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Effects of *N,N'*-Dicyclohexylcarbodiimide and *N*-(Ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline on Hydride Ion Transfer and Proton Translocation Activities of Mitochondrial Nicotinamidenucleotide Transhydrogenase†

Donna C. Phelps and Youssef Hatefi*

ABSTRACT: *N,N'*-Dicyclohexylcarbodiimide (DCCD) inhibits the mitochondrial energy-linked nicotinamidenucleotide transhydrogenase (TH). Our studies [Phelps, D. C., & Hatefi, Y. (1981) *J. Biol. Chem. 256*, 8217–8221; Phelps, D. C., & Hatefi, Y. (1984) *Biochemistry 23*, 4475–4480] suggested that the inhibition site of DCCD is near the NAD(H) binding site, because NAD(H) and competitive inhibitors protected TH against inhibition by DCCD and, unlike the unmodified TH, the DCCD-modified TH did not bind to NAD–agarose. Others [Pennington, R. M., & Fisher, R. R. (1981) *J. Biol. Chem. 256*, 8963–8969] could not demonstrate protection by NADH, obtained data indicating DCCD inhibits proton translocation by TH much more than hydride ion transfer from NADPH to 3-acetylpyridine adenine dinucleotide (AcPyAD), and concluded that DCCD modifies an essential residue in the proton channel of TH. The present studies show that *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) also inhibits TH. The inhibition is pseudo first order at several EEDQ concentrations, and the reaction order with respect to [EEDQ] is unity, suggesting that inhibition involves the interaction of one molecule of EEDQ with one active unit of TH. The EEDQ-modified TH reacts covalently with [^{3}H]aniline, suggesting that the residue modified by EEDQ is a carboxyl group. More significantly, it has been shown that the absorbance change of oxonol VI at 630 minus 603 nm is a reliable reporter of TH-induced membrane potential formation in submitochondrial particles and that TH-catalyzed hydride ion transfer from NADPH to AcPyAD and the membrane potential induced by this reaction are inhibited in parallel by either DCCD or EEDQ.

Vectorial proton translocation by the ATP synthase complexes of prokaryotic and eukaryotic organisms [for a review, see Senior (1983)], by complex III (ubiquinol–cytochrome *c* oxidoreductase) of bovine heart and yeast mitochondria (Esposti et al., 1983; Clejan & Beattie, 1983), and by the bovine cytochrome *c* oxidase (Casey et al., 1980) is inhibited by the carboxyl modifying reagent DCCD.[1] In the mammalian ATP synthase complex, DCCD reacts at two sites, a proteolipid in the membrane sector, F_0, and the β subunit of the catalytic segment, F_1. However, at 0 °C and low concentrations of DCCD, only the proteolipid, which is involved in transmembrane proton translocation, is modified (Kiehl & Hatefi, 1980). In the ATP synthase complex (F_1–F_0), modification of F_0 by DCCD perforce inhibits the catalytic function of F_1, even though the F_1 moiety exhibits full activity when separated from the DCCD-modified F_0. In cytochrome *c* oxidase the primary target for DCCD is subunit III (Casey et al., 1980) while the electron carriers of the enzyme (hemes *a* and a_3, Cu_a, and Cu_{a3}) appear to be contained in subunits I and II (Capaldi et al., 1983). Hence, it can be shown under appropriate conditions that modification of cytochrome *c* oxidase by DCCD inhibits primarily the vectorial proton translocation by the enzyme complex rather than the scalar electron-transfer reaction (Casey et al., 1980). A similar differential effect of DCCD has also been observed in the case of complex III (Clejan & Beattie, 1983; Esposti et al., 1983).

Another mitochondrial energy-transducing enzyme that is inhibited by DCCD is the energy-linked nicotinamidenucleotide transhydrogenase (TH) (Phelps & Hatefi, 1981; Pennington & Fisher, 1981). The former authors showed that SMP-bound TH is protected against DCCD inhibition by NAD(H) and analogues, thus suggesting that the binding site of DCCD might be close to that of NAD(H). By contrast, Pennington & Fisher (1981) could not demonstrate protection by NAD(H) and presented data indicating that DCCD inhibits proton translocation by TH much more than hydride ion transfer from NADPH to AcPyAD. Thus, they concluded that DCCD binds outside the active site and modifies a residue in the proton channel of the enzyme. More recently, we have further confirmed our earlier findings and shown (a) that NAD(H) and analogues, including the NAD(H) competitive inhibitors 5′-AMP and 5′-ADP, protect the purified and the membrane-bound TH against inhibition by DCCD while 2′-AMP and 3′-AMP, which are competitive inhibitors of NADP(H), do not protect and (b) that, unlike the native TH, the DCCD-modified enzyme does not bind to NAD–agarose. (Phelps & Hatefi, 1984).

