The addition of a purified mitochondrial pyridine nucleotide transhydrogenase enzyme preparation to complex I (NADH-CoQ reductase) results in a significant increase in the NADPH-AcPyAD\(^+\) transhydrogenase activity of the complex without influencing the NADH-AcPyAD\(^+\) transhydrogenase activity. When subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of complex I, the purified transhydrogenase enzyme preparation was found to co-migrate with the \(M_r = 150,000\) (130K) subunit of the NADH-CoQ reductase.

Loss of the NADPH-NAD\(^+\) transhydrogenase activity of complex I following limited tryptic digestion was associated with a corresponding loss of the 130K subunit from the complex. These results suggest that the 130K subunit of complex I is the specific peptide responsible for the catalysis of the NADPH-NAD\(^+\) transhydrogenase activity observed in complex I.

Studies have been carried out testing the influence of photoaffinity pyridine nucleotide probes on the NADPH-NAD\(^+\) transhydrogenase activity catalyzed at three levels of resolution, i.e., homogeneous transhydrogenase preparation, a partially resolved membrane preparation (complex I), and an intact mitochondrial membrane preparation (EDTA particles). Such studies have revealed arylazido-\(\beta\)-alanyl NADP\(^+\) (N3'-O-[3-N-(4-azido-2-nitrophenyl)amino]propionyl[NADP\(^+\)]) to be a potent inhibitor and an active site-directed reagent for NADPH-NAD\(^+\) transhydrogenation at all three levels of resolution. On the other hand, arylazido-\(\beta\)-alanyl NAD\(^+\) (A3'-O-[3-N-(4-azido-2-nitrophenyl)amino]propionyl[NAD\(^+\)]) does not produce a significant degree of inhibition of NADPH-NAD\(^+\) transhydrogenase activities prior to or following photolradiation. Nevertheless, the NAD\(^+\) analogue has been found to specifically label, covalently, the transhydrogenase protein following photolradiation of an enzyme-analogue mixture. Arylazido-\(\beta\)-alanyl NAD\(^+\) can as well function as a substrate during transhydrogenation by virtue of being able to accept a hydride ion from NADPH.

An interpretation of the observed nucleotide photoprobe specificity for interaction at the active site for transhydrogenation is advanced. In this interpretation, an ordered binding of substrate involves an initial NADP(H) (or NADP\(^+\)) photoprobe interaction with a hydrophobic region at the transhydrogenation site. This initial reactivity is followed by a positioning of NAD(H) (or the NAD\(^+\)) photoprobe analogue above or periphery to the NADP(H) nucleotide present at the active site region. Supportive evidence for this model for transhydrogenase is presented and discussed.

Ragan and Wider (1) have demonstrated an ATP-dependent reduction of NADPH by NADH in a liposomal system in which complex I was combined with phospholipids, the hydrophobic proteins of bovine heart mitochondria, and the purified mitochondrial F\(_o\)-ATPase protein. This finding indicates that the protein responsible for mitochondrial energy-linked transhydrogenation is a component of complex I.

Utilizing AcPyAD\(^+\) as a hydride acceptor, two types of pyridine nucleotide transhydrogenase activities can be measured directly in complex I: a TD (NADPH-AcPyAD\(^+\)) and a DD (NADH-AcPyAD\(^+\)) transhydrogenation (2). Our previous studies utilizing pyridine nucleotide photoaffinity probes revealed that the two transhydrogenase activity and the NADH dehydrogenase activity of complex I are catalyzed at three independent sites (3-6). First, the NADH dehydrogenase active site was shown to be associated with the 57K subunit of complex I (5). Secondly, we have recently observed that NADH-AcPyAD\(^+\) transhydrogenation is associated with a 42K subunit of complex I (6). We have shown as well that a partially resolved NADH-NAD\(^+\) transhydrogenase preparation isolated from complex I is devoid of NADPH-NAD\(^+\) transhydrogenase activity. The NADPH-AcPyAD\(^+\) transhydrogenase activity of complex I has been assumed to be related to the mitochondrial pyridine nucleotide transhydrogenase because of its sensitivity to palmitoyl-CoA (2), a known potent competitive inhibitor with respect to NADP(H) for this transhydrogenase reaction (7, 8).

In the present paper, the purified transhydrogenase protein is utilized as a reference protein in a demonstration, with the help of partial trypic digestion of complex I, that the 130K subunit of the complex is the peptide responsible for the specific catalysis of NADPH-AcPyAD\(^+\) transhydrogenation, independent of NADH dehydrogenation. One of the major experimental facts supporting the non-identity of the NADPH-AcPyAD\(^+\) transhydrogenation from the NADH-AcPyAD\(^+\) transhydrogenation is advanced.

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\[\text{A3'}-O-[3-N-(4-azido-2-nitrophenyl)amino]propionyl[NADP^+]\]

\[\text{N3'}-O-[3-N-(4-azido-2-nitrophenyl)amino]propionyl[NAD^+]\]
AcPyAD\(^+\) transhydrogenase and NADH dehydrogenase proteins is that arylyazido-\(\beta\)-alanyl NADP\(^+\) is a site-specific labeling reagent for the NADPH-AcPyAD\(^+\) transhydrogenase activity of complex I (4), while arylyazido-\(\beta\)-alanyl NAD\(^+\) has little effect on the same activity (2). The latter reagent is a potent inhibitor of the NADH-AcPyAD\(^+\) transhydrogenation and NADH dehydrogenation of complex I (3).

The present study examines the pyridine nucleotide-dependent activities associated with different types of mitochondrial preparations catalyzing transhydrogenation. The study confirms and elaborates on the conclusion as set forth in our preliminary reports on this subject (9–11).

