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DCCD sensitivity of electron and proton transfer by NADH : ubiquinone oxidoreductase in bovine heart submitochondrial particles – a thermodynamic approach

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The sensitivity of the $H^+/2e^-$ ratio of the redox-driven proton pumping by the NADH : ubiquinone reductase (complex I) of the submitochondrial particles to dicyclohexylcarbodiimide (DCCD) was studied by a thermodynamic approach, measuring the membrane potential and ΔpH across the membrane and the redox potential difference across the complex I span of the respiratory chain. The $\Delta G_r/\Delta\tilde{\mu}_{H^+}$ ratio did not decrease upon additions of 50 or 100 nmol of DCCD per mg protein in the presence of oligomycin although the $H^+/2e^-$ ratio has been demonstrated to decrease upon DCCD addition in kinetic experiments with mitochondria. Complex I then becomes reminiscent of the cytochrome bc_1 complex, which shows DCCD sensitivity of the kinetically but not thermodynamically determined $H^+/2e^-$ ratio.

The lipophilic carboxyl group-reactive agent dicyclohexylcarbodiimide (DCCD) has proved to be an inhibitor of several mitochondrial enzyme systems capable of H^+ translocation across the inner mitochondrial membrane. These include the proton channel of F_0 part of the mitochondrial H^+-ATPase [1] (for further references, see Ref. 2), the cytochrome oxidase complex (complex IV) [3], the cytochrome bc_1 complex (complex III) [4,5], energy-linked NAD(P) transhydrogenase [6] and NADH : ubiquinone oxidoreductase (complex I) [7,8]. The DCCD-sensitivity of complex I has been demonstrated in pulse and initial velocity experiments in rat liver mitochondria by comparing its effects on the complex I plus complex III span on one hand and complex III alone on the other, showing in this way that DCCD abolishes the appearance of vectorial protons at concentrations below those abolishing electron-transfer activity. Two subunits of complex I (13.7 and 21.5 kDa) bind DCCD in submitochondrial particles [8]. A subunit selectivity of DCCD binding to isolated complex I has also been demonstrated, although reports on the identity of the DCCD-binding subunits and the preferential inhibition of proton transfer are at variance [7,9]. It is noteworthy that DCCD sensitivity of complex I is correlated with its capability to energy conservation when various organisms are compared [9].

The selectivity of the DCCD inhibition of complex I with respect to proton transfer could be challenged by a thermodynamic approach in which the free-energy change in the redox reaction across complex I is compared with the electrochemical potential of protons across the mitochondrial membrane [10]. The present study uses this approach in submitochondrial particles to facilitate the estimation of the redox-potential span across complex I.

Chemicals. DCCD, and the routine chemicals were from Merck, Darmstadt, F.R.G. Myxothiazol, NAD, NADH and purified enzymes were from Boehringer-Mannheim GmbH, Mannheim, F.R.G. Succinate (sodium salt) and fumaric acid were purchased from Fluka A.G. Buchs, Switzerland. Hepes, oligomycin, CCCP and phospho*enol*pyruvate (tricyclohexylammonium salt) originate from Sigma Chemical Co., St.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; complex I, NADH : ubiquinone oxidoreductase; complex III, ubiquinol : cytochrome *c* oxidoreductase; complex IV, cytochrome *c* oxidase complex; DCCD, dicyclohexylcarbodiimide; $\Delta\tilde{\mu}_{H^+}$, electrochemical potential of protons; $\Delta\psi$, membrane potential; E_h, actual redox potential; E_m, standard redox potential at pH specified; ΔE, redox potential difference; ETP, bovine heart submitochondrial particles; T, absolute temperature; R, the gas constant; F, the Faraday constant; ΔG, Gibbs free energy; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $MeNH_3^+$, methylammonium cation; TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine.

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Louis, MO. [U-^{14}C]sucrose, potassium [^{14}C]thiocyanate and [^{3}H]methylamine hydrochloride were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. and tritiated water from NEN Chemicals, Dreieich, F.R.G. Sephadex G-50 was from Pharmacia, Uppsala, Sweden.

Mitochondrial preparations. Bovine heart mitochondria were isolated according to Blair [11] and stored at $-70°$ for at least 2 weeks before further processing. Submitochondrial particles (ETP) were prepared in 0.23 M sucrose/10 mM sodium Hepes/0.2 mM EDTA (pH 7.4) and suspended in 0.23 M sucrose/10 mM sodium Hepes (pH 7.4).

