NN'-Dicyclohexylcarbodi-imide-sensitivity of bovine heart mitochondrial NADH:ubiquinone oxidoreductase

Inhibition of activity and binding to subunits

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Dicyclohexylcarbodi-imide (DCCD) inhibition of NADH:ubiquinone oxidoreductase was studied in submitochondrial particles and in the isolated form, together with the binding of the reagent to the enzyme. DCCD inhibited the isolated enzyme in a time- and concentration-dependent manner. Over the concentration range studied, a maximum inhibition of 85% was attained within 60 min. The time course for the binding of DCCD to the enzyme was similar to that of activity inhibition. The NADH:ubiquinone oxidoreductase activity of the submitochondrial particles was also sensitive to DCCD, and the locus of binding of the inhibitor was studied by subsequent resolution of the enzyme into subunit polypeptides. Only two subunits (molecular masses 13.7 and 21.5 kDa) were labelled by [¹⁴C]DCCD, whereas, when the enzyme in its isolated form was treated with [¹⁴C]DCCD, six subunits (13.7, 16.1, 21.5, 39, 43 and 53 kDa) were labelled. Comparison with the subunit labelling of F₁F₀-ATPase and ubiquinol:cytochrome *c* oxidoreductase indicated that the labelling pattern of NADH:ubiquinone oxidoreductase, and enzyme complex with a multitude of subunits, is unique and not due to contamination by other inner-membrane proteins. The correlation between the electron- and proton-transport functions and the DCCD-binding components remains to be established.

INTRODUCTION

Although the chemiosmotic principle of energy conservation is well established (Mitchell, 1966), the stoichiometries of proton transfer by the respiratory chain and by H⁺-ATPase (F₁F₀-ATPase) are under dispute. Deviations from the original stoichiometries are probable on the basis of observations of the magnitude of the protonmotive force generated by the respiratory chain and the free-energy change involved in ATP hydrolysis under the same conditions, since the results call for H⁺/e⁻ stoichiometries higher than can be obtained by the classical redox-loop concept. Likewise measurements of the H⁺/ATP ratio of F₁F₀-ATPase and the known ATP/O ratios of oxidative phosphorylation suggest that H⁺ pumping in the respiratory chain must be in excess of that obtainable with redox loops. High H⁺/e stoichiometries have been observed across the span from NADH to cytochrome *c*, and proton pumping has been demonstrated in the mammalian cytochrome *c* oxidase complex (Wikström, 1977), which is usually not considered to contain redox loops, although such can be devised on the basis of H⁺-conducting 2H₂O/O₂ redox couples (Mitchell *et al.*, 1985).

Proton pumping has been shown to be sensitive to dicyclohexylcarbodi-imide (DCCD) in the cytochrome *bc*₁ complex (complex III) and to cytochrome *c* oxidase (complex IV) (Casey *et al.*, 1980; Beattie & Villalobo, 1982; Degli Esposti *et al.*, 1982). DCCD is also an inhibitor of the H⁺ pumping effected by F₁F₀-ATPase (Cattell *et al.*, 1971), and thus it is thought to have some

specificity for hydrophobic H⁺ channels, which probably contain a carboxy group.

Prompted by observations regarding the DCCD-sensitive pump in the cytochrome *bc*₁ and cytochrome oxidase complexes, an investigation of the NADH:ubiquinone oxidoreductase (complex I) span was performed. It was found that, in rat liver mitochondria, the proton-pumping function of complex I was inhibited by lower DCCD concentrations than was the electron transfer (Honkakoski & Hassinen, 1986). Further experiments presented here show a correlation between DCCD binding and inhibition of the electron-transfer function in complex I isolated from submitochondrial particles derived from bovine heart.

MATERIALS AND METHODS

Reagents

NN'-Dicyclohexyl[¹⁴C]carbodi-imide ([¹⁴C]DCCD) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Non-labelled DCCD and the routine chemicals were from Merck, Darmstadt, Germany. NADH was purchased from Boehringer-Mannheim G.m.b.H., Mannheim, Germany, and ubiquinone-1 (Q₁) was kindly given by Hoffmann-La Roche, Basle, Switzerland. Coomassie Brilliant Blue R-250 was from Serva, Heidelberg, Germany.