MATERIALS AND METHODS

NADH, NADPH, NADP\(^+\), AcPyAD\(^+\), isocitrate, palmityl-CoA, yeast alcohol dehydrogenase, isocitrate dehydrogenase (Type IV), and trypsin (Type I) were obtained from Sigma and NAD\(^+\) was from Boehringer Mannheim. Trypsin inhibitor was obtained from the Worthington Biochemical Co. Arylazido-\(\beta\)-alanyl NAD\(^+\) and arylyazido-\(\beta\)-alanyl NADP\(^+\) were synthesized according to previously described procedures (4, 12).

The EDTA particles (submitochondrial particles) were prepared from ox heart mitochondria as described by Lee and Ernster (13). Complex I (NAD-CoQ reductase) was isolated from the same source according to the procedure described by Hatefi et al. (14). The transhydrogenase protein was a purified preparation provided by Drs. R. Fisher, W. M. Anderson, and S. R. Earle of the Department of Chemistry, University of South Carolina; it was assayed according to the procedure described by Anderson and Fisher (15). Transhydrogenase activities present within EDTA particles were assayed according to published procedures (16, 17). The NADPH-AcPyAD\(^+\) and NADH AcPyAD\(^+\) transhydrogenation of complex I were assayed as previously described (3).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of complex I and the purified transhydrogenase protein was performed according to the procedures described by Weber and Osborn (18) or Laemmli (19). Specific modifications are delineated in the text. Photoirradiation was accomplished as previously described (12). Other conditions are detailed in the legends of the figures and tables.

RESULTS

Identification of the Mitochondrial Pyridine Nucleotide Transhydrogenase Peptide Present in Complex I—Evidence that the 130K subunit of complex I represents the transhydrogenase protein comes from experiments demonstrating the co-electrophoresis of the subunit with a purified transhydrogenase preparation. Fig. 1 shows the sodium dodecyl sulfate gel electrophoretic peptide patterns for the transhydrogenase protein of complex I as well as for a mixture of transhydrogenase protein and complex I. As illustrated, there is a clear enhancement of the concentration of the 130K peptide of the complex when the purified transhydrogenase protein is co-electrophoresed with complex I. All other peptides of the complex remained at the same apparent concentration level except for that peptide adjacent to the 130K subunit (i.e. the 76K molecular weight peptide) which increases to a small degree. This apparent increase in the concentration of the 76K peptide appears to be the result of a broadening of the background staining level in this region due to the major increase in the 130K peptide level. Studies utilizing pyridine nucleotide photoaffinity probes reveal that two transhydrogenation reactions present in complex I, i.e. NADPH-AcPyAD\(^+\) and NADH-AcPyAD\(^+\) transhydrogenations are catalyzed by two distinct and independent active sites (3, 4). The NADPH-AcPyAD\(^+\) transhydrogenase activity is associated with the 130K peptide of complex I on the basis of a direct correlation between the NADPH-AcPyAD\(^+\) transhydrogenase activity and the concentration of the 130K peptide in different complex I preparations (20). In the experiment outlined in

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**Fig. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the transhydrogenase protein and mitochondrial complex I (NADH-CoQ reductase). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (18) at a concentration of 10% acrylamide. A, transhydrogenase protein (10 µg); B, complex I (120 µg); C, complex I (120 µg) together with the transhydrogenase protein (10 µg).

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An examination of Fig. 3 shows that two peptides of molecular weight 55,000 and 38,000 are generated as a result of the loss of the 130K peptide. In addition, the use of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis system of Weber and Osborn (18) modified by using a 9% acrylamide gel reveals that an additional hydrolytic product (70K peptide) is formed as a result of the tryptic cleavage of the 130K peptide.

The above experiments demonstrating a direct correlation of the TD transhydrogenase activity of complex I with the concentration of the 130K subunit of the complex suggests strongly that this peptide is responsible for the catalysis of NADPH-AcPyAD\(^+\) transhydrogenation.
transhydrogenase enzyme of complex I (3). The radioactive analogue labels four peptides of complex I, one of which is the 130K subunit (3).

Palmitoyl-CoA, a potent competitive inhibitor of transhydrogenation (with respect to NADP(H)) at a 3.3-fold excess effects a significant albeit small protection against arylazo-[3-H]-β-alanyl NADP+ labeling of complex I (Table I). By contrast, NADH in slight excess over the NADP+ analogue provided an enhancement (37%) of the photodependent labeling of complex I by arylazo-[3-H]-β-alanyl NADP+. The enhanced labeling is explained on the basis of the formation of a relatively stable NADH-arylazo-β-alanyl NADP+ enzyme complex (see “Discussion”). The results are reversed in the case of the photodependent labeling of complex I by arylazo-[3-H]-β-alanyl-NAD+. In the latter case, NADH effectively prevents labeling (55%) by arylazo-[3-H]-β-alanyl NAD+ while palmitoyl-CoA had but a slight protective influence.

The rationale for the use of palmitoyl-CoA to demonstrate competition for arylazo-β-alanyl-NADP+ at the NADP+ binding site involved three considerations. Firstly, palmitoyl-CoA has been shown to have a stronger affinity for the NADP(H) binding site than NADP(H) (8, 9); secondly, arylazo-β-alanyl NADP+ has a much greater affinity for the NADP(H) binding site than the natural ligand (4); and lastly, related to the above, the amount of natural ligand in excess of the photoprobe concentration required to protect against labeling could result in secondary effects. By way of example, the NADPH-arylazo-β-alanyl NAD+ transhydrogenase activity of complex I complicates the interpretation of arylazo-β-alanyl NAD+ labeling and its protection by NADPH (see below).

