Energy-linked Mitochondrial Transhydrogenation from NADPH to NADP Analogs*

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The mitochondrial energy-linked transhydrogenase enzyme catalyzes hydride ion transfer between NAD and NADP, of which the reaction NADH → NADP is slow in the absence of energy and is accelerated 10-fold or more when the mitochondrial membrane is energized by ATP hydrolysis or respiration. The enzyme is a proton pump and effects proton translocation coupled to hydride ion transfer from NADPH to NAD (Earle, S. R., and Fisher, R. R. (1980) J. Biol. Chem. 255, 827–830). The present studies have shown that submitochondrial particles also catalyze transhydrogenation from NADPH to two NADP analogs, namely 3-acetylpyridine adenine dinucleotide phosphate (AcPyADP) and thionicotinamide adenine dinucleotide phosphate (thioNADP). Both reaction rates are greatly accelerated when the system is energized by ATP hydrolysis (inhibitable by uncouplers or rutamycin) or succinate oxidation (inhibitable by uncouplers or antimycin A). As in the case of NADH → NADP(H) reactions, the transhydrogenations from NADPH to AcPyADP and thioNADP are inhibited by treatment of submitochondrial particles with trypsin or the arginyl residue modifier, butanedione. The $K_m$ values of the above substrates and the $V_{max}$ values under energy-linked conditions have been determined.

The finding that the mitochondrial energy-linked transhydrogenase enzyme catalyzes transhydrogenation from NADPH to NADP analogs has revealed features regarding substrate site specificities and the effect of substrates on the directionality of proton translocation by the enzyme.

*This work was supported by United States Public Health Service Grants GM 24887 and AM 06126 to Y. H. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by United States Public Health Service Training Grant AM 07097.

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§ The abbreviations used are: AcPyAD(P), 3-acetylpyridine adenine dinucleotide phosphate; thioNAD(P), thionicotinamide adenine dinucleotide phosphate; PyrAlaAD, pyridine aldehyde adenine dinucleotide; SMP, phosphorylating submitochondrial particles; CCCP, carbonyl cyanide m-chlorophenylhydrazone; S-13, 5-chloro-3-t-butyl-2′-chloro-4′-nitrosalicylanilide.

NADH to NADP is greatly accelerated when the enzyme system (submitochondrial particles) is energized in the presence of ATP or an oxidizable substrate, while others showed that the reduction of NAD by NADPH was rapid and resulted in membrane energization (for reviews, see Refs. 2–4). Recent studies with the purified enzyme incorporated into liposomes have shown that transhydrogenase is a proton pump, NADPH → NAD transhydrogenation is coupled to proton translocation, and that the reverse reaction is driven by the membrane electrochemical potential of protons (5–7).

Previous studies, briefly reported elsewhere (8), had shown that submitochondrial particles catalyzed NADH → AcPyADP transhydrogenation, and that this reaction appeared to be energy-linked. The present manuscript documents in greater detail energy-driven transhydrogenation from NADPH to two NADP analogs (i.e. AcPyADP and thioNADP), as catalyzed by submitochondrial particles, and provides data indicating that these reactions are also catalyzed by the mitochondrial energy-linked transhydrogenase enzyme discussed above. The results have led to mechanistic clarifications regarding binding site specificities and substrate effects on the directionality of proton translocation by the transhydrogenase enzyme. Other important results obtained from study of energy-linked and non-energy-linked transhydrogenation from NADPH to AcPyADP and thioNADP are presented in the accompanying communication (1).

MATERIALS AND METHODS

SMP was prepared from beef heart essentially according to Low and Vallin (9) as described (1), and protein concentration was measured by the biuret method (10) in the presence of 1 mg of potassium deoxycholate/ml. Spectrophotometric studies were carried out with the Aminco DW-2a, the Aminco-Chance dual wavelength, and Cary 118 spectrophotometers.