Mitochondrial preparations

Bovine heart mitochondria were isolated as described by Blair (1967) and stored at -70 °C for at least 2 weeks

Abbreviations used: DCCD, dicyclohexylcarbodi-imide; complex I, NADH:ubiquinone oxidoreductase complex; complex III, ubiquinol:cytochrome *c* oxidoreductase complex; complex V, F₁F₀-ATPase complex; PAGE, polyacrylamide-gel electrophoresis; Q₁, ubiquinone-1; ETP_H, submitochondrial particles.

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before further processing. Submitochondrial particles (ETP_H) were prepared in the presence of 2.2 mM-EDTA as described by Lee (1979) and suspended in 0.25 M-sucrose.

Enzyme isolation

The NADH:cytochrome *c* oxidoreductase complex was isolated from ETP_H or bovine heart mitochondria (Hatefi & Stiggall, 1978), from which the complex I was isolated as described by Hatefi (1978a). Complex III was isolated from the NADH:cytochrome *c* oxidoreductase complex as described by Hatefi (1978b) and Complex V from ETP_H by the method of Stiggall *et al.* (1978).

Analytical methods

The subunit compositions of the enzyme complexes isolated were determined by SDS/PAGE as described by Laemmli (1970). Proteins were located by staining with Coomassie Brilliant Blue, and the radioactivity of the subunits was determined by fluorography by the methods of Bonner & Laskey (1974) and Laskey & Mills (1975), with 2,5-diphenyloxazole impregnation of the gels, to which Kodak X-Omat AR film was exposed for 2–3 weeks at -70°C . After development, the film was scanned with a Kontes densitometer.

Total radioactivity in the submitochondrial particles and enzyme complexes was determined by liquid-scintillation counting in Instagel.

Protein was determined by the biuret method (Layne, 1957) or as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Determination of enzyme activity

NADH:ubiquinone oxidoreductase activity was assayed by using Q_1 as an electron acceptor (Hatefi, 1978a). The reaction mixture consisted of 20 mM-potassium phosphate, 2 mM- NaN_3 , 0.1 mM- Q_1 , 0.12 mM-NADH and 12 μM -EDTA, pH 8.0, at 37°C . The enzyme was added to a concentration of 5–10 $\mu\text{g}/\text{ml}$.

Labelling experiments

The DCCD-sensitivity of the system was tested in ETP_H suspended at a concentration of 1 mg/ml in 228 mM-sucrose/20 mM-potassium phosphate, pH 8.0. DCCD with a specific radioactivity of 4.6 Ci/mol as a concentrated solution in ethanol was added to a concentration of 20–500 nmol/mg of protein to give a final ethanol concentration of 2% (v/v). The time courses were studied by repetitive sampling from the 37°C reaction mixture up to 60 min. The reaction was terminated by adding a 5-fold excess of glutamate and cooling to 4°C .

The particles were sedimented out at 105000 *g* for 50 min, suspended in 0.25 M-sucrose, centrifuged once more and suspended in 50 mM-Tris/0.67 M-sucrose/1 mM-histidine, pH 8, to a concentration of 23 mg/ml.

This material was used for the isolation of complexes I, III and V as described above, whereupon their subunit compositions and label distributions were determined as described under 'Analytical methods'.

In some experiments the inhibition and labelling were tested by using complex I in its isolated form. In the labelling experiments the protein was precipitated with acetone and processed for SDS/PAGE and fluorography as described above, whereas for the inhibition experi-

Fig. 1. Time course of the inhibition of isolated NADH:ubiquinone oxidoreductase by DCCD

Conditions: 20 mM-potassium phosphate, pH 8.0, 2 mM- NaN_3 , 0.1 mM- Q_1 , 0.12 mM-NADH and 12 μM -EDTA; enzyme concentration, 6 $\mu\text{g}/\text{ml}$. The amounts of DCCD used (nmol/mg of protein) were: □, 20; ○, 50; △, 100; ■, 200. The enzyme was incubated in 43 mM-Tris/HCl (pH 8.0)/576 mM-sucrose/0.86 mM-histidine/0.1 mM-EDTA/2% (v/v) dimethylformamide/enzyme (1 mg of protein/ml) at 37°C in the presence of DCCD, and samples were drawn for activity determination at the time points indicated.

ments DCCD was dissolved in dimethylformamide, which was added to a final concentration of 2% (v/v).

