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Interaction of Purified Nicotinamidenucleotide Transhydrogenase with Dicyclohexylcarbodiimide†

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ABSTRACT: The inhibition of the energy-linked nicotinamidenucleotide transhydrogenase (TH; EC 1.6.1.1) by dicyclohexylcarbodiimide (DCCD) has been further studied because of its important mechanistic implications. We had shown earlier that TH bound to submitochondrial particles from bovine heart is inhibited by DCCD and that NAD(H) protects the enzyme against this inhibition [Phelps, D. C., & Hatefi, Y. (1981) *J. Biol. Chem. 256*, 8217–8221]. By contrast, Pennington and Fisher [Pennington, R. M., & Fisher, R. R. (1981) *J. Biol. Chem. 256*, 8963–8969] working with purified TH concluded that NAD(H) does not protect against DCCD inhibition and that DCCD inhibition involves the TH proton channel rather than the nucleotide-binding active site. The present study shows that NAD(H) as well as AMP and ADP, which are known to bind to the NAD(H) binding site from competitive inhibition studies, protect the purified TH against inhibition by DCCD, whereas 2′-AMP and 3′-AMP, which bind to the NADP(H) site on TH, do not protect. In addition, it is shown that whereas the unmodified TH binds to NAD–agarose such that it is elutable by buffer containing NADH, the DCCD-modified enzyme does not bind to NAD–agarose. These results suggest strongly that DCCD binds at or near the NAD(H) binding site on TH. Another less likely possibility is that NAD(H) and DCCD bind to separate sites, but their bindings are mutually exclusive. With the use of [^{14}C]DCCD, it has been shown that 100% activity inhibition corresponds to 0.5 mol of DCCD binding per mol of TH ($M_r \sim 11 \times 10^4$). Both the inhibition and DCCD binding are pseudo first order with respect to the time of TH exposure to DCCD and follow a parallel course when binding is plotted on the basis of saturation at 0.5 mol of DCCD/mol of TH. Since the purified TH and the membrane-bound TH appear to be dimeric, these results suggest a case of half-of-the-sites reactivity in which only the active protomer is capable of rapid DCCD binding. This conclusion agrees with the possibility of DCCD and NAD(H) binding at the same site. Thus, the protomer that cannot bind DCCD might be inactive because it cannot bind NAD(H) either.

Mitochondrial nicotinamidenucleotide transhydrogenase (TH; EC 1.6.1.1)[1] is a membrane-bound enzyme, which catalyzes the transfer of a hydride ion between NAD and NADP. This reaction is coupled to transmembrane proton translocation as shown in eq 1 where H^+_c and H^+_m denote

$$NADH + NADP + nH^+_c \rightleftharpoons NAD + NADPH + nH^+_m \quad (1)$$

protons on the cytosolic and matrix sides of the inner membrane. The number of protons translocated (n) per mole of hydride ion transferred has been estimated to be close to unity (Earle & Fisher, 1980). The mitochondrial transhydrogenase (TH) has been isolated and highly purified (Hojeberg & Rydström, 1977; Wu et al., 1982). The enzyme has a molecular weight of (11–12) × 10^4 and is considered to occur in the membranes as a dimer (Anderson & Fisher, 1981; Wu & Fisher, 1983).

Previous studies have shown that TH is inhibited by DCCD (Phelps & Hatefi, 1981; Pennington & Fisher, 1981), a property that is shared by all proton-linked ATP synthases (Senior & Wise, 1983), ubiquinol-cytochrome c reductases

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[1] Abbreviations: AcPyAD, 3-acetylpyridine adenine dinucleotide; DCCD, *N,N′*-dicyclohexylcarbodiimide; TH, nicotinamidenucleotide transhydrogenase; SMP, submitochondrial particles; ATPase, adenosinetriphosphatase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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from mammalian and yeast mitochondria (Esposti et al., 1983; Clejan & Beattie, 1983), and cytochrome oxidase (Casey et al., 1980). In these systems DCCD inhibits primarily proton translocation rather than the scalar reactions catalyzed by these enzymes. In the case of TH, data have also been published which indicate that DCCD inhibits proton translocation more than hydride ion transfer (Pennington & Fisher, 1981). Phelps & Hatefi (1981) showed that NAD(H) protects the membrane-bound TH against inhibition by DCCD, while Pennington & Fisher (1981) disagreed with these results and suggested that DCCD inhibits the TH proton channel.