Experiments demonstrating the interaction of arylazo-β-alanyl NADP+ with the purified transhydrogenase protein are outlined in Table II. As indicated, photolabeling of the purified transhydrogenase protein in the presence of arylazo-β-alanyl NADP+ results in a concentration-dependent inhibition of transhydrogenation (experiments 2, 3, and 4). Interestingly, the light-dependent inhibition is but 15 to 20% greater than that observed for the dark control, i.e. the en-

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**Table I**

The influence of palmitoyl-CoA and NADH on arylazo-β-alanyl NADP+ and arylazo-β-alanyl NAD+ labeling of the transhydrogenase peptide of complex I

<table>
<thead>
<tr>
<th>Additions</th>
<th>Arylazo-[3-H]-β-alanyl NAD+</th>
<th>Arylazo-[3-H]-β-alanyl NAD+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radioactivity (cpm)</td>
<td>% of labeling</td>
</tr>
<tr>
<td>None</td>
<td>5875</td>
<td>100</td>
</tr>
<tr>
<td>Palmitoyl-CoA</td>
<td>4420</td>
<td>75</td>
</tr>
<tr>
<td>NADH</td>
<td>8035</td>
<td>137</td>
</tr>
</tbody>
</table>

---

**Fig. 2.** Effect of trypsin treatment on the pyridine nucleotide-dependent activities of complex I. Complex I (1.55 mg) was incubated at 4 °C in 1 ml of 50 mM Tris-HCl, pH 7.5, to which was added 50 μg of trypsin. Subsequent to the addition of trypsin, 5 μl of the incubation medium containing complex I was taken and assayed for NADH-K3Fe(CN)6 reductase ( ), NADH-AcPyAD- transhydrogenase ( ), and NADPH-AcPyAD- transhydrogenase ( ) at those times specified in the figure. The specific activities prior to trypsin treatment were for NADH-K3Fe(CN)6 reductase (270 μmol of K3Fe(CN)6 reduced mg-1 min-1), for NADH-AcPyAD- transhydrogenase (6.4 μmol of AcPyAD- reduced mg-1 min-1), and for NADPH-AcPyAD- transhydrogenase (1.10 μmol of AcPyAD- reduced mg-1 min-1).

**Fig. 3.** The peptide patterns of trypsin-treated complex I. Complex I (1.6 mg) was incubated at 4 °C in 1 ml of 50 mM Tris-HCl, pH 7.5, in the presence of 50 μg of trypsin. At an appropriate incubation time as indicated in the legend above the gel electrophoresis was carried out at 9 mA for 5 h. The position of the 130K (TD transhydrogenase), 57K (NADH dehydrogenase), and 42K (DD transhydrogenase) peptides are indicated in the figure. Channels 11 and 12 illustrate the electrophoretic pattern of trypsin itself at the concentration used in experiments (channel 11) and at a 4-fold higher concentration (channel 12). Channel 13 indicates the electrophoretic pattern of 10 μg of trypsin inhibitor alone.
Pyridine Nucleotide Transhydrogenase Reaction Mechanism

The inhibitory influence of arylazido-β-alanine and its nucleotide derivatives on the transhydrogenase activity of the purified transhydrogenase proteins

The purified transhydrogenase protein (6 μg) was incubated in 0.2 ml of 100 mM Tris-Cl, pH 7.5, 0.05% cholate, containing either arylazido-β-alanine or the pyridine nucleotide derivatives at the indicated concentrations. Enzymatic activity was then measured on a 20-μl aliquot according to the procedure described by Anderson and Fisher (15), prior to and following a 2-min period of photoradiation. The specific activity of the transhydrogenase preparation was 2.7 μmol of AcPyAD reduced min⁻¹ (mg of protein)⁻¹ at 400 μM NADP and 400 μM AcPyAD.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>% inhibition</th>
<th>Non-photo-</th>
<th>Photo-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>irradiated</td>
<td>irradiated</td>
</tr>
<tr>
<td>1</td>
<td>2 Arylazido-β-alanyl NADP⁺ (0.04 μmol)</td>
<td>0</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3 Arylazido-β-alanyl NADP⁺ (0.11 μmol)</td>
<td>35</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4 Arylazido-β-alanyl NADP⁺ (0.17 μmol)</td>
<td>58</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5 Arylazido-β-alanyl NAD⁺ (0.09 μmol)</td>
<td>68</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6 Arylazido-β-alanine (0.11 μmol)</td>
<td>1</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7 Arylazido-β-alanyl NADP⁺ (0.11 μmol) + NADP⁺ (10 μmol)</td>
<td>36</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

Yzyme-analogue mixture not subjected to light irradiation.

Similar concentrations of arylazido-β-alanyl NAD⁺ (experiment 5) or arylazido-β-alanine (experiment 6) do not significantly inhibit transhydrogenation in the dark and result in only low levels of inhibition upon photoradiation. Addition of NADP⁺ to the irradiation mixture (cf. experiments 3 and 7) provides only a small degree of protection against the arylazido-β-alanyl NADP⁺-dependent inhibition for the soluble enzyme. The photodependent inhibition of the transhydrogenase protein activity by arylazido-β-alanyl NADP⁺ was found to be dependent upon the physical state of the transhydrogenase protein. By way of example, 8.2 μM analogue is capable of inhibiting the purified transhydrogenase protein activity some 59% while a similar degree of inhibition for the protein reconstituted into synthetic liposomes requires 19 μM arylazido-β-alanyl NADP⁺ (results not shown). The decreased ability of the NADP⁺ analogue to interact with the liposome-inserted enzyme might indicate that the conformation of the active site for transhydrogenation within the liposome may be different from that of the enzyme in the free state.

