EVIDENCE FOR SUBSTRATE-INDUCED CONFORMATIONAL CHANGES IN MITOCHONDRIAL TRANSHYDROGENASE

John F. BLAZYK and Ronald R. FISHER*
Department of Chemistry, University of South Carolina, Columbia, S.C., 29208 U.S.A.

Received 6 December 1974

1. Introduction

Intramitochondrial pyridine dinucleotide transhydrogenase catalyzes the reversible transfer of a hydride ion equivalent between oxidized and reduced NAD and NADP, eq. 1 [1].

$$NADH + NADP^+ \rightleftarrows NAD^+ + NADPH \qquad \text{(eq. 1)}$$

The rate of this nonenergy-linked reaction is enhanced by several fold in the forward direction and the apparent equilibrium constant increases from 1 to values near 500 when the membrane oxidative phosphorylation system is energized by electron transport or ATP hydrolysis [2]. Three principal mechanisms have been proposed to explain the mode of energy input to the transhydrogenase reaction. (a) The chemical mechanism suggests that one of the transhydrogenase substrates reacts with a nonphosphorylated high-energy intermediate of oxidative phosphorylation to form a high-energy pyridine dinucleotide intermediate. Concomitant with hydride ion transfer, the energized substrate is cleaved exothermally to the corresponding product [3]. (b) The conformational mechanism postulates that energization of the oxidative phosphorylation system results in a conversion of an inactive form of the transhydrogenase (TH) to an active form (TH*) [4,5]. Kinetic data indicate that the relative level of active to inactive conformers may also be determined by the prevailing [NADH] [$NADP^+$]/[NAD^+] [NADPH] ratio, according to eq. 2

$$NADH + NADP^+ + TH^* \rightleftarrows NAD^+ + NADPH + TH \qquad \text{(eq. 2)}$$

(c) The chemiosmotic hypothesis contends that the transhydrogenase functions as a reversible H^+ pump [6,7]. The formation of a pH gradient during membrane energization would allow the exergonic coupling of H^+ translocation to transhydrogenation according to eq. 3 (for submitochondrial particles).

$$NADH + NADP^+ + nH^+_{in} \rightleftarrows NAD^+ + NADPH + nH^+_{out} \qquad \text{(eq. 3)}$$

The stoichiometry of H^+ translocation to hydride ion transfer has been reported to be 2 [8]. It has been proposed that H^+ translocation may be effected by a conformational change in the transhydrogenase [7].

Thus, two of the three mechanisms proposed above may involve a conformational change in the enzyme. In the present study, we have employed thermal and proteolytic inactivation to investigate the possibility of substrate-induced changes in the structure of membrane-bound transhydrogenase. The data reveal that NADPH stabilizes, while $NADP^+$ labilizes, the rat liver submitochondrial particle enzyme to thermal inactivation. NAD^+ and NADH do not significantly influence transhydrogenase stability. In addition, proteolytic inactivation of the enzyme by trypsin is stimulated in the presence of NADPH, but not with $NADP^+$. It is concluded that binding of either $NADP^+$ or NADPH may promote a conformational change in the enzyme, although the conformers obtained are different.

* To whom correspondence and reprint requests should be addressed.

2. Materials and methods

Rat liver mitochondria were prepared according to the method of Schnaitman and Greenawalt [9]. To prepare submitochondrial particles, mitochondria from two livers were suspended in 10 ml of ice-cold 0.25 M sucrose in a 25 ml glass beaker, immersed in ice and sonicated for two 15 sec bursts with a Branson W185 Ultrasonic Sonifier equipped with a W185 probe and operated at full power output. The sonicate was centrifuged at 26 400 *g* for 7 min. The supernatant suspension was decanted and centrifuged at 164 900 *g* for 30 min to sediment the submitochondrial particles which were then diluted with 0.25 M sucrose to a concentration of 20–30 mg protein/ml and stored at −60°C. Protein determinations were performed by the Biuret method [10], using crystalline bovine serum albumin as a standard.

Submitochondrial particles were heat-treated in 15 mm × 125 mm Pyrex test tubes in a preincubation medium (0.31 ml) consisting of 16 mM Tris–HCl buffer, pH 7.5, plus the additions indicated. Preincubation was terminated by quenching the suspensions in ice and the particles were then assayed for nonenergy-linked transhydrogenase activity.