The present studies show that a highly purified preparation of TH is inhibited by DCCD and that, as in the case of the membrane-bound enzyme, NAD(H) and analogues (including AMP and ADP) offer strong protection against this inhibition. In agreement with these results, it has also been shown that, in contrast to the native enzyme, the DCCD-inhibited enzyme does not bind to NAD–agarose. These results suggest either that DCCD binds at or near the NAD(H) binding site or that NAD(H) and DCCD bind at different sites but have mutually exclusive binding properties. Our results have also indicated that full inhibition of TH activity correlates with covalent binding of only one DCCD per 2 mol of TH. Since TH has been shown to be dimeric in the isolated state, these results suggest that modification of one protomer by DCCD is sufficient to render the dimer inactive. This may be a case of half-of-the-sites reactivity or of cooperativity as reported for F_1-ATPase. This enzyme contains three potential DCCD binding sites, one site per each of the three β subunits. However, the first molecule of DCCD seems to bind much more rapidly and results in >90% inhibition of ATPase activity (Wong et al., 1984). In addition, it is known that the ATP synthases contain multiple copies of the DCCD-binding protein in the membrane sector (F_0), but only 1 equiv of DCCD is sufficient to nearly completely block proton translocation (Kopecky et al., 1981).

Materials and Methods

Preparation of TH. The enzyme was isolated from beef heart SMP by using a modification of the procedure of Wu et al. (1982). SMP (about 400 mg of protein in 10 mL of 0.25 M sucrose containing 10 mM Tris-HCl, pH 8.0) were washed with 2 M NaCl and extracted with 1.5% (w/v) Triton X-100 according to Wu et al. (1982). The extract could be used immediately for further purification or stored overnight on ice without loss of activity. It was divided into six portions of 11 mL each. To each batch was added 7.5 mL of 10 mM NaH_2PO_4 before use. The final pH at this point was 6.8. Immediately after the addition of NaH_2PO_4, the extract was loaded on a 2.5 × 11 cm NAD–agarose column, and the column eluted as follows at a flow rate of 3 mL/min: (a) 25 mL of column buffer (10 mM NaH_2PO_4, 1.0 mM NaEDTA, 0.1% Brij 58, and 10 mM β-mercaptoethanol with the pH adjusted to 7.0 or 7.5 as specified) containing 0.1 mM NADP at pH 7.0; (b) 75 mL of column buffer containing 20 mM NaCl, pH 7.0; (c) 25 mL of column buffer, pH 7.0; (d) 22 mL of column buffer containing 0.54 mM NADH, pH 7.5; (e) 60 mL of column buffer, pH 7.0. After step e, a second batch of extract was loaded on the column and the elution protocol repeated as before.

The active fractions from the NAD–agarose column, which were eluted by the buffer containing NADH, were pooled and added at a rate of 0.6 mL/min to a 1.5 × 15 cm HA-Ultragel column preequilibrated with 5 mM sodium phosphate buffer, pH 7.5, containing 0.01% Brij 58. After all NAD–agarose column fractions from the six batches of extract were bound

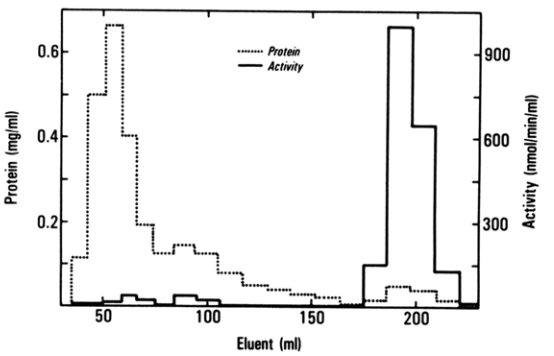

FIGURE 1: Profile of the purification of TH by affinity chromatography on NAD–agarose. The positions at which various elution buffers were experienced coming off the column were the following: buffer containing NADP at 53 mL, buffer containing NaCl at 79 mL, column buffer at pH 7.0 at 149 mL, buffer containing NADH at 174 mL, and buffer at pH 7.5 at 186 mL. The void volume was 37 mL. For other details, see text and Table I.

