle of energy coupling (13). In this scheme, intracomplex cation transfer (driven directly by electron transfer) is made possible by the existence of a cation-transfer chain.

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