Inhibition of nicotinamide nucleotide transhydrogenase in rat liver submitochondrial particles by dicyclohexylcarbodi-imide and butanedione

A. John MOODY* and Robert A. REID

Department of Biology, University of York, York YO1 5DD, U.K.

(Received 11 November 1982/Accepted 21 December 1982)

Nicotinamide nucleotide transhydrogenase in rat liver submitochondrial particles is inhibited by treatment with *NN'*-dicyclohexylcarbodi-imide or butane-2,3-dione. Both inhibitions are pseudo-first-order with respect to enzyme activity. The reaction order with respect to inhibitor is close to unity for butanedione, but is significantly lower than unity for dicyclohexylcarbodi-imide.

Nicotinamide nucleotide transhydrogenase (EC 1.6.1.1), an integral protein of the mitochondrial inner membrane, catalyses the reversible transfer of protons across the membrane according to the equation:

where H_m^+ and H_c^+ represent matrix and cytosolic protons respectively (Mitchell, 1966; Blazyk *et al.*, 1976).

DCCD is known to be an inhibitor of the bovine heart enzyme (Phelps & Hatefi, 1981; Pennington & Fisher, 1981). Butane-2,3-dione, an arginine-residue-modifying reagent (in the presence of borate ions; Riordan, 1973), has also been shown to inhibit the bovine heart enzyme (Djavadi-Ohanian & Hatefi, 1975) and that from *Escherichia coli* (Homyk & Bragg, 1979).

Compared with the bovine heart enzyme, rat liver transhydrogenase has received relatively little attention (Rydstrom, 1981). It was therefore decided to study the effect of these reagents on the rat liver enzyme. The results obtained are comparable with those published for bovine heart enzyme, with certain differences.

Materials and methods

Mitochondria were isolated from the livers of male Wistar-strain rats (about 200 g) by the method of Kielley & Kielley (1951).

Sonic submitochondrial particles were then pre-

Abbreviations used: AcPyAD⁺, oxidized 3-acetylpyridine-adenine dinucleotide; DCCD, *NN'*-dicyclohexylcarbodi-imide; Mops, 4-*N*-morpholinepropanesulphonic acid.

* To whom reprint requests should be addressed.

pared as described by Kielley & Bronk (1958). Mitochondria from two livers were suspended in 80 ml of 30 mM-potassium phosphate buffer, pH 7.0, and centrifuged at 30 000 *g* for 10 min at 4°C. The mitochondria were then resuspended to 10 ml with the same buffer, homogenized by hand, and sonicated for 30 s at 250 W output in a Raytheon 10 kHz magnetostrictive sonicator (Raytheon Co., Waltham, MA, U.S.A.). The sonicated preparation was diluted to 26 ml and centrifuged at 30 000 *g* for 10 min at 4°C. The supernatant was decanted and centrifuged at 30 000 rev./min (*g*_{av.} 115 000; range 68 000–153 000) for 25 min (at plateau speed) at 4°C. Pelleted particles were resuspended to 26 ml and centrifuged as above. The final resuspension depended on their intended use (see below). Particle suspensions were stored at –20°C before use.

Protein was measured by the biuret method (Gornall *et al.*, 1949) in the presence of 1–2 mg of deoxycholate/mg of protein.

Solid DCCD was melted (m.p. 35–36°C), under a stream of warm air, and a 24.8 μ l portion dissolved to 1 ml with *NN*-dimethylformamide to give a 120 mM solution. A 12 mM solution was produced by dilution of 25 μ l of this solution to 0.25 ml with dimethylformamide. Both stock solutions were stored at –20°C. All the experiments with DCCD were performed within 2 weeks of preparing these solutions.

The stock butanedione solution was prepared by dissolving 20 μ l of freshly distilled butanedione (b.p. 88°C) to 1 ml with double-distilled water (final concn. 230 mM). Experiments with butanedione were performed within 8 h of preparation of the stock solution, which was kept on ice during this time.