An investigation of the action of arylazido-β-alanyl NADP⁺ on an intact membrane preparation (i.e. EDTA particles) (13) showed as well that arylazido-β-alanyl NADP⁺, in the dark, is a potent competitive inhibitor with respect to NADP(H) for the three types of transhydrogenations assayed, i.e. the NADPH-NAD⁺ and NADH-NAD⁺ transhydrogenations, as well as the energy-dependent reduction of NAD⁺ by NADH (Fig. 4). Table II summarizes the results of this kinetic study. In addition to the competitive influence of arylazido-β-alanyl-NADP⁺ with respect to NADP(H) for transhydrogenation, our studies reveal that the NADP⁺ analogue is a noncompetitive inhibitor with respect to NAD(H) for the NADPH-NAD⁺ and NADH-NAD⁺ transhydrogenation reactions. This finding is identical with that observed for the inhibitory profile of the NADP⁺ analogue on the NADPH-AcPyAD transhydrogenase activity of complex I (4).

Table II summarizes the results of this kinetic study. In addition to the competitive influence of arylazido-β-alanyl-NADP⁺ with respect to NADP(H) for transhydrogenation, our studies reveal that the NADP⁺ analogue is a noncompetitive inhibitor with respect to NAD(H) for the NADPH-NAD⁺ and NADH-NAD⁺ transhydrogenation reactions. This finding is identical with that observed for the inhibitory profile of the NADP⁺ analogue on the NADPH-AcPyAD transhydrogenase activity of complex I (4).

The specific activity of the transhydrogenase preparation was 2.7 μmol of AcPyAD reduced min⁻¹ (mg of protein)⁻¹ at 400 μM NADP and 400 μM AcPyAD.

bound to the enzyme during an ordered sequence required for transhydrogenation (3, 4).

The NADP⁺ analogue is, on the other hand, an uncompetitive inhibitor with respect to NADH for the energy-linked NADH-NAD⁺ transhydrogenase activity of EDTA particles (Fig. 4F). A similar inhibition profile for the energy-linked transhydrogenase reaction has been reported for adenylate and has been explained on the basis of a proposed strong shift in the dissociation constant of the binary enzyme-substrate complex upon energization (8). Such a kinetic profile may also be explained as being due to an actual mechanistic change for transhydrogenation upon energization of the mitochondrial membrane. In the simplest case, one might postulate a change in the substrate binding sequence. Upon photoradiation of submitochondrial particles in the presence of aryl-
azido-β-alanyl NADP⁺, 50% inhibition was obtained at 0.48 mM, 1.66 mM, and 1.12 mM concentrations of the NADP⁺ analogue for the NADPH-NAD⁺, NADH-NADP⁺, and energy-linked NADPH-NAD⁺ transhydrogenations, respectively (Fig. 5). Thus, kinetic studies of the inhibitory effect of arylazido-β-alanyl NADP⁺ in the dark and following photoradiation reveal that the NADPH-NAD⁺ transhydrogenase activity is always more sensitive to arylazido-β-alanyl NADP⁺ than the other two pyridine nucleotide-dependent activities. Blzyk et al. (22) have suggested that the transhydrogenase enzyme undergoes conformation changes in the presence of NADPH or NADP⁺. A consequence of this possibility would be that the enzyme would be in an NADPH-enzyme complex conformation during the catalysis of NADPH-NAD⁺ transhydrogenation and in an NADP⁺-enzyme complex conformation during catalysis of NADH-NADP⁺ transhydrogenation. Under such conditions, arylazido-β-alanyl-NADP⁺ would be expected to fix the enzyme in an NADP⁺-enzyme complex conformation which would be less favorable for the catalysis of NADPH-NAD⁺ transhydrogenation (see "Discussion").

Studies of the inhibitory effect of arylazido-β-alanyl NADP⁺ on transhydrogenase activities reveal as well that although the NADP⁺ analogue is a potent competitive inhibitor with respect to NADP(H), a relatively high concentration of the analogue is required in order to provide even moderate photodependent inhibition. Such photodependent inhibition is then always found to be but 10 to 15% greater than that observed for the dark inhibition. By way of example, although the Kᵢ values for arylazido-β-alanyl NADP⁺ inhibition are similar for the TD and DD transhydrogenase activities present in different preparations (i.e., see Table III and Ref. 4), the analogue concentration required for 50% inhibition of enzymatic activity following photoradiation is quite variable. For the pure transhydrogenase, the liposome-inserted enzyme, the partially resolved membrane preparation (Complex I), and an intact membrane preparation (EDTA particles), 50% inhibition of TD transhydrogenase following photoradiation was found at 130 μM, 300 μM, 163 μM, and 0.48 to 1.66 mM, respectively. Such variability might be due to a variable insertion of the analogue at the transhydrogenase active site due to different structural restraints with the different preparations. Among other possibilities, the variability could be due to differing inner filtering effects of the membrane preparations resulting in a differential photoactivation of the probe. In an attempt to demonstrate a photo-dependent inhibitory effect in EDTA particles, the mitochondrial particles were subjected to photoradiation in the presence of the photoprobe followed by a washing protocol as illustrated in Table IV. Four samples were prepared: 1) a control containing only EDTA particles; 2) a sample containing EDTA particles and arylazido-β-alanyl NADP⁺ (i.e., a dark control); 3) a third sample containing EDTA particles and the NADP⁺ analogue which was subjected together to a 4-min photoradiation; and 4) a final sample containing EDTA particles together with previously photoradiated arylazido-β-alanyl NADP⁺. As can be observed from Table IV, transhydrogenase was reduced 68%, 72%, and 53% in experiments 2, 3, and 4 when compared to the control (experiment 1) which was not incubated in the presence of arylazido-β-alanyl NADP⁺. Following centrifugation of each of the mixtures to remove unbound analogue and resuspension of the particles in buffer, the NADPH-NAD⁺ transhydrogenase activity was again measured. In this case, only the activity of that preparation photoirradiated in the presence of the NADP⁺ analogue was inhibited. Thus, in the case of submitochondrial particles, a clear photodependent inhibitory effect for arylazido-β-alanyl NADP⁺ on the NADPH-AcPyAD⁺ transhydrogenase activity is observed. Attempts to utilize similar washing procedures with complex I were unsuccessful due to the strong affinity of the analogue to the preparation and to the inability of complex I to withstand such a washing protocol.

