Inhibition of electrogenic anion entry into rat liver mitochondria by *N,N'*-dicyclohexylcarbodiimide

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The carboxyl group reagent dicyclohexylcarbodiimide inhibits the electrogenic entry of Cl^- and NO_3^- into rat liver mitochondria at alkaline pH. The inhibition is time dependent and 50% inhibition is obtained by the addition of 3–4 nmol DCCD/mg protein. The blockage of the pH-dependent anion-conducting pore appears to be unrelated to the other known actions of DCCD on rat liver mitochondria but seems similar to its effect on the uncoupling protein of brown adipose tissue.

Anion permeability *Mitochondria* *Uncoupling protein* *Dicyclohexylcarbodiimide* *Membrane potential* *pH gradient*

1. INTRODUCTION

The carboxyl group reagent *N,N'*-dicyclohexylcarbodiimide (DCCD) was shown [1] to be a potent inhibitor of mitochondrial oxidative phosphorylation. Since then it has become well-established as an inhibitor of proton-pumping membrane enzyme systems [2–7]. DCCD has been shown to inhibit the high electrogenic Cl^- permeability of brown adipose tissue mitochondria and to bind covalently to the M_r 32000 uncoupling protein found in these mitochondria [8].

A similar, high electrogenic permeability to anions such as Cl^- and NO_3^- has been shown to exist in rat liver mitochondria at alkaline pH [9]. This observation has been rationalized in terms of a pH-dependent anion-conducting pore in the inner mitochondrial membrane. The pore appears to open progressively with increasing pH over pH 7–9, requires intra-mitochondrial Ca^{2+} and is inhibited by local anaesthetics such as nupercaine [10].

Here we report that, like the anion-conducting pore of brown adipose tissue, the pH-dependent anion-conducting pore of rat liver mitochondria is blocked by DCCD.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide-*m*-chlorophenyl hydrazone; FCCP, carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone; EGTA, ethyleneglycol-bis-(β-aminoethyl-ether)-*N,N'*-tetraacetic acid; HEDTA, *N*-(2-hydroxyethyl)ethylenediamine-*N,N',N'*-triacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

2. MATERIALS AND METHODS

DCCD was obtained from Aldrich Ltd (Gillingham, Dorset), FCCP and CCCP were from Boehringer Corp. (London) and HEPES was from Hopkin and Williams Ltd. All other reagents were of AR or highest available grade. Sucrose solutions used in the preparation of mitochondria, prior to the addition of buffers, were passed through a cation chelating column (Dowex chelating resin, dry mesh 50–100 from Sigma Chemical Corp.) to remove endogenous Ca^{2+}, since it has been shown [12] that Ca^{2+} affect the anion permeability of rat liver mitochondria at alkaline pH.

Mitochondria were prepared, and their protein concentration determined, essentially as in [11], except that 1.0 mM EGTA was included in the homogenisation and first wash medium. The mitochondria were finally resuspended in a solution containing 250 mM sucrose, 5 mM Hepes–KOH (pH 7.2).

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Light scattering measurements were made in a continuously stirred cuvette, thermostatted at 25°C as in [11]. Free Ca^{2+} concentrations used in the light scattering experiments were determined using the computer programme in [13]. All additions of FCCP, CCCP, antimycin, rotenone and DCCD were made as small volumes of ethanolic solutions.

Mitochondrial ATPase activity was assayed by measuring P_i release from ATP. The reactions were carried out in a medium which contained, in 1 ml, 100 mM KCl, 20 mM Hepes–KOH (pH 7.5), 3 μM CCCP and 2.5 mM $MgCl_2$. After temperature equilibration at 30°C, 2.5 mM ATP (K salt) was added, followed immediately by 200 μg mitochondrial protein to start the reaction. After 10 min, 0.5 ml 5% (w/v) trichloroacetic acid was added and after centrifugation P_i present in 1.0 ml supernatant was determined as in [14].

Incubations with DCCD were carried out at 35–60 mg mitochondrial protein/ml at 0°C. Details for individual experiments are given in the figure legends. Control incubations contained an equivalent volume of ethanol.

