Conformational Features of Bovine Heart Mitochondrial Transhydrogenase†

David E. Modrak,‡ Licia N. Y. Wu,* Julie A. Alberta,§ and Ronald R. Fisher‖
Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208
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ABSTRACT: Both purified and functionally reconstituted bovine heart mitochondrial transhydrogenases were treated with various sulfhydryl modification reagents in the presence of substrates. In all cases, NAD^+ and NADH had no effect on the rate of inactivation. $NADP^+$ protected transhydrogenase from inactivation by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in both systems, while NADPH slightly protected the reconstituted enzyme but stimulated inactivation in the purified enzyme. The rate of *N*-ethylmaleimide (NEM) inactivation was enhanced by NADPH in both systems. The copper–(*o*-phenanthroline)$_2$ complex [$Cu(OP)_2$] inhibited the purified enzyme, and this inhibition was substantially prevented by $NADP^+$. Transhydrogenase was shown to undergo conformational changes upon binding of $NADP^+$ or NADPH. Sulfhydryl quantitation with DTNB indicated the presence of two sulfhydryl groups exposed to the external medium in the native conformation of the soluble purified enzyme or after reconstitution into phosphatidylcholine liposomes. In the presence of $NADP^+$, one sulfhydryl group was quantitated in the nondenatured soluble enzyme, while none was found in the reconstituted enzyme, suggestng that the reactive sulfhydryl groups were less accessible in the $NADP^+$–enzyme complex. In the presence of NADPH, however, four sulfhydryl groups were found to be exposed to DTNB in both the soluble and reconstituted enzymes. NEM selectively reacted with only one sulfhydryl group of the purified enzyme in the absence of substrates, but the presence of NADPH stimulated the NEM-dependent inactivation of the enzyme and resulted in the modification of three additional sulfhydryl groups. The sulfhydryl group not modified by NEM in the absence of substrates is not sterically hindered in the native enzyme as it can still be quantitated by DTNB or modified by iodoacetamide. Treatment of purified transhydrogenase with iodoacetamide resulted in the modification of both exposed sulfhydryl groups in the absence of substrates. In the presence of NADPH, only one exposed plus three additional buried sulfhydryl groups were modified by iodoacetamide. In all cases, sulfhydryl groups were more accessible in the NADPH–enzyme complex. A new aspect discovered here is the formation of an intramolecular disulfide cross-link in transhydrogenase upon $Cu(OP)_2$ and DTNB inactivation as evidenced by an increased electrophoretic mobility. Reversal of the disulfide cross-link was demonstrated with the addition of dithiothreitol. Sulfhydryl titration studies on $Cu(OP)_2$-modified soluble enzyme show that the two exposed DTNB-reactive sulfhydryl residues are cross-linked by $Cu(OP)_2$. In addition to protection of activity, $NADP^+$ nearly completely prevented cross-linking by $Cu(OP)_2$. These results indicate that vicinal cysteines are present at the NADP-binding site.

Mitochondrial inner membrane bound pyridine dinucleotide transhydrogenase catalyzes the reversible transfer of a hydride ion between oxidized and reduced forms of intramitochondrial NAD and NADP (Fisher & Earle, 1982; Rydstrom, 1981). Hydride ion transfer is specific for the 4A locus of NADH and the 4B locus of NADPH (Lee et al., 1965) and is coupled to the formation of an electrochemical gradient, positive on the cytosolic side of the mitochondrial inner membrane (Moyle & Mitchell, 1973; Earle & Fisher, 1980).

Bovine heart transhydrogenase is a transmembrane protein of approximately 110 kDa/monomer (Anderson & Fisher, 1978) and exists as a dimer in the purified enzyme, in reconstituted liposomes, and in the submitochondrial particle membrane (Anderson & Fisher, 1981; Wu & Fisher, 1983). Recently, transhydrogenase primary structure has been deduced from the sequence of corresponding cDNA (Yamaguchi et al., 1988). Transhydrogenase functionally incorporated into phosphatidylcholine liposomes has been shown directly to translocate protons into the vesicles accompanying the reduction of NAD^+ by NADPH (Earle & Fisher, 1980). The mechanism of proton translocation is unknown. Several models have been proposed including pump (Fisher & Earle, 1982; Rydstrom, 1981; Skulachev, 1974; Blazyk et al., 1976; Galante et al., 1980) and loop (Mitchell & Moyle, 1965) mechanisms. On the basis of thermal and proteolytic inactivation studies in submitochondrial particles, Blazyk et al. envisioned that transhydrogenase exists in at least three different conformations: a native (unliganded) enzyme and an $NADP^+$– and an NADPH–enzyme complex. Fisher and Earle put forth the concept of ligand-induced conformational changes and proposed a proton pump mechanism (Fisher & Earle, 1982) wherein transhydrogenase contains a proton-binding domain, separate from the active site, that is accessible to the matrix side of the membrane when NADPH and NAD^+ are bound and accessible to the cytosolic side of the membrane when $NADP^+$ and NADH are bound. Alternatively, a loop mechanism employing a reduced enzyme intermediate located on the side of the membrane opposite to the active site has been suggested (Mitchell & Moyle, 1965).