The effect of NADH on DCCD inhibition and enzyme stability was studied in separate experiments. NADH and DCCD were added to the enzyme solution in appropriate concentrations, and a 6 μl sample was drawn from the reaction mixture after incubation for 30 min for NADH:ubiquinone reductase assay in a total volume of 1 ml.

The time course of [^{14}C]DCCD binding to the isolated enzyme was studied by precipitating the enzyme with 10% (w/v) trichloroacetic acid, filtering on a Whatman GF/F glass-microfibre filter and washing with diethyl ether/ethanol (3:1, v/v). The precipitate plus filter were counted for radioactivity in Instagel.

RESULTS

DCCD-sensitivity of enzyme activity

Isolated enzyme. The isolated NADH:ubiquinone oxidoreductase was inhibited by 90% with rotenone, which indicates intactness of the enzyme. This was inhibited by DCCD in a time- and concentration-dependent manner. Over the concentration range studied, the inhibition attained a stable value within 60 min. Maximum inhibition at 100–200 nmol of DCCD/mg of protein was about 85% (Fig. 1).

Inclusion of NADH in the preincubation mixture over the concentration range 30–120 μM afforded no protection against the inhibitory effect of DCCD on the isolated enzyme. On the contrary, preincubation of the enzyme with NADH at 37°C caused a concentration-

Fig. 2. Time course of the inhibition of NADH:ubiquinone oxidoreductase in submitochondrial particles by various concentrations of DCCD

The conditions of assay were as in Fig. 1. The particles (1 mg of protein/ml) were incubated in 215 mM-sucrose/20 mM-potassium phosphate (pH 8.0)/2% (v/v) ethanol in the presence of DCCD, and samples were drawn for activity determination at the time points indicated. DCCD concentrations (nmol/mg of protein) were: □, 20; ○, 50; △, 100; ■, 200; ●, 500.

dependent inactivation of the enzyme by 15–40% in the absence of DCCD. This occurred concomitantly with a KCN-insensitive oxidation of the NADH.

Submitochondrial particles. The complex I activity was also DCCD-sensitive in submitochondrial particles, a time- and concentration-dependent inactivation of the NADH:ubiquinone oxidoreductase activity being observed over the concentration range of 20–500 nmol of DCCD/mg (Fig. 2).

DCCD binding

Time course. The time course of the binding of [¹⁴C]DCCD to the isolated enzyme was similar to that of the inhibition of enzyme activity, and a stable value was reached in 60 min (Fig. 3).

Comparison of the subunit [¹⁴C]DCCD-labelling profiles of complexes I, III, IV and V. The complex I preparation purified from [¹⁴C]DCCD-treated ETP_H contained more than 27 distinct polypeptide subunits, of which only two, of size 13.7 and 21.5 kDa, contained significant [¹⁴C]DCCD radioactivity. On a protein basis the labelling of the 21.5 kDa subunit was high (Fig. 4). There was a clear enrichment of these radioactive polypeptides in the complex I during the purification steps. The labelling is different from that of complexes III and V, and indicates that these labelled subunits of complex I are not due to contamination. Although enriched in complex I, the labelled subunits must represent a small fraction of the total labelled peptides of ETP_H. Comparison of the Coomassie Blue staining patterns of the SDS/PAGE gels derived from native and DCCD-treated ETP_H indicates that there is no cross-linking of the subunits.