3. RESULTS

The electrogenic uniport of anions across the mitochondrial inner membrane can be followed by measuring the rate of decrease in light scattering when mitochondria are added to an isotonic solution of the ammonium salt of the anion in the presence of an uncoupler to allow H^+ movement to accompany the entry of the freely permeant NH_3. Respiratory inhibitors are added to abolish any energy-linked transport processes. Here, the uncoupler was present in the medium prior to the addition of mitochondria to minimise any effects of Ca^{2+} leakage from the mitochondria before the addition of uncoupler, since intra-mitochondrial Ca^{2+} appears to be required for the operation of the anion pore [9,12]. Because extramitochondrial free Ca^{2+} concentration also affects the rate of anion entry [12], all experiments were carried out in the presence of Ca-HEDTA buffers to give 1 μM free Ca^{2+} at the appropriate pH value. Under these conditions, rapid swelling is obtained in NH_4Cl at pH 8.0, while at pH 7.0 swelling is slow and limited in extent (fig. 1).

Fig. 1 shows that preincubation of rat liver

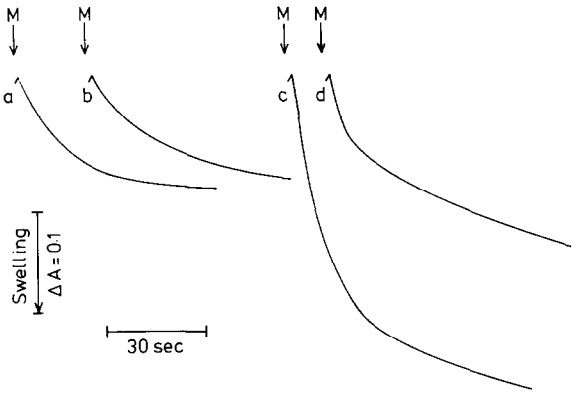

Fig. 1. Effect of DCCD on the osmotic swelling of mitochondria in NH_4Cl at pH 7.0 and 8.0. In each case a total of 4.0 ml medium contained 100 mM NH_4Cl, 2 mM HEPES (adjusted to either pH 7.0 or 8.0 with NH_3), 5 μM FCCP, 1 mM HEDTA (Tris salt), 6 μM (pH 7.0) or 50 μM (pH 8.0) $CaCl_2$ to give a free extramitochondrial [Ca^{2+}] of 1 μM, and 2 μg each of rotenone and antimycin. Preincubation of the mitochondria with DCCD was done at 45 mg protein/ml for $\geqslant$ 3 h at 0°C: (→) addition of 2 mg mitochondrial protein; (a) pH 7.0, no DCCD; (b) pH 7.0, after preincubation with 5 nmol DCCD/mg protein; (c) pH 8.0, no DCCD; (d) pH 8.0, after preincubation with 5 nmol DCCD/mg protein.

mitochondria with 5 nmol DCCD/mg protein causes a pronounced inhibition of swelling in isotonic NH_4Cl (+ uncoupler) at pH 8.0, but has relatively little effect on the slow swelling observed at pH 7.0. Although NO_3^- enter quite rapidly at pH 7.0 compared with Cl^- [9], DCCD has little effect on NO_3^- entry at pH 7.0, although there is a pronounced inhibition at pH 8.0 (fig. 2). Parallel experiments (not shown) with NH_4 acetate where entry of acetate is as the undissociated acid, showed that 5 nmol DCCD/mg protein had no effect on swelling in this medium in the presence or absence of uncoupler at pH 7.0 or 8.0.

In the case of proton translocation by the mitochondrial ATPase, inhibition by DCCD has been found to be time-dependent [6]. This is also true of its effect on anion permeability (fig. 3).

