Effects of Substrate and Inhibitor Binding on Thermal and Proteolytic Inactivation of Rat Liver Transhydrogenase†

John F. Blazyk,‡ Dominique Lam, and Ronald R. Fisher*

ABSTRACT: The thermostability and proteolytic inactivation of rat liver submitochondrial particle transhydrogenase was studied in the presence of pyridine dinucleotide substrates and a variety of divalent metal and nucleotide inhibitors. Relative to the unliganded enzyme, the NADPH–enzyme complex was more thermostable and showed a twofold greater rate of tryptic inactivation, while the $NADP^+$–enzyme complex was more thermolabile and only slightly more susceptible to tryptic inactivation. Neither NAD^+ nor NADH significantly affected thermostability or proteolysis. Similar effects of these ligands were observed for the non-energy-linked and energy-linked transhydrogenase reactions, indicating that both activities are catalyzed by the same enzyme. In thermal experiments, acetyl-CoA, 2′-AMP, and NMNH stabilized, palmitoyl-CoA labilized, and dephospho-CoA, CoA, NMN^+, and 5′-AMP had little effect on enzyme stability. Tryptic inactivation was inhibited by 2′-AMP and NMN^+ but was not influenced by the other nucleotide inhibitors. Divalent metal ion inhibitors (Mg^{2+}, Ca^{2+}, Mn^{2+}, Ba^{2+}, and Sr^{2+}) stabilized transhydrogenase against thermal inactivation and promoted tryptic inactivation.

The active site of mammalian mitochondrial membrane-bound pyridine dinucleotide transhydrogenase (EC 1.6.1.1; Kaplan et al., 1953) is oriented to the inner side of the inner membrane (Van Dam and Ter Welle, 1966; Lee and Ernster, 1966). The enzyme thus exclusively catalyzes the reversible transfer of a hydride ion equivalent between oxidized and reduced forms of intramitochondrial NAD and NADP (eq 1).

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH \qquad (1)$$

The equilibrium constant for this reaction under nonenergized conditions is about 1, but shifts to values as high as 480 when linked to electron transport or ATP hydrolysis (Lee and Ernster, 1964; Kaplan, 1972). The mechanism of transhydrogenase coupling to the oxidative phosphorylation system is not well understood. Mitchell (1966, 1972) has suggested that the energy-linked reduction of $NADP^+$ by NADH occurs by reversal of transhydrogenation at an energy coupling site localized between NADPH and NAD^+; i.e., the transhydrogenase functions directly as a reversible proton pump. Van de Stadt et al. (1971) provided evidence for such an energy coupling site by reporting NAD^+ reduction by NADPH to be coupled to ADP phosphorylation, albeit with a low $P/2e^-$ ratio. Dontsov et al. (1972) illustrated that the reverse reaction (eq 1) generates a membrane potential in submitochondrial particles having the same polarity, but of lower magnitude, as that observed in the presence of ATP or succinate. That the membrane potential results from a pH gradient was suggested by the finding that submitochondrial particles translocate protons inward, coupled to NAD^+ reduction by NADPH (Mitchell and Moyle, 1965).

It has often been postulated that conformational changes of transhydrogenase might play an important role in its coupling to the energy-conservation system (Rydström et al., 1970, 1971b; Green and Ji, 1972; Skulachev, 1974; Blazyk and Fisher, 1975). We have recently provided evidence, through studies of differential thermal and proteolytic inactivation, that conformational changes in transhydrogenase are induced by the binding of at least two of the four substrates (Blazyk and Fisher, 1975). NADPH stabilizes the enzyme to thermal inactivation and markedly increases the degree of tryptic inactivation. On the other hand, $NADP^+$ increases the thermolability of the enzyme, while inducing no significant effect on proteolysis. Neither NAD^+ nor NADH influences the thermal inactivation of transhydrogenase. It was concluded from these results that the binding of $NADP^+$ or NADPH results in the formation of different transhydrogenase conformers, which in turn are unique with respect to the unliganded enzyme.

In this paper, we describe a more detailed investigation of the effects of substrates, as well as selected nucleotide and divalent metal inhibitors, on the differential thermal and proteolytic inactivation of transhydrogenase.

Materials and Methods

Rat liver mitochondria were prepared according to the method of Schnaitman and Greenawalt (1968). Submitochondrial particles were prepared as described previously (Blazyk and Fisher, 1975) and stored at −60 °C. Protein was analyzed by the biuret procedure (Jacobs et al., 1956) using recrystallized bovine serum albumin as a standard.