Transhydrogenase activity from NADPH to AcPyAD⁺ (an NAD⁺ analogue) was assayed in the

absence of an energy source at 35°C by the method of Stein *et al.* (1959), as described by Rydstrom (1979). The assay mixture contained 200 μ M-NADPH, 200 μ M-AcPyAD⁺ and 2 μ g of rotenone/ml in 100 mM-Tris/acetate buffer, pH 7.5. The reduction of AcPyAD⁺ by NADPH was followed at 375 nm, and a value of 5100 was used for the molar absorption coefficient of reduced 3-acetylpyridine-adenine dinucleotide at this wavelength.

DCCD treatment of particles was performed as follows. Samples of particles at 1–30 mg of protein/ml of 50 mM-Tris/sulphate buffer, pH 7.5, were preincubated at 25°C for 5 min and then incubated with various amounts of DCCD dissolved in dimethylformamide. The final concentration of dimethylformamide was never above 25 μ l/ml. Control particles received an equivalent volume of solvent. Samples (50–200 μ g of protein) were removed at intervals and assayed for transhydrogenase activity.

Butanedione treatment of particles was similar, except that 50 mM-Mops/50 mM-borate/HCl buffer, pH 7.5, was used. The volume of butanedione solution added never exceeded 50 μ l/ml. Control particles received an equivalent volume of double-distilled water.

DCCD, butane-2,3-dione, Tris (Trizma base), Mops and rotenone were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. H₂SO₄ and Na₂B₄O₇·10H₂O were of analytical grade and came from BDH Chemicals, Poole, Dorset, U.K. Acetic acid and HCl were supplied by Fisons Scientific Apparatus, Loughborough, Leics., U.K., the former of standard laboratory grade and the latter of analytical grade. NADPH and AcPyAD⁺ came from BCL Ltd., Lewes, Sussex, U.K. *N,N*-Dimethylformamide was of Gold Label standard, as supplied by Aldrich Chemical Co., Gillingham, Dorset, U.K.

Results

A yield of about 9% of the mitochondrial protein was obtained in the particles, i.e. a total of between 25 and 30 mg of protein from two rats. These particles had NADPH:AcPyAD⁺ transhydrogenase activity in the range 80–180 nmol of AcPyAD⁺ reduced/min per mg of particle protein. They also showed energy-linked NADH:NADP⁺ transhydrogenase activity in the presence of oligomycin (1 μ g/mg of particle protein), and with either succinate or ascorbate/tetramethylphenylene-diamine as energy source.

Fig. 1 shows semilogarithmic plots of transhydrogenase-activity-inhibition time courses at various DCCD concentrations. These plots are linear, indicating that the enzyme modification is pseudo-first-order with respect to enzyme. The

Fig. 1. Semilogarithmic plots of the inhibition time course of transhydrogenase activity in the presence of DCCD at 34–1000 nmol/mg of particle protein (solid lines and axes, top and right)

Particles were incubated as described in the Materials and methods section in the presence of 0 (i) (ii), 34 (iii), 40 (iv), 50 (v), 54 (vi), 78 (vii), 222 (viii), 240 (ix), 380 (x), 540 (xi) and 1000 (xii) nmol of DCCD/mg of protein. For clarity, lines have not been drawn for every set of points. The dashed line and axes (lower left and bottom) show a plot of $\log(k - k_0)$ versus $\log(\text{concn. of DCCD})$. The slope of this plot is 0.78 ± 0.04 (S.E.M.). V_0 and V are reaction rates at zero time (30 s after DCCD addition) and at the times shown respectively. k_0 and k are pseudo-first-order rate constants in the absence and presence of inhibitor respectively. The following protein concentrations (mg/ml) were used: 1 (Δ , ∇); 5 (∇ , $\blacktriangle$); 8 ($\bullet$); 12 ($\blacksquare$); 30 ($\square$).

dashed axes and line show a plot of the logarithms of the first-order rate constants against the logarithms of the appropriate DCCD concentrations. The slope of this line, which is the reaction order with respect to inhibitor, was found to be 0.78 ± 0.04 (S.E.M.).

The concentrations of DCCD used have been defined as nmol of DCCD/mg of particle protein. The rate constants correlated well with this index, but not with concentrations in mmol of DCCD/ml of incubation mixture. DCCD is not soluble in the aqueous phase in the amounts used, so it follows that it must dissolve in the membrane phase. However, the index used can only give a measure of the concentration of DCCD in the whole membrane phase, and does not necessarily describe the concentration of inhibitor in the immediate vicinity of the enzyme.