Nicotinamide nucleotides were obtained from P-L-Biochemicals; ATP was from Boehringer; sodium succinate, trypsin inhibitor, and alcohol dehydrogenase were from Sigma; trypsin (grade B) was from CalBiochem; butanedione was from Aldrich Chemical Co.; rotenone was from S. B. Penick & Co.; and rutamycin was a gift from Eli Lilly. Other chemicals used were reagent grade.

RESULTS

Fig. 1 shows the reduction of AcPyADP by NADPH as catalyzed by SMP and energized by ATP hydrolysis (left-hand trace) or succinate oxidation (right-hand trace). The figure also shows inhibition of the ATP-driven transhydrogenation by rutamycin or CCCP, and the inhibition of the respiration-driven transhydrogenation by antimycin A or CCCP. Similar data were obtained for transhydrogenation from NADPH to thioNADP. Fig. 2, A and B, show slope and ordinate intercept replots of double reciprocal Lineweaver-Burk plots against the reciprocal concentrations of the fixed substrate (NADPH, Fig. 2A; AcPyADP, Fig. 2B), each at four concentrations of the variable substrate. From these
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Fig. 1. Transhydrogenation from NADPH to AcPyADP driven by ATP hydrolysis (left-hand trace) or succinate oxidation (right-hand trace). The reaction mixture (1 ml) at 37°C contained 0.25 M sucrose, 10 mM Tris/sulfate, pH 7.5, 10 μM rotenone, 0.5 mM NADPH, and 0.35 mM AcPyADP. The ATP-driven system also contained 5.0 mM MgSO4, while the succicuate-driven system contained 10 μg of rutamycin. Where indicated, 0.25 mg of SMP, 4.8 mM ATP, 10 mM succinate, 20 μg of rutamycin, 25 mM CCP, and/or 50 μM antimycin A were added.

Fig. 2. Slope (O) and ordinate intercept (O) replots against the reciprocal concentrations of fixed substrates (A, NADPH; B, AcPyADP) for determination of Km and Vmax values in the energy-linked transhydrogenation reaction NADPH → AcPyADP. The reaction mixtures contained, in 1 ml at 37°C, 0.25 M sucrose, 50 mM Tris/sulfate (pH 7.5), 0.25 mg of SMP, 0.33 to 2 mM NADPH in the presence of 2 mM AcPyADP, 0.33 to 2 mM AcPyADP in the presence of 2 mM NADPH, 25 μM rotenone, and 3.5 μg of rutamycin. The reactions were started by addition of 10 μl of 2 M sodium succinate, and monitored at 400 minus 450 nm. Specific activities were calculated as nmol/min/mg of protein using a difference absorbance (AcPyADPH minus NADPH) value at the above wavelengths of 2.3 mm⁻¹ cm⁻¹. Data were then plotted in double reciprocal (Lineweaver-Burk) form, and slopes and ordinate intercepts were replotted as shown above. The ordinates shown are in nmol/min/mg of protein for ordinate intercept values or the slope divided by the reciprocal variable substrate concentration in mM for the slope values.

The results, Km and Vmax values for the transhydrogenation reaction NADPH → AcPyADP at 37°C were calculated to be as follows: KmNADPH = 0.4 mM, KmAcPyADP = 1.0 mM, and Vmax = 37 nmol/min/mg of protein. ThioNADP was found to inhibit at high concentrations. Therefore, only apparent Km values for the NADPH → thioNADP transhydrogenation were calculated, as shown by the double reciprocal plots of Fig. 3. These values were KmNADPH = 1.4 mM, KmAcPyADP = 38 μM, and Vmax = 19 nmol/min/mg of protein. Different preparations of SMP differ somewhat in their energy-linked transhydro-