Differential thermal inactivation studies were performed in matched 15 × 125 mm Pyrex test tubes containing a preincubation mixture (0.31 ml) consisting of 16 mM Tris-HCl buffer, pH 7.5, 0.25–0.75 mg of submitochondrial particle protein, and other additions as indicated. In studies using palmitoyl-CoA, the preincubation volume was increased to 0.61 ml with no greater than 0.25 mg of submitochondrial particle protein in order to minimize the effects of nonspecific binding of the inhibitor to the membrane (Rydström, 1972). An excess of fatty-acid-free bovine serum albumin (6 mg) was added after preincubation and before assaying the mixture to

† From the Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208. *Received October 13, 1975.* This work was supported by National Institutes of Health Grant No. GM 22070.

‡ Recipient of a National Institutes of Health Postdoctoral Fellowship (1F32 GM-05256). Present address: Department of Chemistry, Ohio University, Athens, Ohio 45701.

remove palmitoyl-CoA from solution (Rydström, 1972). Heat treatment was terminated by quenching the tubes in ice after the specified preincubation period. The particles were then assayed for either non-energy-linked or energy-linked transhydrogenase activity.

Differential proteolytic inactivation studies were performed similarly, except that 10–20 μg of trypsin was present in the preincubation mixture at 23 °C. Tryptic digestion was generally allowed to progress for 1–2 min, after which excess trypsin inhibitor was added, and the particles were immediately assayed for either non-energy-linked or energy-linked transhydrogenase activity. Controls were performed identically except that excess trypsin inhibitor was added to the preincubation mixture initially along with trypsin.

Non-energy-linked transhydrogenase activity was assayed without NADPH or NAD^+ regenerating systems by the method of Stein et al. (1959). The reduction of the 3-acetylpyridine analogue of NAD^+ ($AcPyAD^+$)[1] by NADPH was monitored continuously at 375 nm, with an assumed millimolar extinction coefficient of 5.1 for reduced 3-acetylpyridine adenine dinucleotide (AcPyADH). The assay mixture (3.0 ml) contained 80 mM phosphate buffer, pH 6.8, 0.19 mM $AcPyAD^+$, 0.15 mM NADPH, and 0.5 μM rotenone.

The assay for energy-linked transhydrogenase utilized alcohol dehydrogenase to continuously regenerate NADH from NAD^+ during the course of the reaction (Ernster and Lee, 1967). The increase in NADPH concentration was measured spectrophotometrically at 340 nm, using a millimolar extinction coefficient of 6.2. The final reaction mixture (3.0 ml) contained 3.4 μM rotenone, 2 μg of oligomycin, 0.2 mg of alcohol dehydrogenase, 26 μM NADH, 250 μM $NADP^+$, 3 mM succinate, 0.25 M sucrose, 6 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 8.0.

$AcPyAD^+$, bovine serum albumin (fatty acid depleted), yeast alcohol dehydrogenase, rotenone, oligomycin, bovine trypsin (type I), soybean trypsin inhibitor (type I-S), α-chymotrypsin, chymopapain, diisopropyl fluorophosphate treated carboxypeptidase A, and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Sigma Chemical Company. All other biochemicals were purchased from P-L Biochemicals.

Results

Rat liver pyridine dinucleotide transhydrogenase can be partially inactivated by means of exposure to high temperature or by proteolysis. We reported previously that the degree of inactivation is influenced by the presence of substrates of the enzyme (Blazyk and Fisher, 1975). In thermal experiments, NADPH stabilized while $NADP^+$ labilized the enzyme to heat inactivation. The thermostability of transhydrogenase was unaffected by the presence of NAD^+ or NADH. In proteolytic experiments with trypsin, NADPH stimulated by at least twofold, while $NADP^+$ only slightly stimulated the rate of inactivation of transhydrogenase.

Effects of Substrates on Proteolytic Inactivation. In agreement with earlier observations on bovine heart submitochondrial particle transhydrogenase by Juntti et al. (1970), we find the rat liver enzyme to be quite susceptible to proteolytic inactivation by trypsin. The degree of tryptic inactivation is linearly dependent on trypsin concentration and is completely inhibited in the presence of excess trypsin inhibitor. Moreover, the inactivation of the enzyme with a fixed trypsin concentration is linear with respect to time (Blazyk and Fisher, 1975).

[1] Abbreviation used: $AcPyAD^+$, oxidized 3-acetylpyridine adenine dinucleotide.

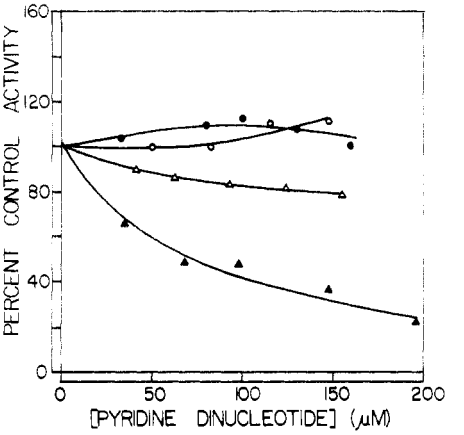


FIGURE 1: The effect of pyridine dinucleotides on tryptic inactivation of transhydrogenase. Submitochondrial particles were preincubated with 10 μg of trypsin for 2 min in the presence of NADPH (▲), $NADP^+$ (△), NADH (●), or NAD^+ (○). Control activity represents transhydrogenation after trypsin treatment in the absence of substrate.