Proteolytic inactivation was performed by preincubating submitochondrial particles (0.5 mg protein) in a medium (0.31 ml) consisting of 16 mM Tris–HCl buffer, pH 7.5, and 5 μg trypsin, plus the additions indicated. A 10-fold excess of trypsin inhibitor (0.1 ml) was added at the end of the preincubation, and the tubes were placed in an ice bath until assayed for nonenergy-linked transhydrogenase activity.

Nonenergy-linked transhydrogenase activity was assayed by the method of Stein et al. [11], in which the 3-acetyl pyridine analog of NAD^+ ($AcPyAD^+$) is reduced by NADPH. This allows the reaction to be continuously monitored in the absence of NADPH or NAD^+ regenerating systems. A value of 5.1 at 375 nm was assumed for the millimolar extinction coefficient of reduced 3-acetyl pyridine adenine dinucleotide. The assay mixture (3.0 ml) contained 0.19 mM $AcPyAD^+$, 0.15 mM NADPH, and 0.5 μM rotenone in 80 mM phosphate buffer, pH 7.0.

NAD^+, NADH, $NADP^+$ and NADPH were obtained from P-L Biochemicals. Rotenone, sodium succinate, 3-acetyl pyridine adenine dinucleotide, bovine serum albumin, trypsin (bovine pancreas, type I), trypsin inhibitor (soybean, type I-S), and carbonyl cyanide *m*-chlorophenyl hydrazone were products of Sigma Chemical Co. All other chemicals were reagent grade of commercial origin.

3. Results

The susceptibility of rat liver submitochondrial transhydrogenase to heat inactivation is shown in fig.1. The presence of 0.2 mM NADPH or 0.2 mM $NADP^+$ in the preincubation medium had a profound effect on the degree of thermostability of the enzyme. The enzyme was stabilized considerably against heat inactivation in the presence of NADPH, while $NADP^+$ tended to potentiate inactivation. Half-maximal activity was observed at 57°C in the presence of NADPH, at 46°C with $NADP^+$ and at 49°C for control preincubations.

Thermostability curves determined for preincubations with each of the four transhydrogenase sub-

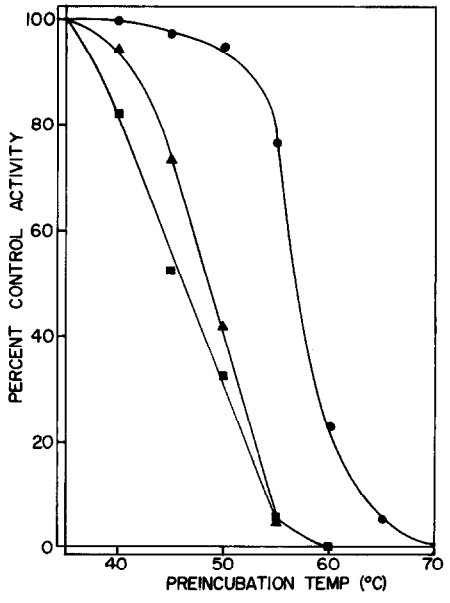

Fig.1. The effect of preincubation temperature on the activity of transhydrogenase. Submitochondrial particles (0.5 mg protein) were preincubated for 1 min with no additions (▲), 0.2 mM NADPH (●), or 0.2 mM $NADP^+$ (■) in the particle suspension.