Fig. 5 shows that palmitoyl-CoA is a stronger inhibitor of the NADPH-AcPyAD⁺ transhydrogenase activity of complex I than is arylazido-β-alanyl NADP⁺. The concentrations required for 50% inhibition of the NADPH-AcPyAD⁺ transhydrogenase activity are 1.15 mM and 5.4 mM for palmitoyl-CoA and arylazido-β-alanyl NADP⁺, respectively. The possibility that both compounds interact at an identical site is supported by the fact that their combined inhibitory action is an additive function of their individual effects as indicated in Table V (23). The experiments outlined in Table V were carried out at nonsaturating concentrations of palmitoyl-CoA and arylazido-β-alanyl NADP⁺ in order to minimize possible nonspecific interactions and to optimize the additive action of the inhibitors.

Interaction of Arylazido-β-alanyl NAD⁺ with the Pyridine Nucleotide Transhydrogenase—Although arylazido-β-alanyl NAD⁺ is a strong competitive inhibitor with respect to NADP⁺ for the transhydrogenase, it cannot substitute for NAD⁺ in accepting a hydride ion from NADH or from
Pyridine Nucleotide Transhydrogenase Reaction Mechanism

The effect of arylazido-$\beta$-alanyl NADP$^+$ on the pyridine nucleotide transhydrogenase activities of submitochondrial (EDTA) particles

Activities were assayed as indicated for Fig. 4.

<table>
<thead>
<tr>
<th>Transhydrogenation reaction</th>
<th>Concentration of fixed substrate $\mu$M</th>
<th>Concentration of arylazido-$\beta$-alanyl NADP$^+$ $\mu$M</th>
<th>Inhibitory kinetic effect</th>
<th>$K_{m,app}$ $\mu$M</th>
<th>$K_{rev}$ $\mu$M</th>
<th>Fig. 4 reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH-NAD$^+$</td>
<td>100 (NADPH)</td>
<td>5.6</td>
<td>Noncompetitive</td>
<td>100</td>
<td></td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>144 (NAD$^+$)</td>
<td>11.3</td>
<td>Competitive</td>
<td>28</td>
<td>0.7</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>200 (NAD$^+$)</td>
<td>5.6</td>
<td>Competitive</td>
<td>28</td>
<td>0.8</td>
<td>A</td>
</tr>
<tr>
<td>NADH-NAD$^+$</td>
<td>80 (NADH)</td>
<td>22.6</td>
<td>Competitive</td>
<td>67</td>
<td>6.4</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>400 (NADP$^+$)</td>
<td>22.6</td>
<td>Noncompetitive</td>
<td>7</td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>NADH-NADP$^+$</td>
<td>80 (NAD(P$^+$)</td>
<td>22.6</td>
<td>Competitive</td>
<td>34</td>
<td>4.1</td>
<td>E</td>
</tr>
<tr>
<td>energy-linked</td>
<td>400 (NADP$^+$)</td>
<td>22.6</td>
<td>Uncompetitive</td>
<td>17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE IV
Arylazido-$\beta$-alanyl NADP$^+$ inhibition of the NADPH-NAD$^+$ transhydrogenase activity of EDTA particles

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control</th>
<th>% transhydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>Prior to centrifugation 100</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>Following centrifugation 100</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>Prior to centrifugation 32</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>Following centrifugation 103</td>
</tr>
</tbody>
</table>

NADPH. Arylazido-$\beta$-alanyl NAD$^+$, on the other hand, can be utilized as a hydride acceptor from NADPH. For the TD transhydrogenation of EDTA particles, arylazido-$\beta$-alanyl NAD$^+$ was observed to have a $K_{m,app}$ (67 $\mu$M) similar to that for NAD$^+$ (59 $\mu$M) and a $V_{max,app}$ 16% of that for NAD$^+$ (Table VI). In complex I, the hydride acceptor property of arylazido-$\beta$-alanyl-NAD$^+$ in TD transhydrogenation is enhanced over its action in the submitochondrial particle preparation, i.e. the $K_{m,app}$ and $V_{max,app}$ were 164% and 60% that observed for NAD$^+$. A 56% inhibition of NADPH-arylazido-$\beta$-alanyl NAD$^+$ transhydrogenation was observed in the presence of 2.8 $\mu$M palmityl-CoA, a degree of inhibition resembling that observed for NADPH-AcPyAD$^+$ transhydrogenation.

It appears as well that arylazido-$\beta$-alanyl NADH can be utilized as a hydride donor for the transhydrogenation reaction observed in EDTA particles. In EDTA particles, arylazido-$\beta$-alanyl NAD$^+$ is a competitive inhibitor with respect to AcPyAD$^+$ for the NADPH-AcPyAD$^+$ transhydrogenation (Fig. 7A). An evaluated $K_{app}$ of 40 $\mu$M for this inhibition (Fig. 7A, inset) suggests that the NAD$^+$ analogue is a relatively weak inhibitor with respect to AcPyAD$^+$ relative to the NADP$^+$ analogue with respect to NADPH (H). In the latter case, the $K_{app}$ value ranged from 0.81 to 6.36 $\mu$M. The NAD$^+$ analogue was previously shown to be a very weak competitive inhibitor with respect to AcPyAD$^+$ for the NADPH-AcPyAD$^+$ transhydrogenase activity of complex I (Fig. 7B).

