Sensitivity to *NN'*-dicyclohexylcarbodi-imide of proton translocation by mitochondrial NADH:ubiquinone oxidoreductase

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Proton extrusion during ferricyanide reduction by NADH-generating substrates or succinate was studied in isolated rat liver mitochondria with the use of optical indicators. *NN'*-Dicyclohexylcarbodi-imide (DCCD) caused a decrease of 84% in the H^+/e^- ratio of NADH:cytochrome *c* reduction, but a decrease of only 49% in that of succinate:cytochrome *c* reduction, even though electron transfer was decreased equally in both spans. The data indicate that a DCCD-sensitive channel operates in the NADH:ubiquinone oxidoreductase region of the respiratory chain.

INTRODUCTION

According to the chemiosmotic theory of oxidative phosphorylation, ATP synthesis utilizes an H^+ -driven ATP synthetase, which conserves the energy of the proton electrochemical potential difference ($\Delta\bar{\mu}_{H^+}$) developed by the respiratory chain across the mitochondrial inner membrane (Mitchell, 1966). The original hypothesis postulated that the stoichiometry between H^+ pumping and electron transfer was $6 H^+/2e^-$ in the span from NADH to oxygen.

The experimental values obtained for $\Delta\bar{\mu}_{H^+}$ and the free-energy change of extramitochondrial ATP (ΔG_{ATP}) in mitochondria call for H^+/ATP ratios higher than 2 (Nicholls, 1974), and this means that the proton-pumping stoichiometry of the respiratory-chain reactions must be higher than was originally anticipated, and this is difficult to formulate without protons other than those involved in the redox reactions of a classical looped respiratory chain (Nicholls, 1974).

The $H^+/2e^-$ stoichiometry of ubiquinol:cytochrome *c* reductase is well established and adequately explained by the Q-cycle or its analogues (Wikström & Berden, 1972; Mitchell, 1976; Trumpower, 1981). It is also generally agreed that cytochrome *c* oxidase not only consumes protons in oxygen reduction, but extrudes protons from the mitochondria (Wikström, 1977; Mitchell *et al.*, 1985). High $H^+/2e^-$ values approaching 4 have been measured for the NADH:ubiquinone reductase segment of the respiratory chain, although results vary (Lawford & Garland, 1972; Ragan & Hinkle, 1975; Reynafarje & Lehninger, 1978; Wikström, 1984), and some methods have been criticized by Wikström *et al.* (1981) and Krab *et al.* (1984) because of time-resolution limitation.

NN'-Dicyclohexylcarbodi-imide (DCCD), a relatively non-specific carboxyl-group-modifying reagent, has proved useful under certain conditions as a probe for peptides reminiscent of the proton channel of F_1F_0 -ATPase (for references, see Azzi *et al.*, 1984). DCCD inhibition data suggest that similar proton channels may operate in the cytochrome oxidase complex (Casey *et al.*, 1979), although other roles have also been proposed for

the DCCD-binding component and the effect of DCCD binding. The proton-pumping mitochondrial NAD(P) transhydrogenase is also inhibited by DCCD (Pennington & Fisher, 1981), and proton translocation by ubiquinol:cytochrome *c* reductase is also sensitive to DCCD (Degli Esposti *et al.*, 1982; Price & Brand, 1983), although DCCD binding may not be mechanistically linked to protein translocation by this enzyme complex (Brand *et al.*, 1985).

Since the H^+/e^- stoichiometry of the first proton-translocating site is high and cannot be explained by a single redox loop, and since DCCD sensitivity is apparently linked to proton translocation, the present study was undertaken to reveal the contribution of a DCCD-sensitive component to proton translocation in this region of the respiratory chain. It was found that the $H^+/2e^-$ ratio of the NADH:ubiquinone reductase segment is higher than 4 and can be lowered to 2 in the presence of DCCD.

EXPERIMENTAL

Materials

Oligomycin, valinomycin, antimycin A, CCCP, rotenone, NEM and malic acid were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Glutamic acid and sodium succinate were from Fluka AG, Buchs, Switzerland. DCCD and the other chemicals were from E. Merck, Darmstadt, Germany, and were at least of reagent grade.

Experimental conditions

Liver mitochondria were isolated from male Sprague-Dawley rats in 250 mM-sucrose/5 mM-Tris/HCl/0.5 mM-EDTA, pH 7.5, as described previously (Schneider, 1948). The incubation medium consisted of 50 mM-KCl, 200 mM-sucrose and 0.5 mM-EDTA, pH 7.5, and was supplemented with NEM (66 μ M), KCN (1 mM), Phenol Red (19 μ M), rotenone (10 μ M), valinomycin (3.3 μ g/ml) and oligomycin (6.6 μ g/ml) where indicated. The mitochondria were incubated when indicated with DCCD for 3 min before the addition of the oxidizable

Abbreviations used: DCCD, *NN'*-dicyclohexylcarbodi-imide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazide; NEM, *N*-ethylmaleimide; $\Delta\bar{\mu}_{H^+}$, proton electrochemical potential difference.