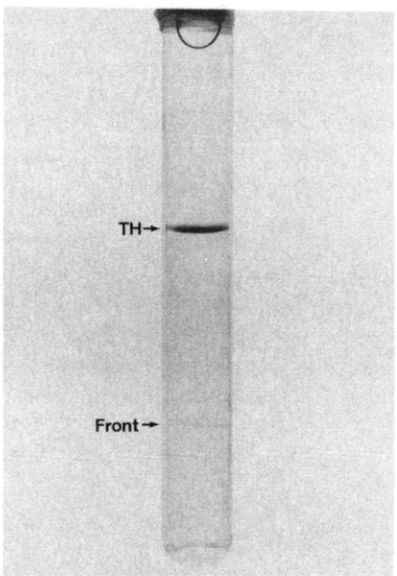

FIGURE 2: Electrophoresis of purified TH. The enzyme, 2 μg, was electrophoresed on a sodium dodecyl sulfate–7.25% acrylamide gel according to the procedure of Laemmli (1970) and stained for protein with Coomassie blue. Comparison with appropriate standards suggested a molecular weight of 11 × 10^4 for TH.

onto HA-Ultragel, the column was washed with the above buffer and eluted with 50 mL of 75–100 mM gradient of sodium phosphate, pH 7.5, containing 0.01% Brij. The flow rate was about 0.9 mL/min. This column concentrates the enzyme (even though the TH peak trails) and separates it from a contaminant of M_r ~35 000 which follows the TH peak. This impurity was also present when we used the procedure of Wu et al. (1982) without modification. Other procedures used for concentrating TH such as ultrafiltration in collodion bags resulted in considerable activity loss. Desalting of TH was achieved by passing 0.5–0.6-mL aliquots through a 3-mL Sephadex G-25 (medium) column according to the procedure of Penefsky (1977). The column buffer was 5 mM sodium phosphate, pH 7.5, containing 0.01% Brij, and the centrifugation was performed in the cold room in a clinical centrifuge for 1 min at top speed. The purified TH was stored on ice.

Figure 1 shows the NAD–agarose column elution profile, Figure 2 shows the purified enzyme electrophoresed on SDS–acrylamide according to the procedure of Laemmli (1970), and Table I shows the progress of purification at each step. Using the procedure of Wu et al. (1982) without modification, we obtained a preparation of TH with a similar

Table I: Purification of TH from SMP

fraction	total protein (mg)	total activity[a]	specific activity[a]
SMP	470	266	
Triton extract	153	178	1.16
active fraction from NAD–agarose	9.5	108	11.4[b]
all other fractions from NAD–agarose	127	7.7	0.06
most active fractions from HA-Ultragel	4.0	75	18.75

[a] Specific activity is expressed as nanomoles of AcPyAD reduced by NADPH per minute per milligram of protein at 37 °C. [b] The peak active fraction (0.55 mg) had a specific activity of 19.

specific activity, but the total yield was less. Wu et al. report, however, a higher specific activity, which was based on protein determination by the Coomassie blue dye binding procedure of Sedmak & Grossberg (1977). In our hands, this procedure gave grossly incorrect values for several proteins when compared with the procedure of Lowry et al. (1951) and gravimetric protein determination.

Transhydrogenase activity of SMP was assayed at 37 °C as before (Phelps & Hatefi, 1981) with NADPH and AcPyAD, respectively, as hydride ion donor and acceptor. Activity of the purified enzyme was assayed similarly, except that the buffer was 100 mM sodium phosphate, pH 6.5, and the nucleotides were 0.3 mM each. The reaction was started by the addition of 1–3 μg of TH.

The extent of covalent labeling of TH by [^{14}C]DCCD was determined as follows. Unless otherwise stated, the enzyme at a protein concentration of 0.25–0.35 mg/mL in 5 mM sodium phosphate, pH 7.5, containing 0.01% (w/v) Brij 58 was incubated with [^{14}C]DCCD (added in a final volume of 1–2.5% ethanol) at room temperature. At appropriate intervals aliquots of 200 μL were withdrawn, chilled in an ice bath, and filtered through two consecutive 1-mL Penefsky-type (Penefsky, 1977) centrifuge Sephadex G-25 (medium) columns equilibrated with the same buffer as above. Then, 80-μL aliquots of the filtered TH, containing 15–20 μg of protein each, were boiled with 20 μL of 5-fold concentrated Laemmli sample buffer (Laemmli, 1970), containing 2% β-mercaptoethanol and 0.002% bromphenol blue, and placed on sodium dodecyl sulfate–7% acrylamide Laemmli rod gels with a $^1/_2$-cm 4% acrylamide stacking gel. After electrophoresis, staining with Coomassie blue, and destaining for 4 days in 10% acetic acid, the gels were scanned at 560 nm in a Gilford densitometer and sliced. The TH bands plus 2 mm of gel on either side were cut, digested overnight at 55 °C in 30% H_2O_2, mixed wih Beta Blend, and counted for radioactivity in a Beckman scintillation counter. The amount of protein in the TH band was usually ≥90% of the total protein placed on the gel, as estimated from the Coomassie blue stain intensities of the densitometric traces. The amount of [^{14}C]DCCD bound to the TH band was then calculated by assuming a molecular weight of 11×10^4 for the TH protomer.