FIG. 6. Inhibition of the NADPH-AcPyAD$^+$ transhydrogenase activity of complex I by palmityl-CoA and arylazido-$\beta$-alanyl NADP$^+$. The assay mixture contained in a total of 1 ml: 0.1 $\mu$M phosphate buffer, pH 7.0, 2 mM NaH$_2$PO$_4$, 91 $\mu$M NADPH, 300 $\mu$M AcPyAD$^+$, together with varying concentrations of palmityl-CoA (A) or arylazido-$\beta$-alanyl NADP$^+$ (B) as indicated in the figure. The assay was initiated by the addition of 10 $\mu$g of complex I. The specific activity for the NADPH-AcPyAD$^+$ transhydrogenase activity of this complex I preparation was 0.87 pmol of AcPyAD$^+$ reduced min$^{-1}$ mg$^{-1}$ of protein.

TABLE III
The effect of arylazido-$\beta$-alanyl NADP$^+$ on the pyridine nucleotide transhydrogenase activities of submitochondrial (EDTA) particles

azido-$\beta$-alanyl NAD$^+$ is a competitive inhibitor with respect to AcPyAD$^+$ for the NADPH-AcPyAD$^+$ transhydrogenation (Fig. 7A). An evaluated $K_{app}$ of 40 $\mu$M for this inhibition (Fig. 7A, inset) suggests that the NAD$^+$ analogue is a relatively weak inhibitor with respect to AcPyAD$^+$ relative to the NADP$^+$ analogue with respect to NADPH (H). In the latter case, the $K_{app}$ value ranged from 0.81 to 6.36 $\mu$M. The NAD$^+$ analogue was previously shown to be a very weak competitive inhibitor with respect to AcPyAD$^+$ for the NADPH-AcPyAD$^+$ transhydrogenase activity of complex I (Fig. 7B).

Arylazido-$\beta$-alanyl NAD$^+$ is an uncompetitive inhibitor with respect to NADPH for the NADPH-AcPyAD$^+$ transhydrogenase activity catalyzed by EDTA particles (Fig. 7B). This result is consistent with our previous findings indicating NADP(H) to be the initial substrate bound at the TD transhydrogenase active site (3, 4).
In summary, arylazido-β-alanyl NAD+ is a relatively good substrate (hydride acceptor) but a weak inhibitor for TD transhydrogenation. A very low photodependent inhibition of transhydrogenase activity of the enzyme, arylazido-β-alanyl NAD+ (0.09 µmol) inhibited enzymatic activity only 13%.

**DISCUSSION**

Hatefi and Hanstein (2) found the NADPH-NAD+ transhydrogenase activity of mitochondria to be fractionated into complex I rather than into complexes II, III, or IV. In spite of this apparent specific fractionation, studies in this laboratory utilizing arylazido-pyridine nucleotide analogues have revealed that there is no functional linkage between the NADH dehydrogenase and the NADPH-NAD+ transhydrogenase of the complex I preparation (3, 4). The 57K subunit of complex I isolated by Hatefi and Hanstein (5) and in this paper we have demonstrated that the 130K subunit of complex I is the peptide responsible for the NADPH-NAD+ (and NADPH-AcPyAD+) transhydrogenase reaction. The fact that the dehydrogenase and transhydrogenase activities are catalyzed by different peptides eliminates the possibility of their being identical entities.

Studies of TD transhydrogenation at three different levels of resolution: the purified enzyme, complex I (a partially resolved membrane preparation), and EDTA particles (an intact submitochondrial membrane preparation) utilizing ar-

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**TABLE V**

*The combined effects of palmitoyl-CoA and arylazido-β-alanyl NAD+ on the NADPH-AcPyAD+ transhydrogenase activity of complex I.*

<table>
<thead>
<tr>
<th>Inhibitor concentrations</th>
<th>% control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitoyl-CoA I&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Arylazido-β-alanyl NAD+ I&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>0.3 µM</td>
<td>1.26</td>
</tr>
<tr>
<td>0.3 µM</td>
<td>2.52</td>
</tr>
<tr>
<td>0.3 µM</td>
<td>4.06</td>
</tr>
<tr>
<td>0.6 µM</td>
<td>3.78</td>
</tr>
<tr>
<td>0.9 µM</td>
<td>2.52</td>
</tr>
</tbody>
</table>

* (i<sub>i</sub>), the fractional inhibition of transhydrogenation following the combined addition of the inhibitors at the concentrations indicated (i<sub>i</sub> and I<sub>i</sub>).

**TABLE VI**

The hydride acceptor ability of arylazido-β-alanyl NAD+ in the TD transhydrogenation reactions catalyzed by EDTA particles and complex I.

<table>
<thead>
<tr>
<th>Transhydrogenase reaction</th>
<th>Concentration of fixed substrate (NADPH)</th>
<th>K&lt;sub&gt;app&lt;/sub&gt; second substrate</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; app</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA particles&lt;sup&gt;a&lt;/sup&gt;</td>
<td>µM</td>
<td>nmoles min&lt;sup&gt;-1&lt;/sup&gt; mg protein</td>
<td>µmol min&lt;sup&gt;-1&lt;/sup&gt; mg protein</td>
</tr>
<tr>
<td>NADPH-NAD+</td>
<td>200</td>
<td>59</td>
<td>295</td>
</tr>
<tr>
<td>NADPH-arylazido-β-alanyl NAD+</td>
<td>200</td>
<td>67</td>
<td>48</td>
</tr>
<tr>
<td>Complex I&lt;sup&gt;b&lt;/sup&gt;</td>
<td>µM</td>
<td>nmoles min&lt;sup&gt;-1&lt;/sup&gt; mg protein</td>
<td>µmol min&lt;sup&gt;-1&lt;/sup&gt; mg protein</td>
</tr>
<tr>
<td>NADPH-NAD+</td>
<td>200</td>
<td>78</td>
<td>0.445</td>
</tr>
<tr>
<td>NADPH-arylazido-β-alanyl NAD+</td>
<td>200</td>
<td>128</td>
<td>0.267</td>
</tr>
</tbody>
</table>

<sup>a</sup> The assay procedure used was that described by Lee and Ernster (13) with the reaction being initiated by the addition of 0.11 mg of EDTA particles.