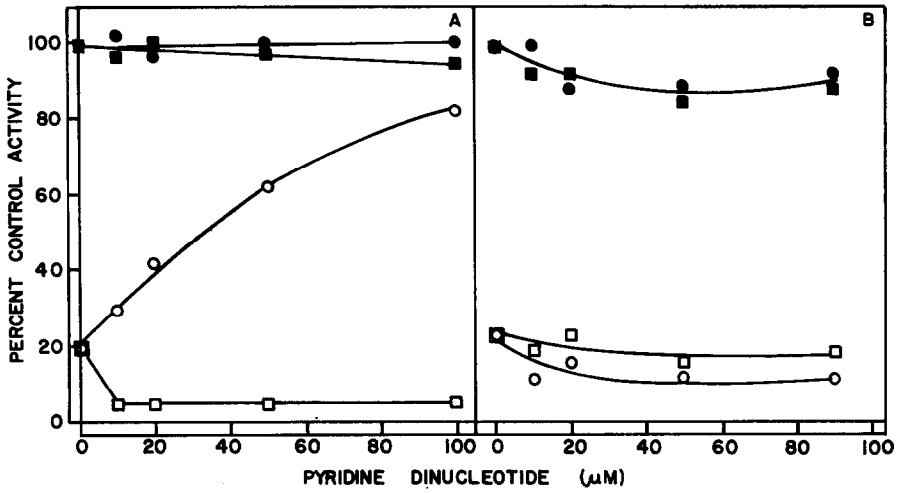

Fig.2. Transhydrogenase activity as a function of pyridine dinucleotide concentration. Submitochondrial particles (0.5 mg protein) were preincubated for 3 min at either 22°C or 50°C. (A) NADPH at 22°C (●) and at 50°C (○), and $NADP^+$ at 22°C (■) and at 50°C (□). (B) NADH at 22°C (●) and at 50°C (○), and NAD^+ at 22°C (■) and at 50°C (□).

strates at concentrations between 0 and 100 μM are shown in fig.2. Nonenergy-linked transhydrogenase activity was assayed after preincubation for 3 min. at 22°C or at 50°C. The degree of protection from inactivation during preincubation at 50°C increased with increasing NADPH concentration, with about 80% activity remaining after preincubation with 100 μM NADPH. Complete protection was afforded at higher NADPH concentrations. On the other hand, $NADP^+$ destabilized the enzyme to heat treatment at 50°C. NAD^+ and NADH caused a slight inactivation of the transhydrogenase at 22°C. Neither NAD^+ nor NADH protected the enzyme against inactivation at 50°C.

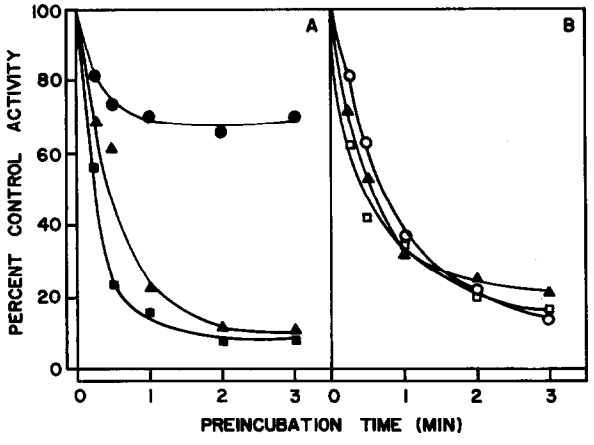

Fig.3. The effect of preincubation time on transhydrogenase inactivation at 50°C. Additions to the preincubation mixture containing submitochondrial particles (0.5 mg protein) were: (A) none (▲), 0.1 mM NADPH (●), or 0.1 mM $NADP^+$ (■), and (B) none (▲), 0.1 mM NADH (○), or 0.1 mM NAD^+ (□).

To determine the relative rates of inactivation of the enzyme in the presence of the four pyridine dinucleotide substrates, the preincubation time at 50°C was varied. Fig.3A illustrates that in the presence of 100 μM NADPH, a rapid initial phase of inactivation is observed, followed by stabilization at approx. 70% of the control activity. It is likely that the initial rapid inactivation corresponds to the fraction of the transhydrogenase not binding NADPH, since higher NADPH concentrations completely protect against inactivation. $NADP^+$, however, increased the inactivation rate rather markedly over the first 30 sec of preincubation. NADH and NAD^+ exerted no significant influence on thermal inactivation when compared to the control which lacked substrate in the preincubation medium (fig.3B).