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substrates, which were malate (1.65 mM) plus glutamate (1.65 mM), 3-hydroxybutyrate (2.5 mM) or succinate (1.65 mM). $K_3Fe(CN)_6$ was used as electron acceptor.

Methods

The rate of ferricyanide reduction was recorded spectrophotometrically (408 nm versus 469 nm) in a laboratory-built triple-beam spectrophotometer (Hassinen & Jämsä, 1982). Extramitochondrial proton concentration changes were simultaneously monitored by observing Phenol Red spectrum changes (540 nm versus 469 nm) and calibrated by the addition of known amounts of HCl. The pH_0 -pulse-height measurements were corrected for ΔpH decay as described by Reynafarje *et al.* (1979).

The rapid-mixing apparatus of Strittmatter (1964) was used to observe the initial reaction rates. The final tests were performed by the dual-wavelength technique with the rapid-mixing apparatus to allow optimization of the spectrophotometry separately for Phenol Red (540 nm versus 480 nm) and $Fe(CN)_6^{3-}$ (415 nm versus 455 nm), in order to eliminate interference between the spectral changes observed. The $Fe(CN)_6^{3-}$ reduction was apparently of zero order in the initial phase of the $Fe(CN)_6^{3-}$ pulse, and the accuracy of the graphic estimation of the initial rate of proton extrusion was verified by employing semilogarithmic plots of rate of $[H^+]$ change versus time.

RESULTS

Mitochondrial suspensions pulsed with $Fe(CN)_6^{3-}$ in the presence of KCN and an appropriate substrate

showed extramitochondrial acidification with a transient pulse in excess of the $1H^+/2e^-$ of the substrate-level dehydrogenations (Fig. 1). This was monitored simultaneously with the concentration of $Fe(CN)_6^{3-}$ and corrected for proton backflow by extrapolation depicted in Fig. 2. The extrapolated total proton extrusion was 9.46 ± 0.48 (mean $\pm$ S.E.M., $n = 3$) $H^+/2e^-$ with malate plus glutamate as the substrate and 5.05 ± 0.04 (mean $\pm$ S.E.M., $n = 3$) with succinate.

To minimize the effect of proton backflow, the experiments on the effect of DCCD were performed by using the rapid-mixing apparatus and recording the initial reaction rates. The initial extrusion rates in the absence of DCCD were $8.73 \pm 0.51 H^+/2e^-$ (mean $\pm$ S.E.M., $n = 4$) for malate plus glutamate and $3.93 \pm 0.07 H^+/2e^-$ (mean $\pm$ S.E.M., $n = 4$) for succinate (Fig. 3). The difference, $4.80 \pm 0.51 H^+/2e^-$, is due to the first phosphorylation site.

Preincubation with DCCD reduced the $H^+/2e^-$ ratio in a concentration-dependent manner (Fig. 3). At DCCD concentration of 64 nmol per mg of protein the $H^+/2e^-$ ratio in the presence of malate plus glutamate was lowered to 3.43 ± 0.09 , although in the presence of succinate (+rotenone) and the same concentration of DCCD the decline was only 16%. At higher DCCD concentrations the $H^+/2e^-$ ratio of succinate:cytochrome *c* reductase approached a value of 2, the number of scalar protons liberated in the succinate dehydrogenase reaction.

Not only the $H^+/2e^-$ ratio but also the electron-transfer activity in NADH and succinate oxidation by ferricyanide was inhibited by DCCD, although inhibition

Fig. 1. Time course of proton extrusion by mitochondria during oxidation of NAD^+ -linked substrates or succinate by ferricyanide

Extramitochondrial acidification (upper tracings in *a-d*) and $Fe(CN)_6^{3-}$ concentration (lower tracings in *a-d*) were monitored simultaneously in a triple-wavelength spectrophotometer by using Phenol Red as a colorimetric pH indicator under the conditions described in the Experimental section. (a) Glutamate plus malate; (b) glutamate plus malate in the presence of DCCD; (c) succinate plus rotenone; (d) succinate plus rotenone in the presence of DCCD. The protein concentration was 3.63 mg/ml; that of DCCD was 92 nmol/mg of protein. The reaction was initiated by the addition of 250 nmol of $K_3Fe(CN)_6$.

Fig. 2. Semilogarithmic plots of the decay of extramitochondrial acidification after the oxidation of NAD-linked substrates or succinate by $\text{Fe}(\text{CN})_6^{3-}$

The decays of the same acidification pulses as those depicted in Fig. 1 are shown. The stable (scalar) portion of the acidification has been subtracted. The ordinate is the logarithm of the absorbance change of Phenol Red and the abscissa the time after ferricyanide addition. The regression lines were fitted by the least-squares method. '+' is the extrapolated point corresponding to the time at which one-half of the ferricyanide was reduced. The $\text{H}^+/\text{2e}^-$ values by extrapolation were: (a) glutamate plus malate, 7.8; (b) glutamate plus malate and DCCD, 4.4; (c) succinate 3.3; (d) succinate and DCCD, 3.2.

of the latter was small with 64 nmol of DCCD per mg of protein (Fig. 4).