TABLE I: Proteolytic Inactivation of Transhydrogenase by α-Chymotrypsin, Chymopapain, and Carboxypeptidase A.[a]

Preincubation Additions	% Control Act. α-Chymotrypsin	Chymopapain	Carboxypeptidase
None	67.9	53.8	86.3
NADPH (100 μM)	27.6	19.2	49.1
$NADP^+$ (100 μM)	52.9	26.9	89.6

[a] Submitochondrial particles were preincubated with either α-chymotrypsin (100 μg for 2 min), chymopapain (50 μg for 1 min), or diisopropyl fluorophosphate treated carboxypeptidase A (100 units for 8 min) along with the indicated additions. The particles were assayed for non-energy-linked transhydrogenase activity immediately following the completion of the preincubation. The control rates with no additions were 48.0 nmol of AcPyADH min^{-1} mg^{-1} protein for the α-chymotrypsin experiment, 47.8 for the chymopapain experiment, and 35.1 for the carboxypeptidase A experiment.

Proteolytic inactivation in the presence of the four substrates of transhydrogenase is presented in Figure 1. It is clear that NADPH induces a drastic stimulation in the degree of tryptic inactivation. $NADP^+$ slightly stimulates, while NADH and NAD^+ have little significant effect on the proteolysis of the enzyme. Thus, it appears that transhydrogenase, upon binding NADPH, undergoes a conformational change in which one or more essential arginyl or lysyl groups become more accessible to trypsin.

Three other proteolytic enzymes, α-chymotrypsin, chymopapain, and diisopropyl fluorophosphate treated carboxypeptidase A inactivate transhydrogenase. Table I details the degree of inactivation observed in the presence of $NADP^+$ or NADPH. A universally large stimulation of inactivation is manifested by NADPH. The inactivation by carboxypeptidase A in the presence of NADPH suggests that one or more C-terminal amino acid residues become more accessible due to NADPH binding, and that the amino acids that are cleaved are essential for enzymatic activity. A slight stimulation caused by $NADP^+$ is observed with α-chymotrypsin similar to that observed with trypsin, but this stimulation is much more pronounced with chymopapain. Therefore, the conformational

TABLE II: Thermal and Proteolytic Inactivation of the Non-Energy-Linked and Energy-Linked Transhydrogenase Reactions in the Presence of NADPH or $NADP^+$.[a]

		Non-Energy-Linked			Energy-Linked		
	Preincubation Additions	Rate (nmol of AcPyADH min^{-1} mg^{-1}) 22 °C	Rate (nmol of AcPyADH min^{-1} mg^{-1}) 50 °C	% Control Act.	Rate (nmol of NADPH min^{-1} mg^{-1}) 22 °C	Rate (nmol of NADPH min^{-1} mg^{-1}) 50 °C	% Control Act.
Thermal	None	40.9	30.9	75.6	23.9	14.0	58.3
	NADPH (200 μM)	48.9	51.8	106.1	25.9	24.9	96.2
	$NADP^+$ (200 μM)	39.9	24.9	62.5	21.9	10.0	45.5
		−Trypsin	+Trypsin		−Trypsin	+Trypsin	
Proteolytic	None	46.9	31.9	68.1	21.9	13.0	59.1
	NADPH (200 μM)	53.8	13.0	24.1	24.9	3.0	12.0
	$NADP^+$ (200 μM)	41.9	26.9	64.3	21.9	11.0	50.0

[a] Submitochondrial particles (0.59 mg of protein) were preincubated at either 22 or 49 °C for 2 min (thermal) or were preincubated with 10 μg of trypsin for 1 min (proteolytic). After quenching in ice (thermal) or adding excess trypsin inhibitor (proteolytic), the particles were assayed for non-energy-linked or energy-linked transhydrogenase activity.

change in transhydrogenase which is apparently induced by NADPH binding causes the enzyme to be labilized to proteolytic enzymes with diverse specificities. Moreover, the conformational change associated with $NADP^+$ binding renders the enzyme much more susceptible only to chymopapain, the least specific of the proteolytic enzymes tested.