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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; AcPyAD, 3-acetylpyridine adenine dinucleotide; AcPyADP, 3-acetylpyridine adenine dinucleotide phosphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; F_1, mitochondrial F_1-ATPase; TH, nicotinamidenucleotide transhydrogenase or the purified transhydrogenase enzyme; SMP, submitochondrial particles.

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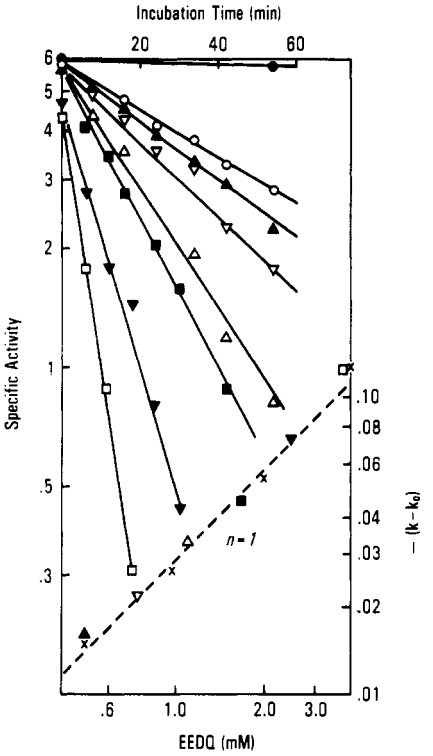

FIGURE 1: Semilogarithmic plot of inhibition of NADPH to AcPyAD transhydrogenation activity (left ordinate) as a function of incubation of purified TH with EEDQ (top abscissa). TH at 0.2 mg/mL in 10 mM sodium phosphate, pH 7.5, containing 0.01% Brij 58 was incubated at room temperature with 0.33 (○), 0.5 (▲), 0.75 (▽), 1.125 (△), 1.69 (■), 2.54 (▼), and 3.8 (□) mM EEDQ and then sampled (2 μg) at the intervals shown (top abscissa) and assayed for activity. The control (●) was incubated with the EEDQ solvent [1.9% (v/v) methanol]. (Lower part) Double logarithmic plot of [EEDQ] (bottom abscissa) vs. the corresponding inhibition rate constants (k) derived from the semilogarithmic plot in the upper part of the figure (right ordinate). k_0 is the activity decay rate constant of the control (●). (×) Data from a separate experiment.

This paper shows that, similar to DCCD, EEDQ also inhibits TH and that the target appears to be a carboxyl group possibly near the NADH binding site of the enzyme. It also demonstrates that both DCCD and EEDQ inhibit in parallel the scalar and the vectorial reactions catalyzed by TH, as monitored respectively by transhydrogenation from NADPH to AcPyAD and by membrane potential formation induced by the transhydrogenation reaction.

Materials and Methods

The preparation of SMP (Löw & Vallin, 1963) and purified TH (Phelps & Hatefi, 1984) and assays for energy-linked and non-energy-linked TH activities of SMP (Phelps & Hatefi, 1981) and the purified enzyme (Phelps & Hatefi, 1984) were essentially the same as described in the references cited. Any changes are indicated in the figure legends. Specific activities are expressed as micromoles of AcPyAD reduced by NADPH per minute per milligram of protein for the purified TH and micromoles of AcPyAD reduced by NADPH or nanomoles of AcPyADP reduced by NADH per minute per milligram of protein for SMP-bound TH. Oxonol VI absorbance change was monitored at 630 minus 603 nm as described elsewhere (Yagi et al., 1984). Protein was estimated by the method of Lowry et al. (1951).