Fig. 2 shows semilogarithmic plots of transhydrogenase-activity-inhibition time courses at various butanedione concentrations. Again, the

Fig. 2. Semilogarithmic plots of the inhibition time course of transhydrogenase activity in the presence of 0.6–9.9 mM-butanedione (solid lines and axes, top and right)

Particles were incubated as described in the Materials and methods section in the presence of 0 (i), 0.6 (ii), 1.2 (iii), 2.9 (iv), 5.3 (v), 5.8 (vi) (vii) (viii), 8.7 (ix) and 9.9 (x) mM-butanedione; 50 mM-borate was present in all cases. For clarity, lines have not been drawn for every set of points. The dashed line and axes (lower left and bottom) show a plot of $\log(k - k_0)$ versus $\log(\text{concn. of butanedione})$. The slope of this plot is 0.99 ± 0.02 (S.E.M.). V_0 , V , k_0 and k have the same meaning as in Fig. 1, except that butanedione was used. The symbols Δ or $\square$ and $\blacksquare$ indicate that the incubation mixture contained 15 and 4 mg of protein/ml respectively. The concn. of protein in the remaining incubations was 8 mg/ml.

enzyme. However, kinetics alone cannot eliminate all other possibilities.

Discussion

The results presented here for the inhibition of transhydrogenase activity in rat liver particles by DCCD are similar to those published elsewhere for bovine heart particles (Phelps & Hatefi, 1981; Pennington & Fisher, 1981). The former workers obtained pseudo-first-order rate constants of about 2.5×10^{-3} – $8 \times 10^{-3} \text{ min}^{-1}$ over the range 40–160 nmol of DCCD/mg of protein (at pH 7.5 and 22°C), and the latter found constants of about 1.8×10^{-3} – $13 \times 10^{-3} \text{ min}^{-1}$ over the range 45–180 nmol/mg (at pH 7.2 and 30°C). With rat liver the rate constants are somewhat higher at similar concentrations of DCCD (assuming that the measure of concentration, nmol/mg, is comparable), i.e. 6×10^{-3} – $22 \times 10^{-3} \text{ min}^{-1}$ over the range 30–160 nmol/mg of protein.

The reaction orders observed for the modification of rat liver enzyme are similar to those for bovine heart. In the latter case it has been concluded from the order with respect to inhibition that one essential residue per catalytic unit is modified by DCCD. However, we do not claim this, since, without more evidence, equating order with the number of residues modified is unjustified.

As well as inhibiting mammalian mitochondrial transhydrogenase, DCCD has been reported to inhibit other systems thought to be involved in proton translocation in: mitochondria, i.e. F_0 -ATPase (Beechey *et al.*, 1967), cytochrome oxidase (Casey *et al.*, 1980), ubiquinone:cytochrome *c* reductase (Lenaz *et al.*, 1982); *Neurospora* and yeast, F_0 -ATPase (Sebald *et al.*, 1979); chloroplasts, CF_0 -ATPase (Nelson *et al.*, 1977); and *E. coli*, BF_0 -ATPase (Fillingame, 1976). This has led to an interest in DCCD over the past few years in connection with the mechanism of these systems, the idea being that it might modify a residue(s) which is part of a ubiquitous proton-pumping structure.

Although this may yet be the case, there are some pointers which indicate that it is not. Firstly, DCCD modifies transhydrogenase at a much lower rate than F_0 -ATPase. The half-life at 1 nmol of DCCD/mg of protein and 0°C for bovine heart particle F_0 -ATPase is about 1 h (Beechey *et al.*, 1967). Inhibition of transhydrogenase would be undetectable at this concentration of DCCD, and this is probably why Beechey *et al.* (1967) observed no inhibition of the enzyme. Secondly, other proteins are inhibited by DCCD, e.g. F_1 , the non-proton-conducting moiety of the mitochondrial ATPase (Pougeois *et al.*, 1979). This is supported by the considerable labelling of particle proteins which occurs during incubation with $[^{14}\text{C}]$ DCCD at the

linearity of these plots indicates that the modifying reaction is pseudo-first-order with respect to enzyme. The dashed axes and line show a plot of the logarithms of the first-order rate constants against the logarithms of the corresponding butanedione concentrations. The reaction order with respect to inhibitor was found to be 0.99 ± 0.02 (S.E.M.) from the slope of this plot.