**FIG. 7.** A kinetic analysis of the inhibition by arylazido-β-alanyl NAD+ of the NADPH-AcPyAD+ transhydrogenase activity of EDTA particles. In the presence of 0 (○), 71.5 µM (●), and 142 µM (△) arylazido-β-alanyl NAD+, A, at a fixed concentration of NADPH of 100 µM, B, at a fixed concentration of AcPyAD+ of 18 µM. Enzymatic activity was assayed as indicated for Fig. 4 and 1/v is given as micromoles of AcPyAD+ reduced min<sup>-1</sup>.

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In summary, arylazido-β-alanyl NAD+ is a relatively good substrate (hydride acceptor) but a weak inhibitor for TD transhydrogenation. A very low photodependent inhibition of transhydrogenase activity is obtained when complex I or the combined addition of the inhibitors at the indicated concentrations (i<sub>i</sub> and I<sub)i</sub>) is evaluated as outlined by Webb (23).
ylazido analogues of NAD$^+$ and NADP$^+$ have provided data applicable to an interpretation of the reaction mechanism for mitochondrial transhydrogenation. The two analogues interact with the enzyme at distinctly different specific sites. Arylazido-$\beta$-alanine NADP$^+$ interacts with the transhydrogenase at the NADP(H) binding site as indicated by the facts that: (a) the analogue is a potent competitive inhibitor with respect to NADP(H) for transhydrogenation; (b) the NADP$^+$ analogue binds to the enzyme at the palmitoyl-CoA interaction site (palmitoyl-CoA is a strong competitive inhibitor of transhydrogenation with respect to NADP(H)); and (c) photodependent inhibition or labeling of the enzyme by the NADP$^+$ analogue can be prevented by the presence of NADPH or palmitoyl-CoA during photolabelling. Arylazido-$\beta$-alanine NAD$^+$ interacts with the TD transhydrogenase at an independent NAD(H)-reactive site based upon the experimental facts that: (a) the NAD$^+$ analogue is a substrate for TD transhydrogenation and the NADP$^+$-arylazido-$\beta$-alanine NAD$^+$ transhydrogenase is sensitive to palmitoyl-CoA treatment; (b) photodependent labeling of the transhydrogenase enzyme by the NAD$^+$ analogue is prevented by NADH but not by palmitoyl-CoA; and (c) preliminary cross-linking experiments with complex I indicate that no cross-linking occurs between the 130K subunit (TD transhydrogenase) and either the 57K subunit (NADH dehydrogenase) or the 42K subunit (NADH-NAD$^+$) transhydrogenase. This latter finding suggests that the photodependent labeling of the 130K peptide by arylazido-$\beta$-alanine NAD$^+$ is not due simply to its juxtaposition to the 57K or 42K peptides. The 57K (NADH dehydrogenase) and 42K subunits (NADH-NAD$^+$ transhydrogenase) are labeled by arylazido-$\beta$-alanine NAD$^+$ in a specific manner.

While the mechanism for TD transhydrogenation appears clearly to involve a ternary complex (16, 17, 24), studies utilizing “site-specific” inhibitors of transhydrogenation have been variously interpreted as indicating either NADP(H) to be the first substrate to bind to the enzyme in an ordered addition sequence (7) or NAD(H) to be the initial reactant in the enzymatic mechanism (8). On the basis of studies with the purified transhydrogenase enzyme, it has now been suggested that a random substrate addition mechanism is involved in the turnover of the enzyme.

The varied interpretation of the reaction mechanism described above is in sharp contrast to that derived from kinetic studies of the transhydrogenase activity observed for both complex I (NADH-CoQ reductase) and submitochondrial particles utilizing pyridine nucleotide photoaffinity probes (20). The latter experiments show arylazido-$\beta$-alanine NAD$^+$ to be a competitive inhibitor with respect to NADP(H) and a noncompetitive inhibitor with respect to NAD(H) while arylazido-$\beta$-alanine NAD$^+$ is a competitive inhibitor with respect to NAD(H) and shows uncompetitive inhibitor with respect to NADP(H) (20). Those studies propagating to show a random substrate addition mechanism have relied heavily on the observation that 5’ AMP is a noncompetitive inhibitor with respect to NADP(H). Arylazido-$\beta$-alanine NAD$^+$ has a $K_i$ value estimated at 40 $\mu$m (this paper) while 5’-AMP has a value estimated to be between 300 and 700 $\mu$m (8). The enhanced inhibitory action of arylazido-$\beta$-alanine NAD$^+$ over that of 5’-AMP is taken to indicate a greater than 10-fold binding specificity of the arylazido-$\beta$-alanine NAD$^+$ analogue over that of 5’-AMP.

On the basis of the above, it is considered likely that the TD transhydrogenase has, as a minimal hypothesis, an ordered ternary complex mechanism with NADP(H) as the initial substrate bound to the enzyme. The finding that NADPH facilitates the binding of the mitochondrial transhydrogenase protein to an affinity column containing covalently bound NAD$^+$ (25) supports the concept that NADP(H) is required to assure binding of NAD$^+$.