Nicotinamide nucleotides were obtained from P-L Biochemicals; EEDQ, sodium succinate, and 5′-AMP were from Sigma; DCCD was from Aldrich; CCCP was from Calbiochem. Oxonol VI (Schuurmans et al., 1978) and [^{3}H]aniline were gifts respectively from Dr. W. G. Hanstein, University of Bochum, and Dr. W. S. Allison, University of California, San Diego. The sources of other chemicals were the same as before (Phelps & Hatefi, 1984).

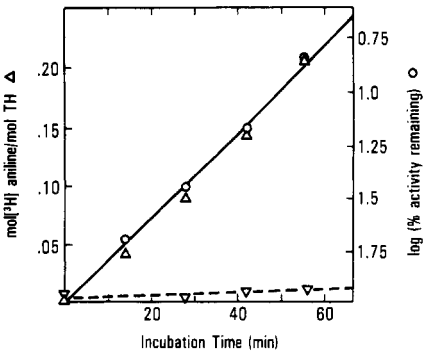

FIGURE 2: Incorporation of [^{3}H]aniline into EEDQ-activated TH (left ordinate) as a function of incubation time and correlation of radioactivity incorporation with enzyme activity inhibition (right ordinate). TH at 0.35 mg/mL in the same medium as in Figure 1 was incubated at room temperature with 2 mM [^{3}H]aniline (163 mCi/mmol) in the presence (△) and absence (▽) of 2 mM EEDQ. At the indicated intervals, 1.75 μg of protein was removed and assayed for activity (○). Also, 0.2-mL aliquots were removed at the same intervals and assayed for protein-bound radioactivity (△, ▽) with the procedure described elsewhere (Phelps & Hatefi, 1984) for determination of protein-bound [^{14}C]DCCD.

Results

Inhibition of Transhydrogenation by EEDQ. Figure 1 shows the time course of inhibition of transhydrogenation from NADPH to AcPyAD as a consequence of incubation of purified TH with several different concentrations of EEDQ. The inhibition was pseudo first order in the EEDQ concentration range used, and the reaction order with respect to [EEDQ] was unity (n = 1, Figure 1 inset), suggesting that inhibition involved the interaction of one molecule of EEDQ with one active unit of the enzyme. Similar results were obtained with SMP-bound TH.

It has been shown that, similar to DCCD, EEDQ can be used to activate appropriate carboxyl groups for the synthesis of peptides and amides (Belleau & Martel, 1968). Satre et al. (1983) were able to incorporate radioactivity from [^{14}C]-glycine ethyl ester into EEDQ-treated F_1 from *Escherichia coli*, and Allison and co-workers (private communication from Dr. W. S. Allison) have used tritiated aniline to make anilide derivatives of the EEDQ-activated carboxyl groups of bovine F_1. Figure 2 shows that inhibition of TH by EEDQ in the presence of [^{3}H]aniline also results in incorporation of radioactivity into the enzyme. The amount of radioactivity incorporated increased linearly with the duration of incubation of TH with EEDQ and [^{3}H]aniline and correlated well with the degree of inhibition of TH (Figure 2, circles and right ordinate). [^{3}H]Aniline in the absence of EEDQ did not inhibit the enzyme, even though it appeared to bind to a small extent to the enzyme preparation. Thus, the results of Figure 2 indicated that, similar to DCCD, the EEDQ inhibition of TH activity is most likely due to covalent modification of a carboxyl group and that the anilide derivative is also inactive. Other studies to be reported elsewhere have suggested that, like DCCD (Phelps & Hatefi, 1984), the site of EEDQ modification is near the active site of TH.

Inhibition of Proton Translocation by DCCD and EEDQ. As mentioned earlier, Pennington & Fisher (1981) have suggested that DCCD inhibits proton translocation by TH much more than hydride ion transfer. For example, they have shown that incubation of proteoliposomes containing TH for 15 min with 1.0 mM DCCD resulted in about 85% inhibition