Complex V purified from the same batch of ETP_H

Fig. 3. Time course of binding of [¹⁴C]DCCD to isolated NADH:ubiquinone oxidoreductase

The incubation mixture was as in Fig. 1. Samples were drawn for protein precipitation at the times indicated and processed for radioactivity determination as described in the Materials and methods section. The concentration of DCCD was 100 nmol/mg of protein.

contained radioactivity in a subunit with an apparent size of 17.8 kDa. The radioactivity of the 'DCCD-binding polypeptide' with a reported *M_r* of 6300 was not resolved in the electrophoresis gel used, because it was swamped by the radioactivity migrating with the lipids. Complex III isolated from the same labelled source showed very little [¹⁴C]DCCD radioactivity, probably for the same reason. An attempt was made to obtain higher resolution in the low-*M_r* range of the electrophoretograms by using the method of Schagger *et al.* (1985), which employs separating gels of 16% (w/v) acrylamide and 20% (v/v) glycerol and a modified buffer system, but this method still left the molecular-mass range below 12 kDa with very little radioactivity (results not shown).

The labelling in isolated complex I incubated with [¹⁴C]DCCD (100 nmol/mg of protein) was weaker than for complex I in submitochondrial particles (Fig. 5), and again the label was found mainly in three subunits (13.7, 16.1 and 21.5 kDa). Labelling was also observed in subunits of size 43 and 53 kDa.

It has been shown recently that porin represents a major DCCD-binding protein of mitochondria and has a molecular mass of 35 kDa (De Pinto *et al.*, 1985). Although porin is a component of the mitochondrial outer membrane and is scarcely present at all in ETP_H, it cannot be excluded as the DCCD-binding component here. It has been previously suggested by the same authors that the 33 kDa component which binds DCCD is identical with the phosphate translocator, an inner-

Fig. 4. Labelling pattern and subunit composition of NADH:ubiquinone oxidoreductase isolated from [^{14}C]DCCD-labelled ETP_H

Submitochondrial particles were incubated in the presence of [^{14}C]DCCD (100 nmol/mg of protein) and complex I was isolated as described in the Materials and methods section. A 100 μg portion of the isolated enzyme was loaded on the SDS/PAGE gel. Protein was stained with Coomassie Brilliant Blue and radioactivity detected by fluorography. Upper trace, radioactivity; lower trace, protein staining. The values against the peaks indicate the sizes of the peptides in kDa.

Fig. 5. Labelling pattern of isolated NADH:ubiquinone oxidoreductase treated with [^{14}C]DCCD

The enzyme was incubated in the presence of 100 nmol of DCCD/mg of protein in 215 mM-sucrose/20 mM-potassium phosphate (pH 8.0)/2% (v/v) ethanol. The protein was precipitated with acetone and analysed as described in the Materials and methods section.

membrane component (Houštěk *et al.*, 1981a). A fraction with DCCD label was observed here in precisely this M_r region, even though neither of these peptides was co-purified with complex I.

The complex III preparation had subunit peptides with apparent molecular-mass values of 9, 12.5, 26.3,

32.6, 43, 45.5 and 71 kDa, and traces of similar bands were also observed in the SDS/PAGE pattern of complex I, but the labelling pattern argues for uniqueness on the part of the complex I polypeptides resolved here. For example, some radioactivity was found in the 12.5 and 9 kDa subunits of complex I, but not in those of complex

III, even though the preparations were from the same labelled batch of ETP_H.

A major peak in radioactivity was found in the 34 kDa fraction of the electrophoretogram of the total ETP_H protein, but this was not co-purified with complexes I, III or V. The labelling pattern of total ETP_H protein was reminiscent of the results of Houštěk *et al.* (1981b). Isolated complex III has been previously shown to be labelled mainly in cytochrome *b* (33–37.5 kDa) and the 8 kDa subunit VIII (Clejan *et al.*, 1984), but the present labelling, *in situ*, is different. The main DCCD-binding component of complex IV is the subunit III (19–25 kDa). The crude complex IV fraction of the present preparation showed label in that region, but the label was clearly enriched during the purification of complex I, so that it is unlikely that this particular radioactive subunit of complex I is a contaminant from complex IV.

DISCUSSION

The molecular mechanism of the energy-conserving function of complex I is still speculative because of the multitude of functional components observed in this enzyme. Its proton-pumping activity as well as electron transfer is affected by DCCD in rat liver mitochondria (Honkakoski & Hassinen, 1986).