The observations that NAD(H) has a protective influence on tryptic inactivation (15, 26) and on dicyclohexylcarbodiimide inactivation of transhydrogenase activity (27), as well as the finding that arylazido-$\beta$-alanine NAD$^+$ can covalently label the transhydrogenase enzyme independent of the addition of NADP(H) (this paper) indicates that NAD(H) binds to the enzyme in the absence of NADP(H). However, these results cannot be used to interpret a sequential binding order of the substrates for transhydrogenation. Several lines of evidence indicated that the binding region for NADP(H) is rather restricted and hydrophobic in nature: (a) the binding site is restricted to NADP(H) and cannot be utilized by NAD(H), i.e. the TD transhydrogenase enzyme does not catalyze a DD transhydrogenation reaction (15); (b) both palmitoyl-CoA and arylazido-$\beta$-alanine NADP$^+$ bind to the enzyme with greater affinity than NADP(H). In comparison to NADPH(H), both inhibitors are more hydrophobic due to the presence of the hydrophobic side chains; and (c) an increase in chain length of the fatty acyl moiety of fatty acyl-CoA derivative has a significant influence on the inhibitory ability of fatty acyl-CoA compounds (5). While dephospho fatty acyl-CoA compounds are competitive inhibitors with respect to NAD(H), the chain length of the fatty acyl moiety has little influence on the inhibitory ability of these compounds. There is as well a less restricted specificity for NAD(H) substitution compared to that for NADP(H) illustrated by the fact that NAD(H) can be substituted for NADP(H), i.e. the transhydrogenase catalyzes a NADPH-NADP$^+$ transhydrogenation reaction (28, 29). These results indicate that NAD(H), in contrast to NADP(H), binds to a less restricted and comparatively more hydrophilic region of the active site. The proposed model could explain the stronger binding of arylazido-$\beta$-alanine NAD$^+$ to the transhydrogenase (as compared to NADP(H)) on the basis of its hydrophilic side chain, i.e. the arylazido group.

Very minor modifications of the NADP$^*$ molecule are known to result in complete inactivity as a substrate for the transhydrogenase. Arylazido-$\beta$-alanine NAD$^+$ analogue may disorient the molecule, preventing its interaction with NAD(H) and thus limiting the hydride transfer reaction. In contrast to the NADP$^*$ analogue, arylazido-$\beta$-alanine NAD$^*$ is a reasonably good substrate for the transhydrogenation, consistent with the NAD$^*$ analogue binding in a less restricted manner to the active site. Blazyk et al. (22) have presented evidence suggesting that the rat liver mitochondrial transhydrogenase enzyme undergoes conformational changes induced by NADPH or NADP$^*$.

On the basis of the above analysis and the postulated NADP(H)-induced conformational change, a sequential model is outlined (Scheme 1) which illustrates the influence of the conformational change on a postulated sequence of nucleotide binding during transhydrogenation. In the presence of arylazido-$\beta$-alanine NAD$^*$, the enzyme assumes the conformation of the NADP$^*$-enzyme complex (Scheme 1, structure 2). Under these conditions, one might expect NADP$^*$ to compete more effectively with arylazido-$\beta$-alanine NAD$^*$ than NADPH. This mechanism is consistent with the greater effectiveness of NADP$^*$ relative to NADPH in protecting against arylazido-$\beta$-alanine NAD$^*$ inhibition of TD transhydrogenations in submitochondrial particles. In

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S. Chen and R. J. Guillery, unpublished results.
Table III, the $K_{\text{app}}$ values for arylazido-$\beta$-alanyl NADP$^+$ in the presence of varying NADPH were shown to average 0.74 $\mu$M for NADPH-NAD$^+$ transhydrogenation. During the NADH-NADP$^+$ transhydrogenation under conditions in which NADP$^+$ was varied, the $K_v$ value for the NADP$^+$ analogue was evaluated at 4.15 $\mu$M for the energy-linked reaction and 6.36 $\mu$M for the non-energy-linked reaction. The 10-fold difference in $K_v$ explains why the NADP$^+$ analogue inhibits the NADPH-NAD$^+$ transhydrogenation to a greater degree than NADH-NADP$^+$ transhydrogenation. The photodependent inhibition of TD transhydrogenation by arylazido-$\beta$-alanyl NADP$^+$ is thus effectively masked due to excess non-covalently but tightly bound analogue (Table IV).

An enhancement of arylazido-$\beta$-alanyl NADP$^+$ labeling in the presence of NADH (Table I) is explained as a result of the formation of a stable enzyme complex in the presence of NADH (Scheme 1, structure 4). The active site for transhydrogenase would be expected to remain in an unmodified conformational state upon photolabeling of the NADP$^+$ analogues (Scheme IV).

This mechanism explains how the NAD$^+$ analogue might be expected to have only a minor photodependent inhibitory effect on transhydrogenation while it nevertheless labels the enzyme at a specific NAD(H) site (Table I) and has substantial reactivity under non-photoirradiated conditions.

The experiments outlined in Figs. 2 and 3 clearly show that illumination of complex I with trypsin results in the loss of TD transhydrogenase activity associated with the disappearance of the 130K subunit (i.e., the TD transhydrogenase peptide). Preliminary experiments have shown that both of the pyridine nucleotide analogues are, upon photolysis, bound to the 70K peptide formed as a result of the tryptic cleavage of the TD transhydrogenase peptide. Isolation of this cleavage product and characterization of the pyridine nucleotide-labeled region(s) are currently under investigation. It is of interest that both arylazido-$\beta$-alanyl NAD$^+$ and arylazido-$\beta$-alanyl NADP$^+$ at 0.1 mM have little influence on the rate of trypsin-dependent inactivation of the TD transhydrogenation of complex I.

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