The sources of the chemicals used were as follows. AMP, ADP, NAD(H), NADP(H), AcPyAD, and NAD–agarose were obtained from P-L Biochemicals; 2′-AMP/3′-AMP mixed isomers, 2′,5′-ADP, Brij 58, and Triton X-100 were from Sigma; DCCD was from Alrich; [^{14}C]DCCD was from Research Products, Inc. All other chemicals used were reagent grade or of the highest quality available.

Results

Our previous studies showed that DCCD inhibits the TH activity of submitochondrial particles and that NAD(H) and AcPyAD offer strong protection against DCCD inhibition (Phelps & Hatefi, 1981). Subsequently, Pennington & Fisher (1981) showed that the purified enzyme was also inhibited by DCCD, but they failed to demonstrate the NAD(H) protection. Their data also suggested that DCCD inhibited vectorial proton translocation more than hydride ion transfer in the scalar reaction. Thus, they concluded that DCCD modification occurs outside the active site and involves a proton-binding group concerned with proton translocation by TH. This point is of mechanistic significance, since in ATP synthase (F_0), complex III, and complex IV the primary inhibitory effect of DCCD has been shown to be on transmembrane proton translocation rather than on the scalar reactions catalyzed by these enzyme complexes. However, as discussed earlier, in F_1-ATPase DCCD also inhibits the scalar reaction by binding to the β subunit at or near the catalytic site.

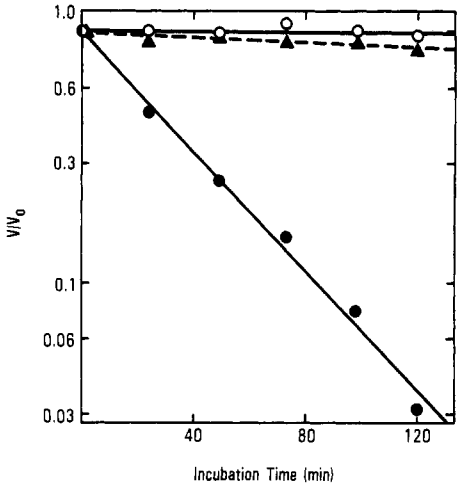

FIGURE 3: Inhibition of TH by incubation with DCCD (●) and the protective effect of AcPyAD (▲) against DCCD inhibition. TH at 0.34 mg/mL was incubated at 22 °C in a buffer containing 5 mM sodium phosphate, pH 7.5, and 0.01% (w/v) Brij 58. Where indicated, 84 μM DCCD in 0.9% final volume of ethanol (●), 0.9% ethanol (○), or 84 μM DCCD plus 2.2 mM AcPyAD (▲) was added. At the incubation intervals shown, samples were withdrawn and assayed at 37 °C for transhydrogenation from NADPH to AcPyAD. V_0 and V are, respectively, the reaction rates at zero time and at the various times of incubation shown.

In agreement with our previous findings, it is seen in Figure 3 that DCCD inhibition of the purified enzyme is also completely prevented by the NAD analogue AcPyAD. Similar results were obtained with either NAD or NADH as the protective agent. Since Pennington & Fisher (1981) were unable to see this protection, we also performed our experiments under their assay conditions which differed from ours mainly in the use by them of choline chloride instead of phosphate as buffer. The results showed again nearly complete protection by AcPyAD against DCCD inhibition. However, the use of the assay conditions of Pennington & Fisher (1981) lowered the pseudo-first-order rate constant of DCCD inhibition by >40%. Also in agreement with our previous findings (Phelps & Hatefi, 1981), NADPH enhanced the rate of DCCD inhibition by 3–4-fold or more. The pseudo-first-order plots of TH activity as a function of time of incubation with DCCD in the presence of NADPH showed a break after about 80% inhibition. Thereafter, a slower rate of inhibition comparable to that in the absence of NADPH was observed. The results of Pennington & Fisher (1981) on both the membrane-bound and purified TH agree with our previous finding that NADP(H) stimulates the DCCD inhibition of TH. In addition, we have shown that competitive inhibitors of NAD(H), namely, 5′-AMP and 5′-ADP, protected the trans-