Since the modification reaction is apparently first-order with respect to both reactants, it follows that the reaction has a bimolecular interaction as the rate-limiting step in its mechanism. The reaction of butanedione with free arginine is of this type (Riordan, 1973), so it seems likely that the inhibitor reacts with one essential arginine residue in the

concentrations of DCCD used in the present work (A. J. Moody & R. A. Reid, unpublished work).

The results presented for butanedione inhibition of transhydrogenase from rat liver show some differences from those published for bovine heart (Djavadi-Ohanian & Hatefi, 1975) and *E. coli* (Homyk & Bragg, 1979) enzymes. Djavadi-Ohanian & Hatefi (1975) observed a time course consisting of two first-order phases. The rate of the first of these is comparable with that reported here. In contrast, Homyk & Bragg (1979) observed single-phase first-order time courses, which had lower rates (approx. 25% of those reported). However, the general similarity in the rates of inhibition of transhydrogenases from the three sources may indicate that the arginine residue is a universal component of the enzymes' active site. This arginine residue is also known to be susceptible to trypsin and phenylglyoxal in *E. coli* (Homyk & Bragg, 1979), and to trypsin in bovine heart (Juntti *et al.*, 1970).

A. J. M. acknowledges the financial support of the Department of Education for Northern Ireland during this work.

References

- Beechey, R. B., Robertson, A. M., Holloway, C. T. & Knight, I. G. (1967) *Biochemistry* **6**, 3867–3879
- Blazyk, J. F., Lam, D. & Fisher, R. R. (1976) *Biochemistry* **15**, 2843–2848
- Casey, R. P., Thelen, M. & Azzi, A. (1980) *J. Biol. Chem.* **255**, 3994–4000
- Djavadi-Ohanian, L. & Hatefi, Y. (1975) *J. Biol. Chem.* **250**, 9397–9403
- Fillingame, R. H. (1976) *J. Biol. Chem.* **251**, 6630–6637
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Homyk, M. & Bragg, P. D. (1979) *Biochim. Biophys. Acta* **571**, 201–217
- Juntti, K., Torndal, U. B. & Ernster, L. (1970) in *Electron Transport and Energy Conservation* (Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C., eds.), pp. 257–271, Adriatica Editrice, Bari
- Kielley, W. W. & Bronk, J. R. (1958) *J. Biol. Chem.* **230**, 521–533
- Kielley, W. W. & Kielley, R. K. (1951) *J. Biol. Chem.* **191**, 485–500
- Lenaz, G., Esposti, M. D. & Castelli, G. P. (1982) *Biochem. Biophys. Res. Commun.* **105**, 589–595
- Mitchell, P. (1966) *Biol. Rev. Cambridge Philos. Soc.* **41**, 445–502
- Nelson, N., Eytan, E., Notsani, B. E., Sigrist, H., Sigrist-Nelson, K. & Gitler, C. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2375–2378
- Pennington, R. M. & Fisher, R. R. (1981) *J. Biol. Chem.* **256**, 8963–8969
- Phelps, D. C. & Hatefi, Y. (1981) *J. Biol. Chem.* **256**, 8217–8221
- Pougeois, R., Satre, M. & Vignais, P. (1979) *Biochemistry* **18**, 1408–1413
- Riordan, J. F. (1973) *Biochemistry* **12**, 3915–3923
- Rydstrom, J. (1979) *Methods Enzymol.* **55**, 261–275
- Rydstrom, J. (1981) in *Mitochondria and Microsomes* (Lee, C. P., Schatz, G. & Dallner, G., eds.), pp. 317–335, Addison-Wesley, Reading, MA
- Sebald, W., Graf, T. & Lukins, H. B. (1979) *Eur. J. Biochem.* **93**, 587–599
- Stein, A. M., Kaplan, N. O. & Ciotti, M. M. (1959) *J. Biol. Chem.* **234**, 979–986