Inactivation of Non-Energy-Linked and Energy-Linked Transhydrogenation. It has often been postulated that the non-energy-linked and energy-linked transhydrogenation reactions are catalyzed by a single enzyme. They are both A specific for NADH oxidation and B specific for NADPH oxidation (Lee et al., 1965), inhibited by palmitoyl-CoA (Rydström et al., 1971a), and an antibody against solubilized transhydrogenase inhibits both reactions in submitochondrial particles (Kawasaki et al., 1964). On the other hand, the pH optima are 6.0 for the non-energy-linked and 7.5 for the energy-linked reactions (Lee and Ernster, 1966), and Mg^{2+} is an inhibitor of the non-energy-linked, but not of the energy-linked reaction (Hommes, 1963; Rydström, et al., 1970). If, indeed, the two reactions are catalyzed by the same enzyme, then it might be predicted that both activities would respond similarly to the influence of $NADP^+$ or NADPH on thermal and proteolytic inactivation. Table II illustrates such an experiment. Although the energy-linked reaction is somewhat more susceptible to thermal inactivation and slightly more labile to proteolysis than the non-energy-linked reaction, the effects of $NADP^+$ and NADPH are strikingly similar. These data lend further support for the notion that a single enzyme is responsible for both activities. The substrates $NADP^+$ and NADPH have been shown by kinetic studies to be competitive for the same binding site on transhydrogenase for both the non-energy-linked and energy-linked reactions (Rydström et al., 1971b). Consistent with this observation, NADPH completely and competitively reverses the thermolability of the enzyme induced by $NADP^+$ (not shown).

Effects of Nucleotide Inhibitors on Inactivation. In order to ascertain whether the adenine or the nicotinamide moiety is primarily responsible for the substrate-induced conformational changes of transhydrogenase, the effects of 2′-AMP, 5′-AMP, ADP, NMN^+, and NMNH on the thermal and proteolytic inactivation of the enzyme were studied. These results are presented in Table III. In thermal experiments, 2′-AMP and NMNH stabilized, ADP and NMN^+ labilized,

TABLE III: Effects of 2′-AMP, NMN^+, and NMNH on Thermal and Proteolytic Inactivation of Transhydrogenase.[a]

Preincubation Additions	Thermal % Control Rate	Proteolytic % Control Rate
Expt 1		
None	59.8	45.5
2′-AMP	70.1	62.5
NMNH	65.0	46.9
2′-AMP, NMNH	75.3	62.9
Expt 2		
None	47.4	54.7
2′-AMP	67.9	62.2
NMN^+	43.1	89.5
2′-AMP, NMN^+	61.5	89.5

[a] Prior to assay for non-energy-linked transhydrogenase activity, submitochondrial particles (expt 1, 0.3 mg of protein; expt 2, 0.5 mg of protein) were preincubated at 49 °C for 2 min (thermal) or were preincubated with 6 μg of trypsin for 2 min (proteolytic). Additions were 2 mM 2′-AMP, 2 mM NMN^+, and 2 mM NMNH. Control activities for thermal experiments 1 and 2, 48.4 and 34.5 nmol of AcPyADH min^{-1} mg^{-1} protein and for proteolysis experiments 1 and 2, 49.8 and 56.6 nmol of AcPyADH min^{-1} mg^{-1} protein, respectively.

while 5′-AMP had no effect on the inactivation of the enzyme. In proteolytic experiments, transhydrogenase was protected from inactivation to varying degrees by all these nucleotides. The effects on the thermal inactivation of the enzyme were very slight except in the case of 2′-AMP which afforded significant protection. A combination of 2′-AMP and NMNH at optimal concentrations exerted no synergistic effect on the thermostability of transhydrogenase as is observed in the presence of NADPH, while a combination of 2′AMP and NMN^+ induced no stimulation of thermal inactivation as observed in the presence of $NADP^+$. Rather, the effects of these combinations were simply additive. Furthermore, 2′-AMP in combination with NMN^+ or NMNH did not stimulate the tryptic inactivation of the enzyme as was observed with both $NADP^+$ and NADPH. In fact, substantial protection against proteolysis was afforded by these combinations. It is evident, therefore, that the entire pyridine dinucleotide molecule is important in

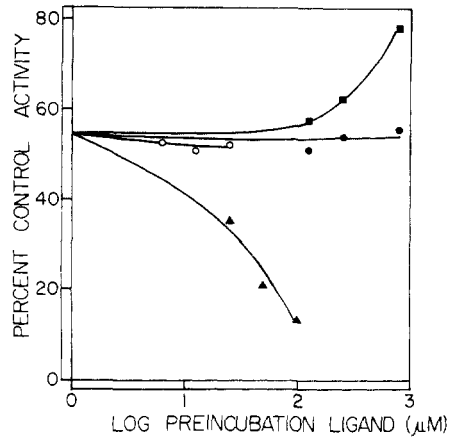


FIGURE 2: The effect of CoA derivatives on the thermal inactivation of transhydrogenase. Submitochondrial particles (0.24 mg of protein) were preincubated at 22 or 50 °C for 90 s in a volume of 0.61 ml in the presence of CoA (●), dephospho-CoA (○), acetyl-CoA (■), or palmitoyl-CoA (▲). Bovine serum albumin (fatty acid free; 6 mg) was added after the preincubation to tubes containing palmitoyl-CoA. The control rate without preincubation additions was 57.8 nmol of AcPyADH min^{-1} mg^{-1} protein.