Table II: Protection by AMP and ADP against DCCD Inhibition of Transhydrogenase Activity[a]

additions	$k - k_0$ SMP	$k - k_0$ TH
none	0.027	0.023
5′-AMP	0.002	0.002
5′-ADP	0.003	0.003
2′-AMP + 3′-AMP	0.024	0.024
2′,5′-ADP	0.024	0.027

[a] SMP at 0.28 mg/mL was suspended in a medium containing 0.25 M sucrose and 50 mM Tris–sulfate, pH 7.5, and was treated with 100 μM DCCD in 0.5% final concentration of ethanol. The control in the absence of DCCD contained the same volume of ethanol. The tubes were incubated at 23 °C and sampled at intervals for assay of transhydrogenase activity. Where indicated, the concentration of added nucleotides was 4 mM, except for the 2′- and 3′-AMP mixed isomers which were present at the combined concentration of 5.7 mM. The assay of NADPH to AcPyAD transhydrogenase activity was performed in the same buffer as above in the presence of 25 μM rotenone, 14 μg of SMP, and 0.3 mM each of NADPH and AcPyAD. The purified TH at 0.34 mg/mL in the same buffer as in Figure 3 was incubated at 23 °C with 150 μM DCCD in the presence or absence of the same concentrations of nucleotides as above and sampled at intervals for activity assay. The control lacked DCCD but was otherwise similarly treated. All data were plotted semilogarithmically as in Figure 3, and inhibition rate constants were calculated from the slopes of the pseudo-first-order plots. k and k_0, pseudo-first-order inhibition rate constants (min^{-1}) in the presence and absence of DCCD, respectively.

hydrogenase activities of SMP and the purified enzyme against inhibition by DCCD. By contrast, 2′-AMP and 3′-AMP, which bind to the NADP(H) site as shown by competitive inhibition studies (Rydström, 1972), as well as 2′,5′-ADP were without effect (Table II). It should be added, however, that occasionally we have obtained TH preparations that are only partially (e.g., ≥50%) protected by NAD(H). We do not know the reason for this, but it is possible that the TH preparations of Pennington & Fisher (1981), which do not exhibit NAD(H) protection against DCCD inhibition, are an extreme case of this occasional problem.

As mentioned above, Pennington & Fisher (1981) concluded that DCCD binding does not involve the active site of TH, whereas the strong protection by NAD(H) (also AMP and ADP) that we have observed in both the membrane-bound and purified TH against DCCD inhibition suggested a possible overlap of the NAD(H) and DCCD binding sites. Thus, it was of interest to see (a) whether the NAD-protected enzyme would bind less DCCD as compared to the unprotected control and (b) whether the DCCD-modified TH would bind NAD. In order to check the former point, we deliberately chose a preparation of TH that exhibited partial NAD(H) protection against DCCD inhibition, so that partial substrate protection could also be correlated with the extent of DCCD binding. The results are shown in Figure 4. It is seen that both in the absence and presence of partial protection, there was an excellent correlation between the degree of inhibition and extent of [^{14}C]DCCD incorporation (note the parallel course of inhibition and DCCD binding in the pseudo-first-order plots of Figure 4). It may be noted that, in the unprotected system, complete inhibition involves the binding of only about 0.5 mol of [^{14}C]DCCD/mol of TH. This point will be discussed further.

In order to see whether the DCCD-modified TH would bind NAD, advantage was taken of the fact that unmodified TH binds to NAD–agarose, a property which was used by Wu et al. (1982) for purification of Triton-solubilized TH. Figure 5 shows that unmodified TH (full trace) becomes bound to an NAD–agarose column and is eluted in a sharp band after inclusion of NADH in the elution buffer. By contrast, the