The results in fig. 3 were derived from experiments similar to those shown in fig. 1. The mitochondria were preincubated at ~50 mg protein/ml with DCCD for the required length of time and were then diluted by 100-fold on addition to

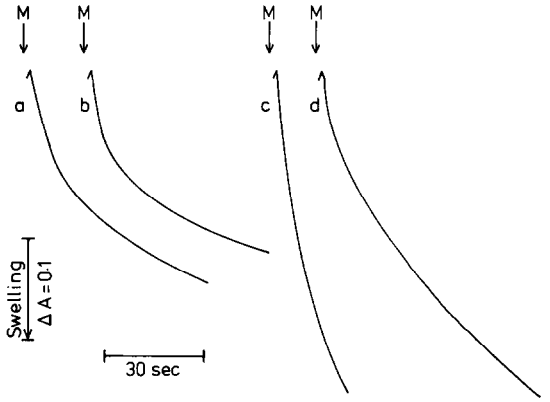

Fig. 2. Effect of DCCD on the osmotic swelling of mitochondria in NH_4NO_3 at pH 7.0 and pH 8.0. Conditions were as for fig. 1 except that 100 mM NH_4NO_3 replaced 100 mM NH_4Cl. Preincubation with DCCD was done at 60 mg protein/ml: (a) pH 7.0, no DCCD; (b) pH 7.0 after preincubation with 5 nmol DCCD/protein mg; (c) pH 8.0, no DCCD; (d) pH 8.0 after preincubation with 5 nmol DCCD/mg protein.

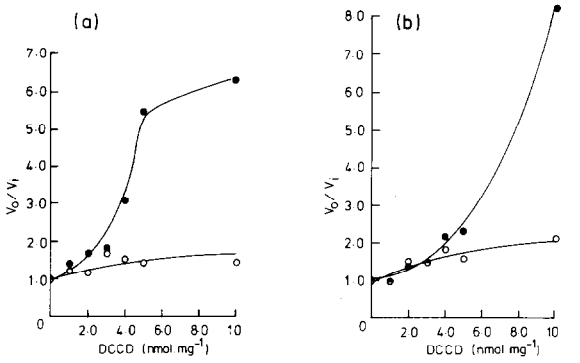

Fig. 4. Effect of [DCCD] on the inhibition of Cl^- and NO_3^- uniport at pH 7.0 and pH 8.0. Cl^- and NO_3^- uniport were measured as in fig. 1 and 2 and the rate of swelling was calculated as in fig. 3. Preincubation conditions were as in fig. 1, with varying [DCCD]: v_0 is the rate in the absence of DCCD; v_i is the rate in the presence of DCCD. Each point is the mean of at least 3 determinations. (a) Cl^- entry: (○) pH 7.0; (●) pH 8.0. (b) NO_3^- entry: (○) pH 7.0; (●) pH 8.0.

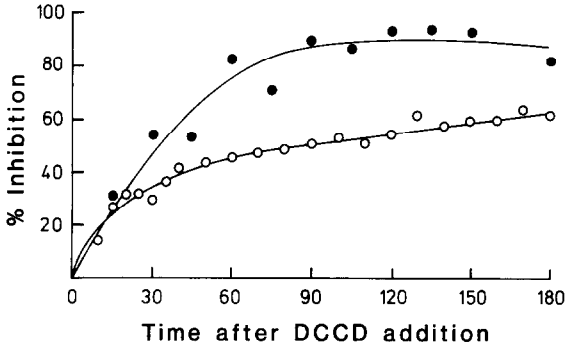

Fig. 3. Time course of inhibition of electrogenic Cl^- uniport and ATPase activity by DCCD. Preincubation conditions (except for incubation time, which is as shown) and conditions for measurement of Cl^- entry were as for fig. 1. Rate of swelling and ATPase activity were estimated as in the text. Each point represents the mean of 4 expt on different batches of mitochondria: (○) time course of inhibition of Cl^- entry by 5 nmol DCCD/mg protein; (●) time course of inhibition of ATPase activity by 5 nmol DCCD/mg protein.

the NH_4Cl medium, thereby preventing any significant further reaction with the inhibitor. The rate of swelling was taken as the reciprocal of the time taken for the absorbance to decrease by 0.08 absorbance units from the value 12 s after addition of mitochondria. The first 12 s of the trace was ignored to avoid inclusion of mixing artefacts. Also included in fig. 3 is data obtained under similar conditions for the time course of inhibition of the ATPase activity. By comparison with the ATPase, anion permeability is inhibited more slowly (time to 50% inhibition is 30 min for ATPase and 90 min for anion permeability). The progress curve for inhibition of anion entry is biphasic under these conditions, while that for the ATPase appears to be monophasic.