Ernster and coworkers have demonstrated that bovine heart transhydrogenase is particularly susceptible to proteolytic inactivation [12]. Differential proteolytic inactivation of enzymes in the presence

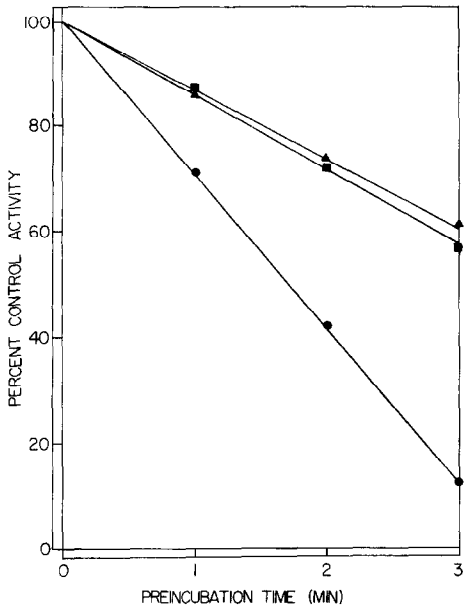

Fig.4. The effect of preincubation time on transhydrogenase inactivation by trypsin. Additions to the preincubation mixture containing submitochondrial particles (0.5 mg protein) were: none (▲), 0.25 mM $NADP^+$ (■), or 0.25 mM NADPH (●).

and absence of ligands has often been used to detect conformational transitions [13,14]. Fig.4 illustrates an experiment in which submitochondrial particles were preincubated with trypsin in the presence and absence of $NADP^+$ or NADPH. NADPH significantly promoted proteolytic inactivation of the transhydrogenase, while $NADP^+$ had little effect on the rate of inactivation as compared to the control.

An attempt was made to relate membrane energization to a transhydrogenase conformational change. The data presented in Table 1 shows that the transhydrogenase thermostability of respiring submitochondrial particles is slightly enhanced over that of nonrespiring particles. De-energization of the respiring particles by addition of the oxidative phosphorylation uncoupler, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), reversed the protection afforded by succinate alone.

4. Discussion

Both the conformational mechanism [4,5] and the Skulachev [7] version of the chemiosmotic mechanism for energy-linked transhydrogenation between NADH and $NADP^+$ invoke a reversible transition between two conformers of the enzyme. Techniques for exploring conformational changes in specific membrane-bound enzymes are limited. In the present study, we have employed thermostability and proteolytic inactivation of the transhydrogenase to probe for possible structural alterations in the enzyme occurring upon substrate binding or membrane energization.

The binding of NADPH enhances thermostability, while $NADP^+$ labilizes the enzyme to thermal inactivation. NADH and NAD^+, on the other hand, had little influence on the thermal inactivation profile of the transhydrogenase. These data suggest that the binding of NADPH promotes a conformational change in the native enzyme to a thermostable conformer, and that the binding of $NADP^+$ induces the formation of a thermolabile conformer. Consistent with this notion is the observation that NADPH, but not $NADP^+$, promotes proteolytic inactivation of the enzyme by trypsin.

Ligand-dependent stabilization and labilization to thermal and proteolytic inactivation of enzymes have frequently been used as evidence for conformational changes [13]. Stabilization to heat inactivation can alternatively be explained by the ligand either protecting heat-labile groups on the enzyme or helping to maintain a native conformation. On the other hand, ligand-induced labilization to thermal inactivation more strongly supports the occurrence of a change in enzyme conformation. Independent methods, such as circular dichroism [15], optical rotatory dispersion [16], fluorescence [17], proton exchange [18], and X-ray diffraction [19] have been used to confirm the presence of conformative transitions in enzymes which exhibit ligand-induced thermostability. Moreover, the stimulation of proteolytic inactivation of enzymes by binding of ligands offers direct evidence for a change in enzyme structure. It is apparent in this case that NADPH binding to the transhydrogenase causes a conformational rearrangement where at least one arginyl or lysyl residue becomes more accessible to trypsin.

From kinetic studies on the bovine heart submitochondrial particle enzyme, Rydström et al. [5] concluded that the transhydrogenase conformation

Table 1
The effect of membrane energization on the thermostability of transhydrogenase

Additions to preincubation mixture	Rate (nmoles AcPyADH/mg protein · min)		Percent of Control Activity
	22°C	50°C	
none	29.4	4.0	13.7
NADPH (0.2 mM)	31.5	25.7	81.7
succinate (3 mM)	30.6	7.5	24.5
CCCP (5 μM)	29.4	4.0	13.7
Succinate (3 mM) + CCCP (5 μM)	28.8	4.6	16.0

Submitochondrial particles (0.5 mg protein) were preincubated for 3 min at 22°C or 50°C, then CN^- was added to a final concentration of 1 mM. Percent control activity indicates the relative rates of the transhydrogenase reaction of particles preincubated at 50°C to that of particles preincubated at 22°C.