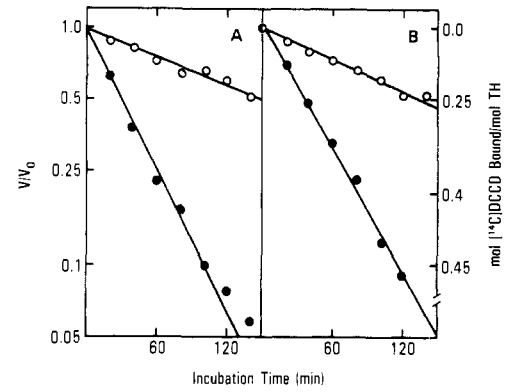

FIGURE 4: Comparison of the degree of labeling of TH (panel B) with activity inhibition (panel A) upon incubation of the enzyme with [^{14}C]DCCD in the absence (●) and presence (○) of AcPyAD. The experimental conditions were the same as Figure 3, except that in the incubation mixture the concentrations of TH, [^{14}C]DCCD, and AcPyAD were respectively 0.28 mg/mL, 90 μM, and 3.8 mM. The ordinate of panel B was calculated as 0.5 − (moles of DCCD bound per mole of TH protomer). V_0 and V are the same as in Figure 3.

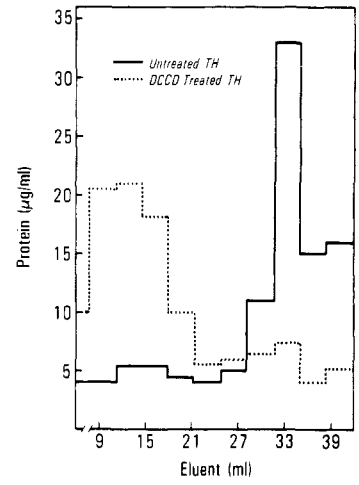

FIGURE 5: Chromatography of purified TH and DCCD-treated TH on NAD–agarose. TH at 0.2 mg/mL of 80 mM sodium phosphate, pH 7.5, containing 0.01% (w/v) Brij 58, was divided into halves. To one half was added 200 μM [^{14}C]DCCD (26 μCi/μmol) in 2.5% final volume of ethanol. The second half was treated with the same amount of ethanol, and both samples were incubated at room temperature until the DCCD-treated enzyme had lost 90% of its activity. The samples were then cooled in an ice bath and filtered through Sephadex columns equilibrated with the same buffer as above. They were then placed on 1 × 10 cm NAD–agarose columns equilibrated with the column buffer (pH 7.0) described under Materials and Methods. The positions at which various elution buffers (see Materials and Methods) were experienced coming off the column at a flow rate of 0.5 mL/min were the following: buffer containing NADP at 14 mL, buffer containing NaCl at 18 mL, column buffer at pH 7.0 at 29 mL, and buffer containing NADH at 32 mL. For other details, see Table III.

DCCD-inhibited enzyme (dotted trace) does not bind to NAD–agarose and is washed through with buffer alone. Table III gives the details of the experiment regarding protein, TH activity, and [^{14}C]DCCD radioactivity applied to the NAD–agarose column and eluted with buffer alone followed by buffer containing NADH. The results show that whereas all the active TH was bound to the column and was eluted with the NADH-containing buffer, about 90% of the protein and radioactivity of the [^{14}C]DCCD-treated TH was washed from the column with buffer alone. Thus, on the one hand, the results of Figures 3 and 4 indicate that, in the presence of NAD, TH does not bind and cannot be inactivated by DCCD, and on the other hand the results of Figure 5 and Table III show that the DCCD-inactivated TH cannot bind NAD. These results suggest strongly that DCCD and NAD bind to

Table III: Effect of DCCD Treatment on TH Binding to NAD–Agarose[a]

parameter measured	control TH	[^{14}C]DCCD-treated TH
(1) TH placed on columns		
total protein (μg)	290	280
total TH activity (μmol/min)	2.6	0.24
(2) buffer wash		
total protein (μg)	56	260
total TH activity (μmol/min)	0.045	
total radioactivity (cpm)		85 400
(3) NADH wash		
total protein (μg)	228	26
total TH activity (μmol/min)	2.86[b]	
total radioactivity (cpm)		8400

[a] For details see Figure 5 and the text. [b] The increase in specific activity upon chromatography of TH on NAD–agarose was also observed in other experiments.