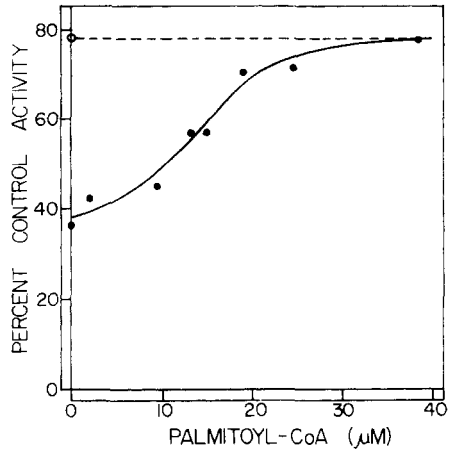


FIGURE 3: The effect of palmitoyl-CoA on NADPH-induced stimulation of proteolytic inactivation of transhydrogenase by trypsin. Submitochondrial particles (0.25 mg of protein) were preincubated with 4 μg of trypsin alone (○), or with 4 μg of trypsin, 53 μM NADPH, and palmitoyl-CoA (●) for 2 min. Bovine serum albumin (fatty acid free; 6 mg) was added before assaying. The control rate without additions was 72.2 nmol of AcPyADH min^{-1} mg^{-1} protein.

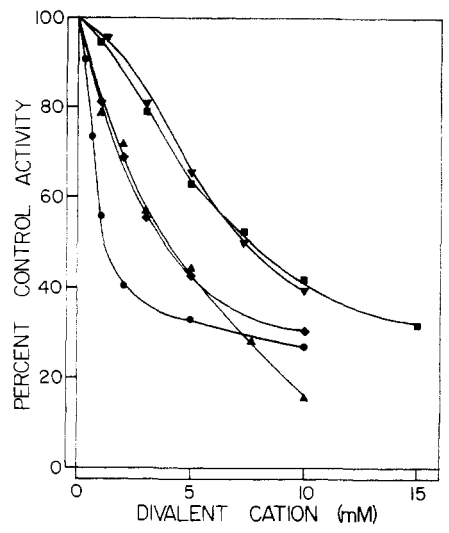


FIGURE 4: Inhibition of non-energy-linked transhydrogenase by divalent cations. Transhydrogenase was assayed in the presence of $MnCl_2$ (●), $MgCl_2$ (▲), $CaCl_2$ (♦), $BaCl_2$ (■), or $SrCl_2$ (▼) as described under Materials and Methods, except 100 mM Tris-HCl buffer, pH 8.0, was substituted for phosphate buffer.

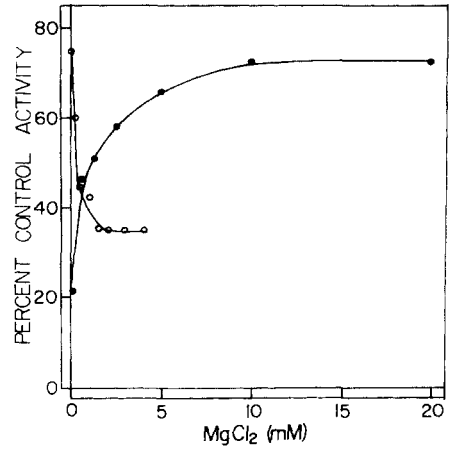


FIGURE 5: The effect of Mg^{2+} on the thermal and tryptic inactivation of transhydrogenase. For the thermal preincubation (●), a mixture containing submitochondrial particles in 16 mM Tris-HCl, pH 7.5, with a varying $MgCl_2$ concentration, was heated at 22 (control) or 50 °C for 2 min. In the proteolytic experiment (○), submitochondrial particles (0.7 mg of protein) were preincubated with 15 μg of trypsin and $MgCl_2$ for 2 min. The control rate with trypsin and trypsin inhibitor initially present in the preincubation mixture was 52.4 nmol of AcPyADH min^{-1} mg^{-1} protein.

determining the conformation of the enzyme and that the pyrophosphate linkage between the adenine and nicotinamide moieties is essential.

Coenzyme A, dephospho-CoA, and CoA thioesters of saturated fatty acids are site-specific inhibitors of transhydrogenase (Rydström, 1972). The effects of several CoA derivatives, in their inhibitory concentration ranges, on the thermal and proteolytic inactivation of transhydrogenase were examined. The data in Figure 2 indicate that the NAD-site specific inhibitor, dephospho-CoA, exerted no effect on heat inactivation while the effects of NADP-site specific inhibitors were variable, with acetyl-CoA enhancing, palmitoyl-CoA labilizing, and CoA not affecting the thermostability of transhydrogenase. None of these CoA derivatives showed any effect on the tryptic inactivation of the enzyme.