DISCUSSION

The present results corroborate previous reports of $\text{H}^+/\text{2e}^-$ ratios reaching values of 4 or higher in the NADH:ubiquinone reductase segment of the respiratory chain (Reynafarje & Lehninger, 1978; Wikström, 1984). In contrast with experimentation with an oxygen electrode (Reynafarje & Lehninger, 1978), the method used here is not subject to the criticism (Krab *et al.*, 1984) of a long time constant in the method used for measuring electron flux. In fact the values obtained with the methodology used here give $\text{H}^+/\text{2e}^-$ values for sites 1 + 2 that very closely obey the prediction of Lemasters and his group (Freedman & Lemasters, 1984; Lemasters *et al.*, 1984) arrived at on the basis of thermodynamic data.

To our knowledge, there are no previous reports on the effect of DCCD on proton pumping by site 1. The

Fig. 3. Effect of on the the $\text{H}^+/\text{2e}^-$ ratio of proton extrusion during $\text{Fe}(\text{CN})_6^{3-}$ reduction in rat liver mitochondria

Substrates were: ●, malate plus glutamate; ○, succinate. Initial rates of the extramitochondrial acidification and reduction were determined in separate experiments as described in the Experimental section.

Fig. 4. Effect of DCCD on the rate of $\text{Fe}(\text{CN})_6^{3-}$ reduction in rat liver mitochondria

Substrates used were: ●, malate plus glutamate; ○, succinate. Experimental conditions were as in Fig. 3.

$\text{H}^+/\text{2e}^-$ stoichiometry of site 1 was taken here as being the difference between the stoichiometries at sites (1 + 2) and 2, as the use of external ubiquinone-1 as an electron acceptor resulted in erratic proton translocation (results not shown), so that the stoichiometries observed by Lawford & Garland (1972) with external quinones could not be reproduced. A stoichiometry of $4\text{H}^+/\text{2e}^-$ was found here for the span from succinate to cytochrome *c*, in accordance with previous data (Reynafarje &

Lehninger, 1978). It is our experience, however, that the $H^+/2e^-$ ratio in the succinate:cytochrome *c* reductase region declines in the presence of DCCD, with a concomitant decrease in electron-transfer activity, even though Price & Brand (1983), Degli Esposti *et al.* (1982) and Beattie & Villalobo (1982) observed a decline in this ratio with no specific effect on electron-transfer activity. One should also note that Nałecz *et al.* (1983) observe DCCD to be an inhibitor of ubiquinol:cytochrome *c* reductase without bringing about any change in the H^+/e^- ratio, and that the inhibition of electron-transfer activity is related to cross-linking between its subunits V and VII, although all the subunits bind [^{14}C]DCCD. The 8 kDa protein, which is preferentially labelled with DCCD (Lorusso *et al.*, 1983; Clejan *et al.*, 1984), has recently been isolated from ubiquinol:cytochrome *c* reductase and characterized (Borchart *et al.*, 1985). A mechanistic role of the DCCD-binding component in H^+ translocation by the cytochrome *bc*₁ complex has not been corroborated (Brand *et al.*, 1985). The DCCD-binding component in the cytochrome *c* oxidase complex is subunit III, which has been considered to be involved in proton translocation (Casey *et al.*, 1980).

The molecular mechanism of proton pumping by NADH:ubiquinone reductase and its inhibition by DCCD remain to be established. It has recently been shown in this laboratory that two subunits of complex I isolated from submitochondrial particles treated with DCCD bind this inhibitor and that isolated NADH:ubiquinone reductase is inhibited by DCCD (P. T. Vuokila and I. E. Hassinen, unpublished work). Hence this segment of the mitochondrial respiratory chain can be included in the category of DCCD-sensitive proton-translocating enzymes.

Since NADH-dependent H^+ extrusion was inhibited at the low end of the concentration range of DCCD used (64 nmol/mg of protein), whereas succinate-dependent extrusion was not, it is improbable that the effect on the $H^+/2e^-$ ratio simply due to a DCCD-induced increase in the proton backflow in the membrane. This aspect of the effect of DCCD has been studied previously (Al-Shawi & Brand, 1981; Price & Brand, 1983).

The mechanism of H^+ translocation by NADH:ubiquinone reductase is still speculative, not least because of the multitude of functional components observed in this enzyme complex. There are two pH-sensitive iron-sulphur centres in NADH:ubiquinone reductase which could be involved in H^+ translocation (Ohnishi & Salerno, 1982; Hatefi, 1985), but the subunit allocation of centre N-2 is not known and the subunit corresponding to the molecular mass of the N-1a centre subunit does not bind DCCD (P. T. Vuokila & I. E. Hassinen, unpublished work).

Assignment of a function of the DCCD-sensitive component of NADH:ubiquinone reductase remains a task for the future, as the effects of DCCD on the relationship between the proton electrochemical potential and the redox span across site 1 may reveal mechanistic involvement of the DCCD-sensitive component in proton translocation, and DCCD binding experiments may show its subunit specificity. The present data indicate that the NADH:ubiquinone oxidoreductase complex can be added to the list of DCCD-sensitive mitochondrial proton-translocating enzymes.

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