genase activity for the above reactions. Therefore, the Vmax values given above would be expected to change somewhat from one SMP preparation to another. Also, as will be seen in the accompanying communication (1), the rate of energy-linked transhydrogenation from NADPH to AcPyADP and thioNADP is highly pH-dependent. In the pH range 6.5 to 8.5, the rate is highest at pH 7.5 and diminishes considerably on both the acid and the alkaline sides of pH 7.5. However, at pH ≤ 6.0, the rate increases sharply and does not seem to require membrane energization. The accompanying communication (1) also shows that, in the transhydrogenation reactions, NADH → AcPyADP and NADH → thioNADP at pH 7.5, the Km values for both substrate pairs decrease severalfold in going from non-energy-linked to energy-linked conditions. We have checked this point for the reaction NADPH → thioNADP, and have found that the apparent KmNADPH under non-energy-linked conditions at pH 7.5 is about 0.5 mM, i.e., more than 10-fold greater than the Km of this substrate at pH 7.5 under energy-linked conditions. These results are in excellent agreement with the interpretations offered in the accompanying communication (1) regarding the mechanisms of energy-linked and non-energy-linked transhydrogenations.

In general, submitochondrial particles catalyze not only transhydrogenations from NADPH to NADP analogs as shown above, but also the following types of transhydrogenation reactions (Table I): NADH → NADP(H) as in Fig. 2. Two separate enzymes are responsible for these reactions. The energy-linked transhydrogenase enzyme has been known to catalyze NADH → NADP(H) transhydrogenation, of which the rate of NADP reduction is accelerated 10-fold or more by membrane energization. The other enzyme is NADH dehydrogenase, which catalyzes NADH → NAD transhydrogenation at a rapid rate, and NADP → NAD transhydrogenation at pH ≤ 6.0 and at a relatively slow rate. Our finding that submitochondrial particles catalyzed NADPH → AcPyADP and thioNADP transhydrogenations posed two important questions that needed to be clarified. These questions concern (a) the validity of the assays for NADPH → AcPyADP and thioNADP transhydrogenations, and (b) the nature of the enzymes which catalyzed these reactions.

Fig. 3. Double reciprocal plots of specific activity versus substrate concentration for the energy-linked transhydrogenase reaction NADPH → thioNADP. The reaction mixtures contained in 1 ml at 37°C the same concentrations of sucrose, buffer, rotenone, succinate, rutamycin, and SMP as in Fig. 2. The fixed concentrations of NADP and thioNADP were, respectively, 3.0 and 0.1 mM. The variable concentrations for NADP (O) and thioNADP (O) were as shown in the figure. Rates were monitored at 400 minus 450 nm, and specific activities were calculated in nmol/min/mg of protein, using an absorbance value for reduced thioNADP at 400 minus 450 nm of 11.5 mm⁻¹ cm⁻¹.
TABLE I

Effect of mitochondrial transhydrogenation reactions on membrane energization

| Reaction          | Specific activitya         |
|-------------------|---------------------------|
|                   | Nonenergized | Energized |
| NADH → AcPyADP    | 9.7          | 113       |
| NADH → thioNADP   | 18           | 177       |
| NADPH → AcPyAD    | 198          | 185       |
| NADPH → thioNAD   | 11.4         | 8.5       |
| NADPH → PyrAldAD  | 55           | 55        |
| NADH → AcPyAD     | 648          | 665       |
| NADH → thioNAD    | 361          | 332       |
| NADH → PyrAldAD   | 385          | 408       |

a Specific activity is expressed as nanomoles/min/mg of protein at 30°C. Reductions of AcPyAD(P), thioNAD(P), and PyrAldAD were measured as described respectively, at 37°C, 400 nm, and 365 nm. The absorbance values used [reduced analog minus NAD(P)H] at the above wavelengths for calculation of specific activities were 6.3 mm⁻¹ cm⁻¹ for AcPyAD(P), 11.9 mm⁻¹ cm⁻¹ for thioNAD(P), and 5.54 mm⁻¹ cm⁻¹ for PyrAldAD.