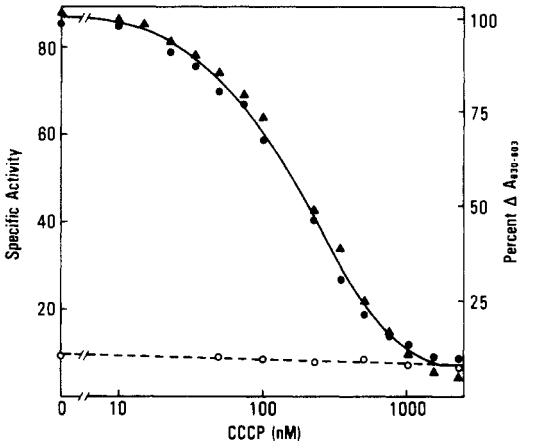

FIGURE 3: Effect of incremental uncoupling with increasing concentrations of CCCP on the rate of energy-linked transhydrogenation from NADH to AcPyADP (●) and the energy-induced absorbance change of oxonol VI (▲). The assay mixtures at 37 °C contained 0.25 M sucrose, 50 mM Tris–sulfate, pH 7.5, 25 μM rotenone, 1 μg of rutamycin/mL, and 21 μg of SMP/mL. The membranes were energized by addition of 10 mM sodium succinate. The transhydrogenase assay mixtures also contained 0.2 mM NADH and 0.5 mM AcPyADP, and the reactions were followed at 375 minus 425 nm (Phelps & Hatefi, 1981). The oxonol VI assay mixtures contained 4 μM oxonol VI, and the absorbance change of oxonol VI was monitored at 630 minus 603 nm. CCCP was added at the indicated concentrations in a final volume of ethanol not exceeding 2% v/v. (○) Rate of transhydrogenation from NADH to AcPyADP in the absence of succinate.

of proton translocation and only 30% inhibition of transhydrogenation from NADPH to AcPyAD. Since proton translocation in TH is coupled to hydride ion transfer from NADPH to AcPyAD, these results would suggest that DCCD has two effects: (a) inhibition of proton translocation and (b) partial decoupling of the proton translocation from the hydride ion transfer reaction. Since, unlike the ATP synthase complex, TH appears to be a homodimer and does not contain one set of polypeptides concerned with catalyzing the scalar reaction and another set involved in proton translocation, it was of interest to reexamine the differential effects of DCCD reported by Pennington & Fisher (1981). This was particularly important since our previous studies (Phelps & Hatefi, 1984) had shown that the binding of 1 mol of [^{14}C]DCCD/2 mol of TH (one dimer) resulted in complete activity inhibition, thus indicating that the differential inhibitions observed by Pennington & Fisher (1981) would have to be effected somehow by one molecule of DCCD reacting with a single site on the enzyme.

For reasons that will be discussed below, we selected to work with SMP rather than TH proteoliposomes, and we monitored proton translocation by the more sensitive means of measuring the formation of a membrane potential induced by transhydrogenation from NADPH to AcPyAD. As a reporter of the membrane potential, the absorbance change of oxonol VI was used, which in other studies in this laboratory was shown to be a reliable procedure (Yagi et al., 1984). Nevertheless, additional tests were necessary in order to be able to correlate TH activity with the dye response. First, as seen in Figure 3, it was shown that the rate of respiration-induced transhydrogenation from NADH to AcPyADP and the magnitude of the oxonol VI absorbance change at 630 minus 603 nm decreased in parallel when the system was uncoupled by addition of incremental amounts of CCCP. Second, it was shown that transhydrogenation in the reverse direction, i.e., from NADPH to AcPyAD, did induce an oxonol VI absorbance change and that the extent of this change was correlated with