was converted from an inactive to an active form, either by energization of the oxidative phosphorylation system or by binding of NADPH and NAD^+ to the enzyme. It was further proposed that the extent of transhydrogenase activation is related to the [NADH] [$NADP^+$] / [NAD^+] [NADPH] ratio. Later studies were interpreted to indicate that the kinetic mechanism for both the energy-linked and nonenergy-linked transhydrogenase involves an ordered binding of substrates where NADH (or NAD^+) is bound obligatorily before $NADP^+$ (or NADPH) is bound, to form a Chance–Theorell type ternary complex intermediate [20].

Clearly, the present studies show that NADPH and $NADP^+$ are bound, at least to the liver transhydrogenase, in the absence of NAD^+ and NADH, respectively. This indicates that the conformation of the enzyme may be under the control of the [$NADP^+$] / [NADPH] ratio, rather than that ratio incorporating all four substrates. Table 1 illustrates that some protection against heat inactivation is conferred upon membrane energization by succinate-dependent respiration. However, the optimal protection afforded by energization is much less than that observed in the presence of NADPH. These thermostability and proteolytic inactivation studies strongly suggest that the binding of NADPH or $NADP^+$ results in the formation of different transhydrogenase conformers which are in turn different than those of the de-energized, as well as the energized membrane.

Acknowledgements

The authors wish to thank Ms. Tina Poucher for her technical assistance. This work was supported in part by the American Cancer Society grant BC-135 and by a grant from the Research Corporation.

References

[1] Kaplan, N. O., Colowick, S. P. and Neufeld, E. F. (1953) J. Biol. Chem. 205, 1–15.
[2] Lee, C.-P. and Ernster, L. (1964) Biochim. Biophys. Acta 81, 187–190.
[3] Lee, C.-P. and Ernster, L. (1966) in: Regulation of Metabolic Processes in Mitochondria, B.B.A. Library, Vol. 7, pp. 218–234, Elsevier, Amsterdam.
[4] Rydström, J., Teixeira da Cruz, A. and Ernster, L. (1970) Eur. J. Biochem. 17, 56–72.
[5] Rydström, J., Teixeira da Cruz, A. and Ernster, L. (1971) Eur. J. Biochem. 23, 212–219.
[6] Mitchell, P. (1974) Bioenergetics 3, 5–24.
[7] Skulachev, V. P. (1974) in: 'Annals of the New York Academy of Sciences', Vol. 227, pp. 188–202, The New York Academy of Sciences, New York.
[8] Moyle, J. and Mitchell, P. (1973) Biochem. J. 132, 571–585.
[9] Schnaitman, C. and Greenawalt, J. W. (1968) J. Cell Biol. 38, 158–175.
[10] Jacobs, E., Jacob, M., Sanadi, D. R. and Bradley, L. B. (1956) J. Biol. Chem. 223, 147–156.
[11] Stein, A. M., Kaplan, N. O. and Ciotti, M. M. (1959) J. Biol. Chem. 234, 979–986.

[12] Juntti, K., Torndal, U. B. and Ernster, L. (1970) in: Electron Transport and Energy Conservation (J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater, eds.) pp. 257–271, Adriatica Editrice, Bari.
[13] Citri, N. (1973) in: Advances in Enzymology, (pp. 397–648 (Meister, A., ed.), Vol. 37, Wiley, New York.
[14] Adelman, R. C., Morse, D. E., Chan, W. and Horecker, B. L. (1968) Arch. Biochem. Biophys. 126, 343–352.
[15] DeVijlder, J. J. and Harmsen, B. J. (1969) Biochim. Biophys. Acta 178, 434–440.
[16] Ulmer, D. D., Li, T.-K. and Vallee, B. L. (1961) Proc. Natl. Acad. Sci. U.S. 47, 1155–1165.
[17] Telegdi, M. and Keleti, T. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. 3, 131–140.
[18] Kirtley, M. E. and Koshland, D. E. (1970) J. Biol. Chem. 245, 276–286.
[19] Havsteen, B. H. (1965) Acta Chem. Scand. 19, 1643–1651.
[20] Rydström, J. (1972) Eur. J. Biochem. 31, 496–504.