Validity of the Assay—Regarding (a), a serious complication would be the presence of NAD(H) either in the nucleotides used as substrate or bound to the added submitochondrial particles. The presence of NAD would result in the following transhydrogenation reactions, i.e. NADPH → NAD followed by the energy-requiring reaction NADH → NADP analogs. Both reactions are known to be catalyzed by the mitochondrial energy-linked transhydrogenase, and the sum would appear as though one is studying an energy-requiring transhydrogenation from NADPH to AcPyADP and thioNADP. That NADPH, NADP, and AcPyADP obtained from the source mentioned under “Materials and Methods” do not contain detectable amounts of NAD(H) was carefully tested and ascertained as in our previous studies (11). Both AcPyADP and thioNADP were also checked by thin layer chromatography and found not to contain detectable amounts of NAD. Submitochondrial particle preparations can contain, however, up to about 0.2 nmol of bound NAD/mg of protein (11, 12), and in the assays employed we could have added up to 0.11 μM NAD together with the particle preparations. This point and the possible presence of enzymically effective levels of NAD in the samples of AcPyADP and thioNADP were checked, therefore, under the assay conditions used. As seen in Fig. 4, left-handed trace, AcPyADP is reduced in a reaction mixture containing rotenone-treated SMC, succinate as an oxidizable substrate to provide energy, and NADPH as reductant. When succinate oxidation was interrupted by antimycin A, or an uncoupler was added, the reduction of AcPyADP was inhibited. The right-hand trace of Fig. 4 shows a similar experiment, except that the reductant NADPH was replaced by β-hydroxybutyrate, the substrate of the NAD-linked α-β-hydroxybutyrate dehydrogenase enzyme, which is bound to SMP. It is seen that there was no reduction of AcPyADP until NAD (2.67 μM) was also added. As seen in Fig. 4, this concentration of added NAD resulted in a rate of AcPyADP reduction close to that in the left-hand trace. Higher amounts of added NAD resulted in somewhat higher rates of AcPyADP reduction, and the rates at lower levels of added NAD are shown in Fig. 5. In this figure, the highest point shown represents the rate (ΔA/min) of the right-hand trace of Fig. 4, the lower rates are for smaller amounts of added NAD as shown, and the rate shown by the arrow at the lower left of the figure is at a concentration of added NAD equivalent to the amount of bound NAD that might have been added together with SMP (i.e. 0.2 nmol of bound NAD/mg of SMP) in this experiment. These results indicated, therefore, that any contamination by NAD in the assay system used would have had to be less than 0.13 μM (the lowest level of added NAD in Fig. 5), since the addition of this amount of

FIG. 4. Energy-linked reduction of AcPyADP by NADPH (left-hand trace) and dt-β-hydroxybutyrate plus NAD (right-hand trace). The assay mixture in a total volume of 3 ml at 37°C contained 0.25 M sucrose, 50 mM Tris/sulfate (pH 6.8), 83 mM MgSO4, 0.6 mM sodium succinate, 1.3 μg/ml of rutamycin, 5 μM rotenone, 0.33 mM AcPyADP, and 0.35 mg of SMP/ml. Where indicated, 0.9 mM NADPH, 16.7 mM dt-β-hydroxybutyrate, 2.67 μM NAD, and 30 μM antimycin A (or 25 μM CCCP) were added. The reduction of AcPyADP was monitored at 400 minus 450 nm.

FIG. 5 (left). Energy-linked reduction of AcPyADP by dt-β-hydroxybutyrate and increasing concentrations of added NAD as shown. The reaction conditions were the same as in Fig. 4.