As in the case of $NADP^+$, NADPH completely and competitively reversed the labilization of transhydrogenase induced by palmitoyl-CoA. An analogous experiment to show reversal of NADPH-induced stimulation of tryptic inactivation by palmitoyl-CoA is portrayed in Figure 3. Palmitoyl-CoA is capable of completely reversing the effect of NADPH, with half-maximal reversal at about 14 μM.

Effect of Cations on Inactivation. It has been well established that Mg^{2+} is an inhibitor of the non-energy-linked transhydrogenase reaction. Other divalent cations including Ca^{2+}, Mn^{2+}, Ba^{2+}, and Sr^{2+} can also inhibit the reaction, as seen in Figure 4. The most potent inhibitor of those tested was Mn^{2+}. The specific energy-linked accumulation of Ca^{2+} by mitochondria is well known (for review, see Lehninger, 1970). It is noteworthy, therefore, that Ca^{2+} in the 1–5 mM range inhibited non-energy-linked transhydrogenation to about the same extent as Mg^{2+}.

Since these divalent cations are capable of binding to and inhibiting transhydrogenase, the influence of the ligands on the thermal and proteolytic inactivation of the enzyme was

TABLE IV: Thermal and Proteolytic Inactivation of Transhydrogenase in the Presence of Divalent Cations.[a]

Preincubation Additions (1 mM)	Thermal			Proteolytic		
	Rate (nmol AcPyADH min^{-1} mg^{-1}) 22 °C	50 °C	% Control Act.	Rate (nmol of AcPyADH min^{-1} mg^{-1}) −Trypsin	+Trypsin	% Control Act.
None	42.3	18.7	44.1	40.8	23.7	58.0
$MgCl_2$	39.8	25.5	64.1	38.4	10.6	27.7
$CaCl_2$	40.4	26.7	66.2	41.7	12.3	29.4
$MnCl_2$	41.1	32.9	80.0	37.6	8.2	21.7
$BaCl_2$	39.2	24.9	63.5	41.7	14.7	35.5
$SrCl_2$	41.7	27.4	65.7	41.7	15.5	37.3

[a] Submitochondrial particles (0.72 mg of protein) were preincubated at either 22 or 50 °C for 1 min (thermal) or were preincubated with 10 μg of trypsin for 1 min (proteolytic). After quenching in ice (thermal) or adding excess trypsin inhibitor (proteolytic), the particles were assayed for non-energy-linked transhydrogenase activity.

investigated. The thermostability of transhydrogenase was enhanced in the presence of Mg^{2+}, as shown in Figure 5. Half-maximal protection against thermal inactivation was afforded at a Mg^{2+} concentration of about 0.8 mM. In addition, the presence of Mg^{2+} stimulated the proteolytic inactivation of transhydrogenase (Figure 5), with half-maximal stimulation at about 0.5 mM. In control experiments, Mg^{2+} did not enhance the hydrolysis rate of the synthetic trypsin substrate, *p*-toluenesulfonyl-L-arginine methyl ester (Hummel, 1959). The difference in the apparent Mg^{2+} dissociation constants obtained in the thermal and proteolytic experiments was not unexpected since anomalously high values are often obtained in the thermal inactivation studies (Citri, 1973). An explanation for this behavior is that the apparent dissociation constant may represent the interaction of the ligand with a thermally induced transition conformation of the enzyme (Citri, 1973). These values compare with a K_I for Mg^{2+} of 1.7 mM (not shown). The effect of Mg^{2+}, then, on the thermal and tryptic inactivation of the enzyme is qualitatively identical with that of NADPH. Other divalent cations which were shown to inhibit transhydrogenase were tested similarly, and the results are presented in Table IV. In general, all the divalent cations exhibited behavior similar to that of Mg^{2+}. It is interesting that Mn^{2+}, the most potent inhibitor of the group, showed the largest degree of enhancement of thermostability and stimulation of proteolysis. In the 1–5 mM range, KCl did not influence enzyme thermostability or proteolytic inactivation. Therefore, the effects of the divalent cations are concluded not to be manifestations of the general ionic environment of the transhydrogenase.

Discussion

In this study, differential thermal and proteolytic inactivation were used as probes for conformational changes in membrane-bound rat liver mitochondrial transhydrogenase. At least three different conformers of the enzyme were detected. The unliganded enzyme, the $NADP^+$–enzyme binary complex, and the NADPH–enzyme binary complex exhibited different susceptibilities to thermal and tryptic inactivation. Neither NAD^+ nor NADH influenced the inactivation of the enzyme, suggesting either that these substrates are not bound in binary complexes with transhydrogenase or, if they are bound, they do not induce conformational changes measurable by the techniques employed.