Also the DCCD-sensitivity of the mitochondrial function as a whole is complicated because of the multitude of interaction mechanisms operating between DCCD, proteins and lipids (Azzi *et al.*, 1984). DCCD reacts with carboxy groups with a number of consequences, e.g. the binding of DCCD or formation of a new peptide bond and cross-linking without net binding of DCCD. The latter can also be bound to a thiol group or the phenolic hydroxy group of an aromatic amino acid. At neutral pH the most active DCCD-reactive groups are the carboxyl and thiol ones. The DCCD-binding group in F₁F₀-ATPase has been demonstrated to be a single glutamic acid residue in a hydrophobic domain (Sebald *et al.*, 1979). Cross-linking of the subunits of the isolated enzyme has been observed in complex III by two groups of workers (Lorusso *et al.*, 1983; Nalecz *et al.*, 1983), but this has been challenged by others (Clejan *et al.*, 1984). Complex V on the one hand and complexes I, III and IV on the other differ in their sensitivities to DCCD. Higher inhibitor concentrations are needed for the last three (Casey *et al.*, 1980; Degli Esposti *et al.*, 1982; Honkakoski & Hassinen, 1986).

The assignment of the inhibitory action of DCCD on complex I to a specific amino acid residue or functional group (carboxy or thiol) of one or more of the subunits of the enzyme remains to be established. As far as proton translocation is concerned, the *E*'₀ (midpoint potential) of the iron-sulphur cluster N-2 of complex I is pH-dependent (Ingledew & Ohnishi, 1980), and it has been suggested that this component is involved in H⁺ translocation (Ohnishi & Salerno, 1982; Hatefi, 1985), but its subunit allocation is not known. The other pH-sensitive iron-sulphur cluster is N-1a and it has been assigned to the 75 kDa subunit. Although an exact subunit allocation is lacking, the tetranuclear iron-sulphur cluster N-4 has been assigned to the IP_(II+III) fraction (Ohnishi *et al.*, 1985) that also contains a subunit with a molecular mass (13 kDa) corresponding to that of the peptide which binds DCCD in the present experiments. Complex I contains also in the iron-sulphur

protein fraction a 14 kDa subunit which binds Q (Suzuki & Ozawa, 1986). Preliminary experiments with chaotropic resolution of complex I suggest, however, that the DCCD-labelled subunits (including the 13.7 kDa subunit) are located in the hydrophobic residue of the enzyme (P. T. Vuokila & I. E. Hassinen, unpublished work). It is probable that selectivity of the labelling would increase upon decrease in DCCD concentration, but in this case the binding was investigated under conditions leading to rapid inhibition of the enzyme activity.

It is tempting to draw an analogy between the DCCD-sensitivity of the energy-conserving function of complex I (Honkakoski & Hassinen, 1986) and the documented interactions of DCCD with specific sites in other regions of the respiratory chain, viz. the glutamic acid-90 residue of subunit III of cytochrome oxidase (Casey *et al.*, 1980) and the glutamic acid-58 residue of the subunit 9 of F₁F₀-ATPase (Sebald *et al.*, 1979), which have been implicated in H⁺ translocation. In the case of cytochrome oxidase the relationship between the DCCD-reactive carboxy group and proton pumping is more complex than previously thought, because the H⁺-pumping capability partially survives removal of subunit III (Finel & Wikström, 1986). In each case the DCCD-binding component has been found to be a hydrophobic proteolipid. Also in complex I the hydrophobic fraction is the DCCD-binding moiety, as shown in the present study. This is in line with the fact that formation of a stable conjugate of DCCD with proteins necessitates a hydrophobic environment protecting the initial bond from nucleophilic attack by water. The primary structures of the mitochondrially synthesized six subunits of complex I can be deduced from the known nucleotide sequence of mitochondrial DNA, but none of these corresponds in molecular mass to the subunits binding DCCD in the present experiments (Chomyn *et al.*, 1985).

In conclusion, the present data show that the DCCD-sensitivity of the energy-conserving function of complex I in intact mitochondria is related to rather specific binding of the inhibitor to its subunits in submitochondrial particles and isolated enzyme. That selectivity of the susceptibility to DCCD is dependent on the microenvironments around the reactive site is demonstrated by the finding that selectivity is higher in submitochondrial particles than in the isolated enzyme. The findings await exact identification of the DCCD-binding sites.

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