FIG. 6 (right). Energy-linked reduction of thioNADP by SMP in the presence of alcohol dehydrogenase and ethanol added NAD. The reaction mixtures in 3 ml at 37°C contained 0.25 M sucrose, 50 mM potassium phosphate (pH 7.5), 5 μM rotenone, 8 μg of rutamycin, 20 mM sodium succinate, 0.9 mg of SMP, 60 μg of crystalline alcohol dehydrogenase, 140 mM ethanol, and 6 mM hydrazine. The reaction mixtures represented by Traces 1 to 5 contained the following: Trace 1, 0.5 mM thioNADP and 50 μM NAD; Trace 2, same as Trace 1 in the absence of succinate; Trace 3, same as Trace 1 in the absence of NAD or NADP and succinate; Trace 4, same as Trace 1 in the absence of thioNADP; Trace 5, the reaction mixture in the absence of NAD and thioNADP. The reaction mixtures were incubated for 15 min at 37°C, and vigorously shaken at frequent intervals to reaerate. They were then placed in a boiling water bath for 2 min, and centrifuged in a clinical centrifuge at top speed to sediment the denatured protein. The clear supernatants were withdrawn, diluted 6-fold with water, and their absorbance spectra were recorded in a Cary 116 spectrophotometer.
NAD elicited a measurable rate of AcPyADP reduction even though the $K_m$ for NAD in the $\beta$-hydroxybutyrate dehydrogenase reaction is about 1300 times higher (13). The next experiment (Fig. 6) was carried out with thioNADP in the presence of ethanol + alcohol dehydrogenase + added NAD as the source of reducing power. The amounts of ethanol and alcohol dehydrogenase were the same as those used by Teixeira da Cruz et al. (14) in their NADH → NAD transhydrogenation experiments, and hydrazine was also added as done by these authors to trap the acetalddehyde produced. Trace 1 of Fig. 6 is the absorbance of the deproteinized reaction mixture after 15 min of incubation showing the energy-linked reduction of thioNADP ($\lambda_{\text{max}}$ at 395 nm) in the presence of rotenone-treated SMP, succinate, ethanol, alcohol dehydrogenase, and NAD. Trace 2 is a parallel experiment in the absence of added succinate, which shows considerably less reduction of thioNADP at 395 nm due to non-energy-linked transhydrogenation from NADH. Trace 3 is for the complete system in the absence of added NAD ± succinate showing no detectable reduction of thioNADP. Trace 4 is the absorbance of the complete system in the absence of thioNADP showing the level of reduced NAD at 340 nm, and Trace 5 is the system in the absence of both NAD and thioNADP. These results show also that neither the particles nor thioNADP could have contained amounts of NAD large enough to effect appreciable reduction of thioNADP. Therefore, the data of Figs 4 to 6 allow the conclusion that sub mitochondrial particles do catalyze energy-linked NADPH → AcPyADP and thioNADPH transhydrogenation directly and without intervention by NAD.

The Nature of the Enzyme Catalyzing Transhydrogenation from NADPH to NADP Analogs—It was shown earlier that the mitochondrial energy-linked transhydrogenase contains an essential arginyl residue, highly susceptible to treatment of SMP with trypsin or the specific arginyl modifier, butanedione (15, see also Ref. 16). By contrast, electron transfer from NADH, NADPH, and succinate to oxygen were not so affected (15, 17). As seen in Table II, SMP-catalyzed transhydrogenations involving NADP(H) and analogs are all strongly inhibited by treatment of the particles with trypsin, whereas transhydrogenations involving only NAD(H) and analogs are unaffected by this treatment. This is because the energy-linked transhydrogenase enzyme does not catalyze NADH → NAD transhydrogenation, and NADH dehydrogenase, which catalyzes this reaction (Table II, Reactions 6 to 8), is not inhibited by treatment of SMP with trypsin or butanedione (see also Ref. 18). It might be added, also, that as far as is known these are the only two enzymes in SMP which catalyze nicotinamide nucleotide transhydrogenation.