In addition to NADP-dependent conformational changes, other nucleotides exerted an influence on the thermal and proteolytic inactivation of transhydrogenase. The most striking of these was palmitoyl-CoA, which was shown to be competitive with NADPH for the NADP-binding site in concurrence with an earlier conclusion of Rydström et al. (1971a) based on kinetic data. The effect of palmitoyl-CoA closely resembles that of $NADP^+$ in that both stimulate thermal inactivation and have only a slight effect on proteolytic inactivation of transhydrogenase. It is not possible to determine from these studies whether the conformational change induced by palmitoyl-CoA is identical with that induced by $NADP^+$. Interestingly, acetyl-CoA, which is also specific for the NADPH binding site but with a much higher K_I than palmitoyl-CoA (Rydström, 1972), stabilized the enzyme to thermal inactivation. Rydström has postulated that the NADP-binding site is situated in a more hydrophobic environment than the NAD-binding site due to the much higher affinity of the former for long-chain fatty acyl-CoA derivatives. It is evident from the data presented here that the degree of hydrophobicity of the inhibitor binding to the NADP site profoundly affects the thermostability, and thus the conformation of the enzyme.

A number of divalent cations were shown to be inhibitors of non-energy-linked transhydrogenation. Divalent cations which act as activators or inhibitors have been shown, by a variety of techniques, to cause conformational changes in enzymes, including pyruvate kinase (Kayne and Suelter, 1965), glutamine synthetase (Shapiro and Ginsburg, 1968), and enolase (Hanlon and Westhead, 1969). In the present study, divalent cation binding to transhydrogenase promoted proteolytic inactivation of the enzyme, the order of effectiveness being: $Mn^{2+} > Ca^{2+} \approx Mg^{2+} > Ba^{2+} \approx Sr^{2+}$. This labilization to proteolysis may be interpreted to indicate a cation-induced structural change to a more thermostable conformation. The effects induced by divalent cations on the conformation of transhydrogenase cannot be attributed to the ionic strength of the medium. No change in the thermal or proteolytic inactivation of the enzyme was observed in the presence of monovalent salt concentrations of equal ionic strength to that of preincubation mixtures containing divalent cations which strongly influenced the degree of inactivation of transhydrogenase.

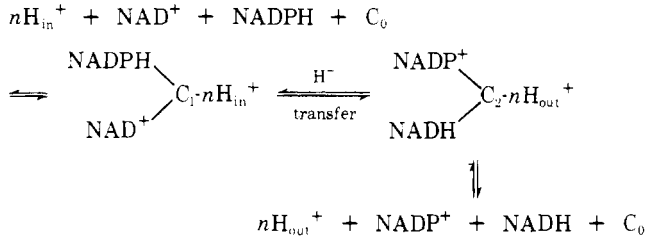
Compared with the non-energy-linked transhydrogenase, the energy-linked reaction is inhibited to a far lesser extent by Mg^{2+} (Hommes, 1963). This indicated to us the possibility that membrane energization either alters the transhydrogenase to a conformation which does not bind Mg^{2+} or prevents a Mg^{2+}-dependent inhibitory structure change. In the former

case, membrane energization would be predicted to reverse Mg^{2+}-dependent thermostability or labilization to tryptic inactivation. No such reversal of these properties was observed when submitochondrial particles were preincubated with Mg^{2+} in the presence of succinate. It would, therefore, appear that energization does not affect the association of Mg^{2+} with transhydrogenase.

Substrate-induced conformers of transhydrogenase are likely to play an important role in the function of the enzyme. For example, conformational changes in transhydrogenase are fundamental to a number of models for its energy coupling to the oxidative phosphorylation energy coupling system (Rydström et al., 1970, 1971b; Skulachev, 1974). A direct coupling of proton translocation across the inner membrane as proposed by Mitchell (1966, 1972) has been suggested by Skulachev (1974) to be effected by a reorientation in the membrane, either by rotation or perhaps by a conformational change, of a transhydrogenase proton translocation subunit controlled by a catalytic subunit. On the other hand, Rydström et al. (1970) have proposed that transhydrogenase undergoes a conformational charge from a nonenergized inactive conformer to an energized activated conformer, which may be induced by membrane energization, by a proper substrate ratio, or by acidification of the medium (Rydström, 1974).

No direct evidence has been presented previously by these workers in support of conformational changes of transhydrogenase being induced by substrate binding. It has been reported, however, that membrane energization alters the affinity of transhydrogenase for its substrates (Rydström et al., 1971b).

A working hypothesis for energy coupling to transhydrogenation can be based on the finding that transhydrogenase can exist in three conformers. The following simplistic equation illustrates one possible coupling mechanism.