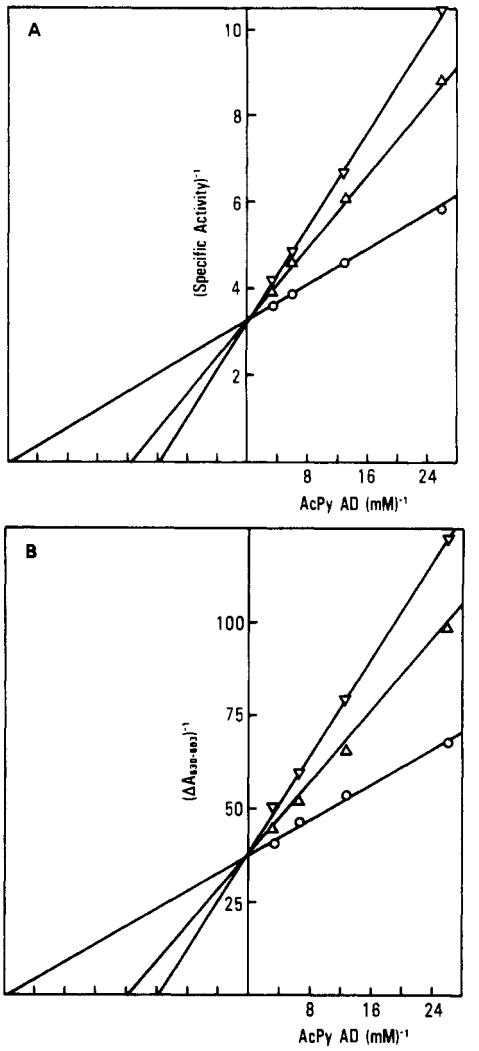

FIGURE 4: Double-reciprocal plots of (A) TH activity vs. [AcPyAD] and (B) TH-driven oxonol VI response vs. [AcPyAD] at 0 (○), 1 (△), and 2 mM (▽) 5′-AMP. The assay mixtures at 37 °C contained 0.25 M sucrose, 50 mM Tris–sulfate, pH 7.5, 25 μM rotenone, 0.2 mM each NADPH and AcPyAD, and the indicated amounts of 5′-AMP. The transhydrogenase reaction was followed at 375 minus 425 nm in the presence of 140 μg of SMP/mL, and the oxonol VI absorbance change was monitored at 630 minus 603 nm in the presence of 4 μM oxonol VI and 100 μg of SMP/mL.

the rate of the transhydrogenase reaction. For the latter purpose, the kinetics of transhydrogenation from NADPH to AcPyAD were assayed as a function of [AcPyAD] in the absence and presence of two concentrations of the competitive inhibitor 5′-AMP. As expected, double-reciprocal plots of the data showed a competitive inhibition pattern (Figure 4A). Then, the same experiment was repeated under identical conditions, except that this time instead of activity the change in the absorbance of oxonol VI was measured at different [AcPyAD] in the absence and presence of the same two concentrations of 5′-AMP. Double-reciprocal plots of these data, in which the ordinate was now the reciprocal of oxonol VI absorbance change, showed again a competitive inhibition pattern with exactly the same abscissa intercepts in the absence and presence of the two different concentrations of 5′-AMP (Figure 4B). These results demonstrated, therefore, that in SMP membrane potential formation as monitored by the oxonol VI absorbance change was well correlated with the activity of the transhydrogenase reaction in the direction NADPH → AcPyAD.

Under similar conditions, the effects of incubation of SMP with DCCD and EEDQ were then studied on the trans-

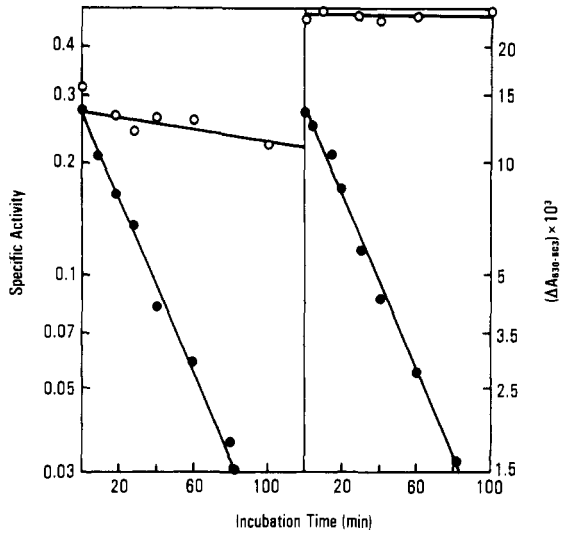

FIGURE 5: Effect of DCCD on inhibition of TH activity (left panel) and TH-driven membrane potential formation (right panel) in SMP. SMP at 0.5 mg/mL in a mixture containing 0.25 M sucrose, 50 mM Tris–sulfate, pH 7.5, 0.5 μg of oligomycin/mL, and 25 μM rotenone was incubated at room temperature with 0.1 mM DCCD. At the indicated intervals, samples containing respectively 25 and 100 μg of protein were withdrawn and assayed for transhydrogenase activity and TH-driven oxonol VI response as in Figure 4. (●) SMP treated with DCCD in a final volume of 1.4% ethanol; (○) SMP treated with 1.4% ethanol.