Using trypsin- or butanedione-treated SMP, it was found that both energy-linked transhydrogenase reactions NADPH → AcPyADP and NADPH → thioNADPH were inhibited (Figs. 7 and 8), while addition of untreated SMP to the same reaction mixtures elicited normal transhydrogenation. In ad-

![Fig. 7. Energy-linked reduction of AcPyADP (left-hand trace) and thioNADP (right-hand trace) by NADPH in the presence of trypsin-treated and untreated SMP. The reaction mixtures, at a final volume of 3.0 ml, contained 0.25 mM succrose, 50 mM Tris/sulfate (pH 7.0), 8.3 mM sodium succinate, 8.3 mM MgSO4, 1.3 μg/ml of ruthenycin A, 5 μM rotenone, 0.83 mM NADPH, 0.83 mM AcPyADP (left-hand experiment), and 0.33 mM thioNADP (right-hand experiment). Where indicated, 0.74 mg of SMP, 0.8 mg of trypsin-treated SMP, 16.7 μM antimycin A, and 16.7 μM S-13 were added. Trypsin-treated SMP was prepared as described in the legend to Table II, and was assayed to show that its succinate oxidase activity was not inhibited.](image_url)

![Fig. 8. Inhibition by butanedione of energy-linked transhydrogenation from NADPH to thioNADP. SMP at 25 mg/ml was incubated in a medium containing 0.25 mM succrose, 50 mM sodium borate (pH 8.0), and 30 mM butanedione for 30 min at 30°C. Control SMP was treated similarly in the absence of butanedione. Assay conditions were the same as in Fig. 1 in the presence of 0.8 mM NADPH and 0.35 mM thioNADP. Each SMP addition was 5 μl (125 μg).](image_url)

TABLE II
Transhydrogenase activities of trypsin-treated sub mitochondrial particles

| Reaction                  | Specific activity |
|---------------------------|-------------------|
| NADH → AcPyADP           | Untreated SMP     |
|                           | Trypsin-treated SMP |
| NADH → thioNADP          | 109               |
|                           | 166               |
| NADPH → AcPyAD           | 218               |
|                           | 346               |
| NADPH → thioNAD          | 11.5              |
|                           | 3.4               |
| NADPH → PyrAldAD         | 82                |
|                           | 21                |
| NADH → AcPyAD            | 765               |
|                           | 854               |
| NADH → thioNAD           | 342               |
|                           | 351               |
| NADH → PyrAldAD          | 382               |
|                           | 345               |

* These reactions were energized by succinate (10 mM) oxidation. Trypsin-treated particles had essentially the same succinate oxidase activity as the untreated SMP.

2 As regards the effects of trypsin and butanedione on the ATP-driven reactions, the following points should be considered. Trypsin does not inhibit the ATPase activity of SMP (16) but butanedione does (19). The transhydrogenase enzyme is more sensitive to inhibition by butanedione than is ATPase (15, 19). Nevertheless, in Fig. 7, the inhibition of ATP-driven transhydrogenation in the presence of butanedione-treated SMP should be considered to be due to inhibition of both the transhydrogenase and the ATPase.
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condition, we have shown elsewhere (18) that NADH dehydrogenase purified from mitochondria does not catalyze NADPH → AcPyADP transhydrogenation at detectable rates. These results suggest, therefore, that the above reactions are catalyzed by the same mitochondrial enzyme which catalyzes energy-linked NADH → NADP transhydrogenation. In confirmation of this conclusion, Anderson and Fisher (19) have shown that antibody to the purified transhydrogenase enzyme inhibits the NADPH → AcPyADP transhydrogenase activity of submitochondrial particles.