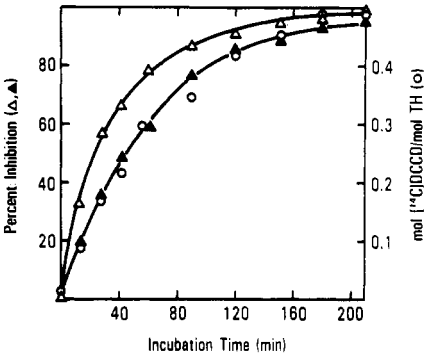

FIGURE 6: Inhibition of TH by DCCD in the absence (▲) and presence (△) of NADPH and correlation of the degree of inhibition with the extent of covalent interaction of DCCD with TH (○). TH at 0.46 mg/mL of 10 mM sodium phosphate, pH 7.5, containing 0.01% (w/v) Brij 58 was treated with 66 μM [^{14}C]DCCD in the absence (▲) and presence (△) of 1.9 mM NADPH. Within experimental error, the same degree of radioactivity was found to be covalently linked to the enzyme (○) during the course of incubation of TH with [^{14}C]DCCD in either experiment.

overlapping sites. A less likely possibility is that DCCD and NAD bind to separate sites, but the binding of each somehow inhibits the binding of the other.

We had previously shown that the presence of NADP accelerates the inhibition of TH by DCCD. With the purified enzyme, this effect was seen better with NADPH (Figure 6). However, the extent of covalent modification of TH by [^{14}C]DCCD was, within the limits of experimental accuracy, very nearly the same in the presence or absence of NADPH (Figure 6). These results may mean that first DCCD binds to the enzyme noncovalently or as the unstable covalent isourea adduct, which is then converted slowly to the stable N-substituted urea, and that NADP(H) binding favors the first step without much influencing the slower second step. It should be pointed out that determination of the extent of [^{14}C]DCCD firmly attached to the enzyme involves cooling of the sample to 0 °C, passage through two successive Penefsky-type Sephadex columns, then treatment with sodium dodecyl sulfate, electrophoresis on polyacrylamide, staining, and destaining of the gels, and finally digestion and measurement of radioactivity associated with the TH band. The use of this prolonged procedure has the inherent danger of underestimating the amount of bound DCCD which causes a certain degree of inhibition, since loosely bound DCCD may also cause inhibition and be removed by the above treatments. However, filtration through two consecutive centrifuge–Sephadex columns (Penefsky, 1977) and gel electrophoresis were both necessary in order to obtain reproducible labeling of TH.

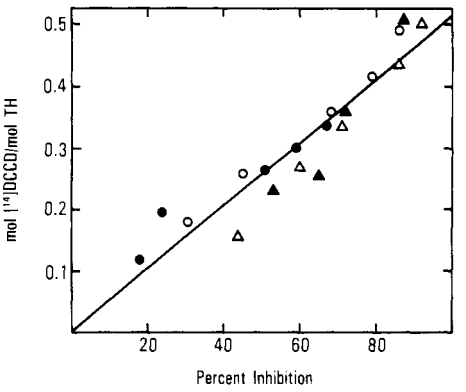

FIGURE 7: Correlation of percent inhibition and extent of labeling of TH with [^{14}C]DCCD. TH at 0.44 mg/mL was incubated for various periods of time with 25 (●), 50 (○), 95 (▲), and 189 (△) μM [^{14}C]DCCD, then sampled, and assayed for activity and bound radioactivity. The line, which extrapolates at 100% inhibition to 0.515 mol of [^{14}C]DCCD/mol of TH protomer, is the result of a least-squares analysis of the experimental data.

Chilling of the [^{14}C]DCCD-treated enzyme to 0 °C and passage through two centrifuge–Sephadex columns were sufficient to stop further interaction of [^{14}C]DCCD with the enzyme. When one Sephadex column plus chilling was used, a slow but considerable labeling continued. The electrophoresis in the presence of sodium dodecyl sulfate was necessary because variable amounts of radioactivity seemed to be trapped by the enzyme preparation, which were not capable of covalent interaction with TH, but which passed through the Sephadex columns together with the protein. This adventitious radioactivity, which was often greater than that which was covalently linked to the enzyme, was removed, however, during electrophoresis and staining and destaining of the gels. A similar problem was encountered elsewhere when the mitochondrial ATP synthase was labeled with [^{14}C]DCCD. There, too, gel electrophoresis plus staining and destaining (or washing of the gels in the destaining medium) was necessary for removal of extraneous radioactivity noncovalently bound to the enzyme complex (Kiehl & Hatefi, 1980).