Determination of internal volume and membrane orientation of submitochondrial particles. Two approaches were used for the former purpose. One was in principle similar to the method used by Oku et al. [12] for liposomes and based on determination of the intravesicular space occupied by sucrose during the sonication step in the preparation of ETP. [^{14}C]sucrose (0.23–0.9 μCi/ml) was present during the sonication whereafter the particles were washed twice in the same tracer-containing medium to eliminate soluble protein and applied onto a Sephadex G-50 column (1 cm × 18 cm) equilibrated with the same buffer without radioactive tracer. The fractions containing particulate material were collected and the radioactivity determined. The other method was essentially the same as used by Sorgato et al. [13] and based on a dual label experiment in the presence of [^{14}C]sucrose and $^{3}H_2O$.

Our initial approach for determination of the intravesicular water volume was an entrapment method which would detect only opened and resealed particles, which probably represent the particle population with the membrane orientation sought. This gave a vesicle space of 0.48 ± 0.05 (mean ± SE) μl per mg protein. The solute exclusion method gave a value of 1.03 ± 0.02 μl per mg protein, in accord with the data of Sorgato et al. [13]. Two explanations for this difference are possible: (1) the exclusion method detects all particles but the entrapment method only those which have been opened and resealed; or (2) the entrapment method is affected by losses of internal solute during harvesting of particles. The proportion of particles with the wrong orientation was 18%. If these had the same volume/protein ratio as the average vesicle population, an 18% error would result in the volume determined by exclusion. A reasonable estimate of the vesicle space with correct orientation would then be 0.84 μl per mg protein. The entrapment method would give the lower limit of the correct space. The capacity to oxidize external ferrocytochrome *c* was used as a test for the presence of particles with mitochondrion-like membrane orientation [14]. The ratio of the rates of cytochrome *c* oxidation in the absence and presence of 0.7% deoxycholate was taken as presenting the proportion of particles with mitochondrion-like membrane orientation. Protein was determined by the biuret method using bovine serum albumin as a standard.

Determination of membrane potential and proton gradient. SCN^- and methylamine were used as probes for $\Delta\psi$ and ΔpH, respectively. Distribution of the probes across the particle membrane was determined by the filtration method described by Berry and Hinkle [15]. Polylysine (20 μg per ml) was present in experiments employing SCN^- and $MeNH_3^+$, and enzyme assays of the filtrate showed practically full retention of the particles on a Whatman GF/F filter. The intravesicular probe concentration was calculated as in Ref. 15. Blank values of probe retention in particles plus filter were obtained by using particles uncoupled with CCCP.

Experimental protocol. The measurements were performed at 37° C in a total volume of 2.1 ml of 150 mM sucrose/50 mM sodium Hepes (pH 7.4). This was supplemented by ETP (2 mg protein), DCCD (in 5 or 10 μl ethanol), oligomycin (13 μg) and myxothiazol (0.4–0.6 μg). The amount of myxothiazol was selected in preliminary experiments to inhibit the oxygen consumption rate by 90% so as to bring the complexes I and II closer to equilibrium. It was assumed that the ubiquinone pool equilibrates with the succinate/fumarate couple under the conditions used. After incubation for 10 min in the presence of varying amounts of DCCD (or corresponding amount of the solvent ethanol) the reaction was started by adding 1 μmol each of NADH, NAD^+, succinate and fumarate. The experiment was terminated by taking 0.4-ml samples into 0.4 ml of 1 M KOH/50%-ethanol for the determination of NADH or into 0.4 ml of 12% $HClO_4$ for the determination of NAD^+, succinate and fumarate [16].

Statistics. An evaluation was performed using analysis of variance and the Bonferroni modification of Student's *t*-test [17].