Fig. 4 shows titration curves for inhibition of anion permeability by DCCD after a long preincubation (>3 h) to ensure essentially complete reaction with the inhibitor. In agreement with the results shown in fig. 1, there is little inhibition of NO_3^- of Cl^- entry by DCCD at pH 7.0, while at pH 8.0 there is a much more potent inhibition. For both anions at pH 8.0, 50% inhibition is obtained by the addition of 3–4 nmol DCCD/mg protein. The small shift in the titration curve for NO_3^- compared to Cl^- is probably accounted for by the superimposition of the higher permeability of NO_3^- at neutral pH on the alkaline pH-dependent permeability [10].

4. DISCUSSION

Previous work from this laboratory has suggested that there is a pH-dependent anion-conducting pore in the mitochondrial inner membrane. Here we show that this pore, but not the lower anion permeability observed at neutral pH, is blocked by low concentrations of DCCD. This effect of DCCD on anion permeability can be distinguished from other effects of DCCD on the mitochondrial inner membrane. Although inhibition of the ATPase is seen at similar, low DCCD concentrations, the time course of inhibition is faster than that observed for the effect on anion permeability. Inhibition of proton translocation by the cytochrome b–c_1 segment and cytochrome oxidase appear to need much higher DCCD concentrations [4,7] but in these cases the preincubation conditions were different from those used here. We conclude that the action of DCCD on the anion-conducting pore is separate from and unrelated to its effects on these other mitochondrial functions.

In contrast, the action of DCCD on the uncoupling protein of brown adipose tissue mitochondria is similar to its action on the anion-conducting pore, when allowance is made for the high concentration of uncoupling protein. The analogy between the two systems is strong and both systems appear to provide an explanation for the non-ohmic relationship between proton conductance and membrane potential [15]. However, the pH-dependent anion-conducting pore may act as a safety valve [12], preventing the development of excessively high ΔpH values or membrane potential, while the uncoupling protein of brown adipose tissue mitochondria catalyzes a specialised physiological control mechanism.

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REFERENCES

[1] Beechey, R.B., Robertson, A.M., Holloway, C.T. and Knight, J.G. (1967) Biochemistry 6, 3867–3879.
[2] McCarty, R.E. and Racker, E. (1967) J. Biol. Chem. 242, 3435–3439.
[3] Harold, F.M., Paulasova, F. and Baarda, J.R. (1970) Biochim. Biophys. Acta 196, 235–244.
[4] Casey, R.P., Thelen, M. and Azzi, A. (1979) Biochem. Biophys. Res. Commun. 87, 1044–1051.
[5] Phelps, D.C. and Hatefi, Y. (1981) J. Biol. Chem. 256, 8217–8221.
[6] Kiehl, R. and Hatefi, Y. (1980) Biochemistry 19, 541–548.
[7] Price, B.D. and Brand, M.D. (1982) Biochem. J. 206, 419–421.
[8] Kolarov, J., Houštěk, J., Kopecký, J. and Kužela, S. (1982) FEBS Lett. 144, 6–10.
[9] Selwyn, M.J., Dawson, A.P. and Fulton, D.V. (1979) Biochem. Soc. Trans. 7, 216–219.
[10] Selwyn, M.J., Fulton, D.V. and Dawson, A.P. (1978) FEBS Lett. 96, 149–151.
[11] Selwyn, M.J., Dawson, A.P., Stockdale, M. and Gains, N. (1970) Eur. J. Biochem. 14, 120–126.
[12] Warhurst, I.W., Selwyn, M.J., Dawson, A.P. and Fulton, D.V. (1982) EBEC Rep. 2, 549–550.
[13] Dawson, A.P. (1982) Biochem. J. 206, 73–79.
[14] Fiske, C.H. and Subba Row, Y. (1925) J. Biol. Chem. 66, 375–400.
[15] Nicholls, D.G. (1974) Eur. J. Biochem. 50, 305–315.
[16] Nicholls, D.G. (1979) Biochim. Biophys. Acta 549, 1–29.