As seen in Figures 4 and 6, the use of the above procedure for determination of covalently bound DCCD revealed that at all stages of inhibition the extent of labeling (i.e., moles of DCCD per mole of the TH protomer) was numerically equivalent to half the degree of inhibition observed, and only 0.5 mol of DCCD was bound per mol of TH protomer when activity was nearly completely inhibited. Figure 7 shows this correlation between inhibition and DCCD labeling of the enzymes for four different preparations of TH, each treated with a different concentration of [^{14}C]DCCD from 25 to 190 μM. The line extrapolating at 100% inhibition to 0.515 mol of DCCD/mol of TH protomer (the molecular weight taken as 11×10^4) was determined by least-squares fit of the experimental data, the correlation coefficient being 0.974. These results are of particular interest in view of the dimeric nature of TH. They suggest either half-of-the-sites activity or a situation analogous to the DCCD modification of F_1-ATPase, where modification by DCCD of one of the three β subunits results in >90% inhibition of ATPase activity (Wong et al., 1984).

Discussion

It has been shown that DCCD inhibits the transhydrogenase activity of purified TH. The inhibition follows pseudo-first-order kinetics with a single slope to nearly complete inhibition, which suggests a single type of activity-related modification. In agreement with our previous findings with the mem-

brane-bound TH (Phelps & Hatefi, 1981), and in contrast to the report of Pennington & Fisher (1981), NAD(H) and analogues [including the NAD(H) competitive inhibitors AMP and ADP] strongly protect the enzyme against inhibition and labeling by DCCD. It has also been shown that whereas purified, unmodified TH binds to NAD–agarose, the DCCD-inhibited enzyme does not. Together, these results suggest strongly that DCCD binds at or near the NAD binding site on TH such that the binding of either NAD or DCCD interferes with the binding of the other. A less likely possibility is the binding of NAD and DCCD at separate and nonoverlapping sites but with mutually exclusive binding properties.

In energy-transducing systems, DCCD is known to inhibit proton translocation in enzyme complexes III, IV, and V (ATP synthase), the latter being a common feature among prokaryotic and eukaryotic ATP synthases where DCCD inhibition involves modifications of a carboxyl group of proteolipid in the membrane sector (F_0). These findings and the results of an experiment which suggested that DCCD inhibits proton translocation more than hydride ion transfer led Pennington & Fisher (1981) to conclude that DCCD modification of TH occurs outside the active site and involves residues concerned with proton translocation. However, it should be remembered that in energy-transducing systems DCCD inhibition is not always concerned with modification of proton-translocating moieties. In the ATP synthase, DCCD inhibits at two distinct sites. One target is the proton-conducting proteolipid discussed above; the other is the β subunit of F_1-ATPase at or near the catalytic site. In the case of TH, our results suggest that DCCD modification involves the NAD binding site. Kinetic data indicate that this site also binds AMP and ADP. Thus, it is possible that the NAD-binding site of TH might have structual features (e.g., a DCCD-reactive carboxyl) similar to the active site of F_1-ATPase.

Another possible similarity to F_1-ATPase is that DCCD modification of one protomer per TH dimer appears to result in nearly complete inhibition. In F_1-ATPase, which contains three β subunits and three potential DCCD-reactive sites, the binding of 1 mol of DCCD/mol of F_1 also results in >90% inhibition (Wong et al., 1984). This extensive degree of inhibition is probably related to the catalytic cooperativity of F_1, meaning that turnover at any one site is greatly facilitated by substrate binding to additional active sites. It is also possible that the above results with TH are indicative of half-of-the-sites activity. In that case, our [^{14}C]DCCD binding data, which indicate saturation at 1 mol of DCCD per TH dimer, might mean that the second site on the inactive TH protomer is not available for DCCD binding. Since DCCD appears to bind at or near the same site as NAD, the catalytic inactivity of the second protomer might be because it is also incapable of binding NAD. It should be pointed out that, in contrast to our results, Pennington & Fisher (1981) found a nearly 1:1 correlation between the extent of labeling of TH with [^{14}C]-DCCD and the degree of inhibition of the enzyme. Indeed, when the activity of TH was about 83% inhibited, the enzyme was found to contain nearly 1.1 mol of [^{14}C]DCCD/mol. However, we too obtained such high levels of labeling when we used a procedure similar to that of Pennington and Fisher, which did not involve immediate filtration of the chilled enzyme through two Sephadex columns in order to stop further interaction of TH with [^{14}C]DCCD.

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Registry No. TH, 9014-18-0; DCCD, 538-75-0; AcPyAD, 86-08-8; NAD, 53-84-9; NADH, 58-68-4; 5′-AMP, 61-19-8; ADP, 58-64-0.

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