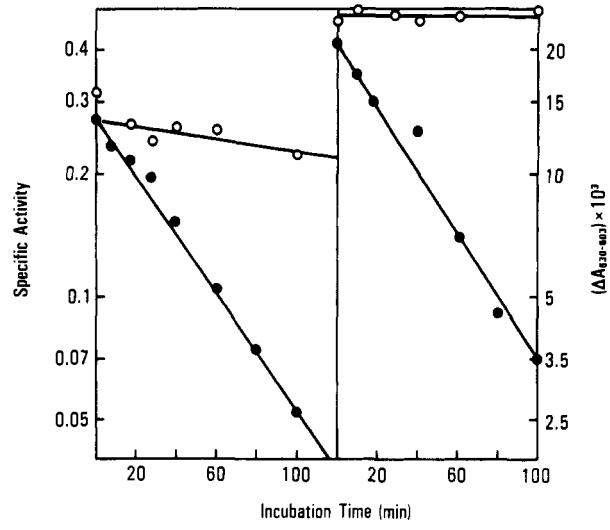

FIGURE 6: Effect of EEDQ on inhibition of TH activity and TH-driven membrane potential formation in SMP. Incubation and assay conditions were the same as those in Figure 5 except that SMP was incubated with 2.0 mM EEDQ (in a final volume of 1.4% ethanol) instead of DCCD. (●) SMP treated with EEDQ; (○) SMP treated with 1.4% (v/v) ethanol.

hydrogenase activity and the resultant membrane potential formation. As seen in Figures 5 and 6, both parameters, i.e., the rate of transhydrogenation from NADPH to AcPyAD and the extent of oxonol VI absorbance change induced by the transhydrogenase reaction, decreased in parallel when SMP was incubated with either DCCD (Figure 5) or EEDQ (Figure 6). These results indicated, therefore, that in SMP these carboxyl group modifiers do not exert differential effects on the rate of transhydrogenation and the extent of membrane potential induced by this reaction.

One point should be discussed here with regard to the oxonol VI absorbance change in the presence of DCCD. As seen in Figure 5, at zero time the oxonol VI absorbance change in the presence of DCCD was considerably lower than that in the absence of DCCD. This is an artifact not related to the transhydrogenase-induced membrane potential. The same effect was observed when membrane potential was induced in SMP by succinate or NADH oxidation in the absence and presence of DCCD.

Discussion

It has been shown that EEDQ inhibits the purified and the membrane-bound TH, apparently by modification of one carboxyl group per active unit of the enzyme. Preliminary experiments have also suggested that the mode of inhibition of EEDQ is somewhat similar to that of DCCD. Previous studies (Phelps & Hatefi, 1981, 1984) have shown that the DCCD inhibition is specifically prevented by NAD(H) and by the NAD(H) competitive inhibitors 5′-AMP and 5′-ADP and that the DCCD-modified enzyme does not bind to NAD–agarose. Therefore, it appears that DCCD, and possibly also EEDQ, binds to TH near the NAD(H) binding site. These findings are in contrast to the results of Pennington & Fisher (1981), who could not demonstrate protection by NAD(H) against DCCD inhibition and concluded that DCCD inhibition does not involve the enzyme active site.

The latter authors have also shown with TH-inlaid liposomes that DCCD appears to inhibit proton translocation much more than hydride ion transfer from NADPH to AcPyAD. In examining the effects of DCCD and EEDQ on the coupled scalar and vectorial reactions catalyzed by TH, we selected to work with SMP rather than reconstituted TH proteoliposomes for the following reasons. First, the measured H^+/H^- stoichiometry data of Fisher and co-workers indicated values appreciably smaller than unity (Earle & Fisher, 1980) and often around 0.5 or less under the conditions used for examining the effects of chemical modifiers (Wu & Fisher, 1982). Thus, we were concerned that such low H^+/H^- values could cause measurement errors, especially when the system was partially inhibited with DCCD or EEDQ. Second, the presence of TH molecules not inserted transmembranously into the liposomes and the possible differential effects of DCCD and EEDQ on transmembranous vs. nontransmembranous TH molecules could complicate the results. We further selected to measure the effect of the above inhibitors on transhydrogenation-induced membrane potential rather than ΔpH, because we were able to ascertain that membrane potential, as monitored by the absorbance change of oxonol VI, was rather precisely correlated with TH activity under both endergonic (NADH → AcPyADP, Figure 3) and exergonic (NADPH → AcPyAD, Figure 4) conditions, whereas direct measurement of small pH changes was problematic, especially when the enzyme was greatly inhibited by DCCD or EEDQ. At any rate, under our experimental conditions DCCD and EEDQ inhibited both transhydrogenation from NADPH to AcPyAD and membrane potential formation induced by this reaction in parallel. These results do not agree with the paper of Pennington & Fisher (1981).