The native conformation (C_0) can be transformed to C_1 by binding NADPH or to C_2 by binding $NADP^+$. In this formulation no distinction is made as to whether NAD(H) or NADP(H) binds first to the enzyme (Rydström, 1972), only that the conformation of the enzyme is directed by the binding of NADPH or $NADP^+$. Thus, the conformation of the enzyme would change concomitantly with hydride ion transfer. If transhydrogenase is linked to proton translocation as suggested by Mitchell (1966) and Skulachev (1974), this might occur if it functions directly as a proton pump with C_1 having a protonation site on the inner side of the inner membrane and C_2 having the protonation site at the outer surface, or indirectly by the transduction of a substrate-induced conformational change in the enzyme to a contiguous proton pumping protein. The direction and extent of transhydrogenation would, therefore, be determined by the ratio of substrates and products as well as the pH (or electrochemical) gradient across the inner mitochondrial membrane.

More detailed information concerning transhydrogenase conformers and the energy coupling mechanism awaits the reconstitution of homogeneous enzyme in synthetic membrane systems.

References

Blazyk, J. F., and Fisher, R. R. (1975), *FEBS Lett. 50,* 227.

Citri, N. (1973), *Adv. Enzymol. 37,* 397.

Dontsov, E. A., Grinius, L. L., Jasaitis, A. A., Severina, I. I., and Skulachev, V. P. (1972), *J. Bioenerg. 3,* 277.

Ernster, L., and Lee, C.-P. (1967), *Methods Enzymol. 10,* 738.

Green, D. E., and Ji, S. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 726.

Hanlon, D. P., and Westhead, E. W. (1969), *Biochemistry 8,* 4247.

Hoek, J. B., Rydström, J., and Ernster, L. (1973), *Biochim. Biophys. Acta 305,* 669.

Hommes, F. A. (1963), in Energy-Linked Functions of Mitochondria, Chance, B., Ed., New York, N.Y., Academic Press, p 39.

Hummel, B. C. W. (1959), *Can. J. Biochem. Physiol. 37,* 1393.

Jacobs, E., Jacob, M., Sanadi, D. R., and Bradley, L. B. (1956), *J. Biol. Chem. 223,* 147.

Juntti, K., Torndal, U. B., and Ernster, L. (1970), in Electron Transport and Energy Conservation, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Bari, Italy, Adriatica Editrice, p 257.

Kaplan, N. O. (1972), *Harvey Lect. 66,* 105.

Kaplan, N. O., Colowick, S. P., and Neufeld, E. F. (1953), *J. Biol. Chem. 205,* 1.

Kawasaki, T., Satoh, K., and Kaplan, N. O. (1964), *Biochem. Biophys. Res. Commun. 17,* 648.

Kayne, F. J., and Suelter, C. H. (1965), *J. Am. Chem. Soc. 87,* 897.

Lee, C.-P., and Ernster, L. (1964), *Biochim. Biophys. Acta 81,* 187.

Lee, C.-P., and Ernster, L. (1966), *Regul. Metab. Mitochondria, Proc. Symp. 1965 7,* 218.

Lee, C.-P., Simard-Duguesne, N., Ernster, L., and Hoberman, H. D. (1965), *Biochim. Biophys. Acta 105,* 397.

Lehninger, A. (1970), *Biochem. J. 119,* 129.

Mitchell, P. (1966), *Biol. Rev. Cambridge Philos. Soc. 41,* 445.

Mitchell, P. (1972), *Bioenergetics 3,* 5.

Mitchell, P., and Moyle, J. (1965), *Nature (London) 208,* 1205.

Rydström, J. (1972), *Eur. J. Biochem. 31,* 496.

Rydström, J. (1974), *Eur. J. Biochem. 45,* 67.

Rydström, J., Panov, A., Paradies, G., and Ernster, L. (1971a), *Biochem. Biophys. Res. Commun. 45,* 1389.

Rydström, J., Teixeira da Cruz, A., and Ernster, L. (1970), *Eur. J. Biochem. 17,* 56.

Rydström, J., Teixeira da Cruz, A., and Ernster, L. (1971b), *Eur. J. Biochem. 23,* 212.

Schnaitman, C., and Greenawalt, J. W. (1968), *J. Cell Biol. 38,* 158.

Shapiro, B. M., and Ginsburg, A. (1968), *Biochemistry 7,* 2153.

Skulachev, V. P. (1970), *FEBS Lett. 11,* 301.

Skulachev, V. P. (1974), *Ann. N.Y. Acad. Sci. 227,* 188.

Stein, A. M., Kaplan, N. O., and Ciotti, M. M. (1959), *J. Biol. Chem. 234,* 979.

Van Dam, K., and Ter Welle, H. F. (1966), *Regul. Metab. Processes Mitochondria, Proc. Symp. 1965 7,* 235.

Van de Stadt, R. S., Niewenhuis, F. J. R. M., and Van Dam, K. (1971), *Biochim. Biophys. Acta 234,* 173.