Thermodynamics. The contribution of the ΔpH component (42 ± 7 mV) to the total Δp (165 ± 6 mV) was higher than in mitochondria but much lower than observed by Rottenberg and Lee [18], who have shown that the pH of EDTA particles gives 130 mV as the chemical component of the proton-motive force $\Delta p = \Delta\tilde{\mu}_{H^+}/F$. The mechanistic $H^+/2e^-$ ratio of proton pumping by complex I can be taken to be approximated by the $\Delta G_r/\Delta\tilde{\mu}_{H^+}$ ratio under near equilibrium conditions, where ΔG_r is the Gibbs free-energy change in the redox reaction between NADH/NAD^+ and ubiquinol/ubiquinone. The redox state of cytochrome *b* in the presence of myxothiazol was used to evaluate the equilibrium in the ubiquinone region of the respiratory chain. The E_m values of cytochromes *b* are in dispute because of the multicomponent analysis involved [19–21], but a redox titration of cytochrome(s) *b* with the succinate/fumarate couple under the present experimental conditions gave a clean curve with an apparent $E_{m7.4}$ of +23 mV for the main component

TABLE I

Effects of DCCD on the energetics of electron transfer and proton pumping by NADH: ubiquinone reductase in submitochondrial particles

The results are means ± S.E. from 7–12 experiments.

DCCD (nmol/mg protein)	$\Delta\psi$ [a] (mV)	$2.3 \cdot RT \cdot \Delta pH/F$ [b] (mV)	Δp [c] (mV)	ΔE_h [d] (mV)	n [e] ($H^+/2e^-$)
–	123 ± 4	42 ± 7	165 ± 6	324 ± 2	3.94 ± 0.41
50	98 ± 3 [f]	46 ± 9	144 ± 7 [f]	331 ± 1	4.69 ± 0.22 [f]
100	70 ± 4 [g]	32 ± 8	102 ± 6 [g]	338 ± 1	6.67 ± 0.45 [g]

[a] From SCN^- distribution.
[b] From $MeNH_3^+$ distribution.
[c] $\Delta p = \Delta\psi + (2.3 \cdot RT \cdot \Delta pH)/F$.
[d] $\Delta E_h = E_h^{succ/fum} - E_h^{NADH/NAD}$
[e] $n = 2 \cdot \Delta E_h / \Delta p$.
[f] $P < 0.05$ compared to controls.
[g] $P < 0.01$ compared to controls.

which is close to the +30 mV reported for one of the cytochrome b_{561} species [20]. Therefore a near-equilibrium most probably prevailed across site I in the absence of DCCD. The $\Delta G_r/\Delta\tilde{\mu}_{H^+}$ ratio was found to be 3.94 ± 0.41 (mean ± S.E. from seven experiments) under control conditions and was increased to 4.69 ± 0.22 by 50 nmol DCCD per mg protein and to 6.67 ± 0.45 by 100 nmol DCCD per mg protein (Table I). The $H^+/2e^-$ ratio under control conditions was comparable to the experimentally determined ratio for complex I in intact rat liver mitochondria [7,22,23]. However, the increasing $H^+/2e^-$ ratio under the influence of DCCD is probably only apparent and results from inhibition of complex I to a degree which no more allows near-equilibrium in the reaction. Particles with mitochondrion-like membrane orientation could be energized in the presence of myxothiazol by feeding in reducing equivalents at the level of cytochrome *c*. In particles like ETP with inverted membrane this necessitates use of membrane-penetrant redox mediators like TMPD. However, it has been shown that these cause shunting of certain spans of the respiratory chain making the measurement of $H^+/2e^-$ ratios at any specific site unreliable [24], and in the case of TMPD low coupling efficiency has been observed [25].

The question is how DCCD is able to decouple the proton transport from electron transfer in complex I in kinetic experiments, as has also been demonstrated in the case of complexes III and IV [3,4]. A tight mechanistic link would cause an equal inhibition of both modalities of the reaction. If there were no effect on the mechanism of coupling, would there be tightly and loosely coupled units in the mitochondrial preparation? The thermodynamic approach seems to give evidence which indicates that the situation in complex I is similar to that observed in complex III, where DCCD does not affect the mechanistic stoichiometry of the reaction estimated by a thermodynamic approach [10], even though kinetic measurements demonstrate an inhibitory effect [4,5]. One should note that the known oligomycin-like effect of DCCD on mitochondria is not involved in the present experiments, because oligomycin was included in all incubation to eliminate the effects of DCCD at the level of the F_1F_0-ATPase [1]. This also excludes the use of ATP-generated $\Delta\tilde{\mu}_{H^+}$ in experimentation.

The dissociation between the data obtained by a kinetic and thermodynamic approach appears to be a persistent phenomenon because in this respect the complexes I and III behave similarly. There might also be a greater analogy between the proton-conducting channels of the various proton-pumping electron-transferring and ATP-hydrolyzing enzyme complexes than previously thought.

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