As was discussed earlier, in both the ATP synthase complex and cytochrome *c* oxidase, the primary target of DCCD (i.e., the proteolipid subunit of the F_0 segment of ATP synthase and subunit III of cytochrome *c* oxidase) is a polypeptide that in ATP synthase is not involved in catalyzing ATP hydrolysis and in cytochrome oxidase does not appear to contain any electron carriers. Under conditions that only the proteolipid of ATP synthase is modified by DCCD, the catalytic activity of the complex is inhibited because its coupled proton translocation through F_0 is prevented by the DCCD modification. In order to realize the hydrolytic activity of F_1 in such a system, one would have to either separate F_1 from the DCCD-inhibited F_0 or create a proton leak between F_1 and the DCCD block

point by removal or structural modification of an appropriate noncatalytic subunit. In DCCD-modified cytochrome *c* oxidase also a somewhat similar decoupling must take place in order to permit electron transfer to proceed while the coupled proton translocation is inhibited. Thus, it is conceivable that DCCD modification of subunit III causes a conformation change in the multisubunit complex that separates a portion of the proton channel from the electron carrier subunits of the enzyme.

In the case of TH, we are concerned with a single type of polypeptide, which presumably contains both the catalytic site and the proton channel (the latter could also be shared between the two protomers). In order to rationalize the results of Pennington & Fisher (1981), one would have to assume that DCCD binds to the proton channel domain of TH and somehow decouples this domain from the catalytic domain of the same protein molecule. Otherwise, similar to the F_1–F_0 complex, inhibition of the proton channel of TH would perforce inhibit its scalar reaction as well. Pennington and Fisher realize this problem and conclude that their results indicate that in TH "hydride ion transfer is not obligatorily coupled to proton translocation". If this were the case, such a lack of control would allow NADPH to become rapidly oxidized, because the rate of the non-energy-linked transhydrogenation from NADPH to NAD is very much faster than the rate of the energy-linked transhydrogenation from NADH to NADP. However, this possibility does not agree with the situation in intact respiring mitochondria in which NADP is found in a highly reduced state (Klingenberg & Slenczka, 1959).

Pennington & Fisher (1981) have also proposed one DCCD reactive site per protomer and a third, which results in cross-linking. The latter is a possibility, and we have experienced some cross-linking of purified TH with EEDQ, although very little under the conditions employed with DCCD. However, we have shown in a previous paper (Phelps & Hatefi, 1984) that total inhibition of the transhydrogenase activity of purified TH corresponded to covalent attachment of only 0.5 mol of [^{14}C]DCCD/mol of TH (i.e., 1 mol of DCCD/TH dimer). Thus, whatever the mechanism, 1 mol of covalently bound DCCD/TH dimer must inhibit both transhydrogenation and the consequent proton translocation. A simple explanation consistent with our previous and present results is that the TH dimer exhibits half-of-the-sites reactivity (or site–site catalytic cooperativity similar to F_1-ATPase) and that DCCD (also possibly EEDQ) binds near the catalytic site and inhibits both transhydrogenation and proton translocation by interfering with NAD(H) binding to the enzyme. In view of the above results and conclusions, we feel that a possible explanation for the data of Pennington & Fisher (1981) might be that under certain conditions the presence of noncovalently bound DCCD might interfere with transmembrane proton movements or proper measurement of the rate of proton translocation.

Acknowledgments

We thank C. Munoz for the preparation of mitochondria.

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