**DISCUSSION**

Previous findings, together with the results of this paper, show that the mitochondrial energy-linked transhydrogenase enzyme catalyzes the following transhydrogenation reactions: NADPH → NAD, AcPyAD, or thioNAD; and NAD(P)H → NADP, AcPyADP, or thioNADP. The latter reactions, i.e. transhydrogenation to NADP and analogs, are energy-requiring, regardless of whether the reductant is NADH or NADPH. Therefore, since transhydrogenation to NADP is energy-driven while transhydrogenation to NAD is not, it seems possible that the energy-requiring feature may not be a hydride ion abstraction from NAD(P)H, but rather the transfer of reducing equivalents to NADP. In these respects, NADH dehydrogenase exhibits similar transhydrogenase properties, except for the lack of energy-linked modulation of its activities. NADH dehydrogenase also catalyzes transhydrogenation from NAD(P)H to NAD, but when NADP is the acceptor, the reaction is either extremely slow (with NADH as donor) or undetectable (with NADPH as donor). (In the case of NADH dehydrogenase, the ability of the enzyme to catalyze hydride ion abstraction from NAD(P)H is easily demonstrated with the use of quinones or ferric compounds as acceptors.) Thus, it appears that an important feature of the transhydrogenase enzyme is facilitation of NADP reduction in the presence of an energy supply. Another feature is that, unlike NADH dehydrogenase, which can catalyze NADH → NADP transhydrogenation, the energy-linked transhydrogenase enzyme must have NADP or NADPH as one of its substrates. This suggests two possibilities. Either one of the two nucleotide binding sites of the enzyme is highly specific for NADP(H), or NAD(H) binding to either site compels the second site to bind NADP(H) for productive transhydrogenation. If the first possibility should be correct, then the second site would have to be less specific, with a strong preference for NAD(H), but still capable of binding NADP(H) productively. This situation would also agree better with the strict stereospecificity of the NADP(H) ⇌ NAD(H) transhydrogenation reaction, i.e. 4B hydrogen of NADPH and 4A hydrogen of NADH, because the possible absolute specificity of one site for NADP(H) would likely dictate which one of the two C-4 hydrogens of NADPH would be available for transfer. It might also impose steric restrictions on how the second nucleotide binds at the second site, thereby resulting in the stereospecificities of hydride ion transfer observed. In this regard, study of the stereospecificity of hydride ion transfer from NADPH to AcPyADP and thioNADP might be useful.

A more important point of interest which arises from discovery of NADPH → AcPyADP and thioNADP transhydrogenation concerns the directionality of proton translocation by the enzyme. In the classical reaction NADPH + NAD = NADP + NADH, it is now clear from the valuable studies of Earle and Fisher (6, 7) and Rydström (5) with the purified enzyme that hydride ion transfer from NADPH to NAD is coupled to proton translocation from the m (matrix) side of the mitochondrial inner membrane to the c (cytosolic) side. By contrast, hydride ion transfer from NADH to NAD is facilitated by the presence of a proton gradient and is driven beyond the equilibrium point dictated by the reduction potentials of the substrates by the coupled transfer of protons from the c side to the m side. Thus, it is clear that directionality of proton translocation by the enzyme is coupled to the directionality of hydride ion transfer between NAD(H) and NADP(H). This being the case, what can we learn from the transhydrogenation reactions NADPH → AcPyADP and thioNADP? Since these reactions are energy-linked, it is clear that proton translocation from the c to the m side drives these transhydrogenations. Thus, it appears that whenever the hydride ion acceptor is NADP and regardless of whether the donor is NADH or NADPH, hydride ion transfer is sluggish under nonenergized conditions because the enzyme is poised preferentially for proton translocation from the c side to the m side. By contrast, when the hydride ion acceptor is NAD, the preferred directionality of proton translocation is reversed. This is consistent with the observation that SMP-catalyzed transhydrogenation from NADPH → NAD is accelerated by acidification of the medium (m side) under non-energy-linked conditions, and less so when the system is energized (see Ref. 1 (Fig. 7) and Ref. 20).

It is important to consider that in mitochondria the transmembrane pH change brought about by membrane energization does not appear to be too far removed from neutrality and, more important, that the proton translocation induced by transhydrogenation from NADPH to NAD occurs (at least initially) in the absence of a proton gradient at neutral pH. Since the transhydrogenase enzyme spans the mitochondrial membrane (4, 5, 21), this means that the groups on the enzyme that are in contact with the aqueous phase on the two sides of the membrane and constitute the initial and the terminal points of the proton pump must have pK values also not far removed from neutrality. We are currently studying this aspect of the problem, and the results will be reported elsewhere.

**Acknowledgments**—We thank Mr. C. Muñoz for the preparation of beef-heart mitochondria.

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