Chemical modification studies on submitochondrial particle transhydrogenase have demonstrated the presence of one class

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* Author to whom correspondence should be addressed.
‡ Present address: Department of Chemistry, Indiana University, Bloomington, IN 47405.
§ Present address: Program of Cell and Developmental Biology, 25 shattuck St., Harvard Medical School, Boston, MA 02115.
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of sulfhydryl group in the nicotinamide adenine dinucleotide phosphate (NADP) site and another peripheral to the active site (Earle et al., 1978). Reaction of the peripheral sulfhydryl group with NEM,[1] or both classes with DTNB, completely inactivated submitochondrial particle transhydrogenase. $NADP^+$ or NADPH almost completely protected against DTNB inactivation. $NADP^+$ partially protected the enzyme from inactivation by NEM while NADPH stimulated inactivation. Although much earlier studies were performed on submitochondrial membrane preparations, the spacial relationship between these two classes of sulfhydryl groups remains uncertain.

In this paper, substrate binding induced conformational changes in purified and reconstituted transhydrogenase were investigated. To evaluate conformational changes, the number of sulfhydryl groups exposed to the medium was quantitated with DTNB in the presence or absence of substrates. Evidence is presented that vicinal sulfhydryl groups are present at the NADP-binding site in both purified and submitochondrial particle transhydrogenase.

Experimental Procedures

Materials. Pyridine dinucleotides were from P-L Biochemicals (Milwaukee, WI) and Pharmacia, Inc. (Piscataway, NJ). NEM, DTNB, IAA, DTT, *o*-phenanthroline, egg yolk phosphatidylcholine (type V-E), bis(acrylamide), and all detergents were purchased from Sigma Chemical Co. (St. Louis, MO). FCCP was obtained from Pierce Chemical Co. (Rockford, IL). Acrylamide was purchased from United States Biochemical Corp. (Cleveland, OH). All other chemicals were of reagent grade.

Methods. Bovine heart mitochondria and submitochondrial particles were prepared as described previously (Sanadi et al., 1967; Low & Vallin, 1963). Transhydrogenase was purified from submitochondrial particles and reconstituted into phosphatidylcholine liposomes (Wu et al., 1982, 1986). Protein concentration was determined with a Coomassie Blue assay (Semak & Grossberg, 1977) using bovine serum albumin as the standard. Polyclonal antibodies were generated against homogeneous bovine heart transhydrogenase as described (Anderson et al., 1981).

Sulfhydryl group quantitation was performed with DTNB (Ellman, 1959). The release of the thionitrobenzoate anion was monitored at 412 nm before and after the addition of SDS (final concentration, 0.2%) to denature the enzyme.

Chemical modifications of transhydrogenase were performed at 50 μg/mL in 100 mM sodium phosphate, pH 7.5, containing 0.05% sodium cholate at room temperature with other additions and modifications as indicated in the figure legends. Stock solutions of the cross-linking reagent $Cu(OP)_2$ were prepared by mixing an equal volume of 4 mM $CuSO_4$ and 8 mM 1,10-phenanthroline before dilution into the reaction mixture. Transhydrogenase activity was assayed by monitoring at 25 °C the reduction of $AcPyAD^+$ (190 μM) by NADPH (150 μM) at 375 nm in a 1-mL reaction mixture containing 80 mM potassium phosphate, pH 6.3 (Blazyk et al., 1976). FCCP (0.49 μM) was also included in assays with liposomes.

Gel electrophoresis was performed using 10% SDS–polyacrylamide gels according to Laemmli (1970) except that the sample buffer contained 5 mM NEM instead of β-mercaptoethanol to block sulfhydryl groups. The gels were stained with 0.25% Coomassie Brilliant Blue R250 in 22.7% methanol/9.2% glacial acetic acid and destained with 18% methanol/9% glacial acetic acid in an automated destaining cell (Canalco II, Miles Laboratories).

In order to identify the location of submitochondrial particle transhydrogenase after SDS–polyacrylamide gel electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper with a Bio-Rad transblot apparatus in a buffer containing 25 mM Tris-HCl, 193 mM glycine, and 20% (v/v) methanol, pH 8.3, for 3 h at 70 V and 0.25 A. To visualize transhydrogenase, the blot was incubated with anti-transhydrogenase antibody and goat anti-rabbit IgG by the procedure given in a Bio-Rad Immunoblot assay kit.

Table I: Sulfhydryl Quantitation of Soluble and Reconstituted Transhydrogenase

enzyme	substrate present[b]	no. of sulfhydryl groups/monomer[a] –SDS	+SDS
soluble		2.2	4.0
	NAD^+	2.3	3.6
	NADH	2.2	3.7
	$NADP^+$[c]	1.2	4.0
	NADPH	3.8	2.0
reconst[d]		2.1	
	$NADP^+$	0.0	
	NADPH	3.8	

[a] Sulfhydryl quantitation was performed on 20–50 μg of soluble or 20 μg of reconstituted transhydrogenase. The soluble enzyme was diluted to 1 mL with 100 mM sodium phosphate, pH 8.0, containing 0.05% sodium cholate, and the reconstituted enzyme was in 1 mL of 1 mM Tricine–sodium hydroxide, pH 8.0, containing 100 mM choline chloride. After a stable base line at 412 nm was obtained, 10 μL of DTNB was added (25 μM final concentration) and the absorbance was monitored until no further increase. SDS was added to a final concentration of 0.2% and the A_{412} was monitored as before. The molar extinction coefficient of the thionitrobenzoate anion used was 13 600 L mol^{-1} cm^{-1}. [b] Where noted, the indicated substrate was included at a final concentration of 1 mM prior to DTNB titration. [c] SDS was added to a final concentration of 0.4%. [d] With the reconstituted enzyme (reconst), the number of sulfhydryl groups present after SDS addition could not be quantitated due to irregular absorbance changes resulting from solubilization of the liposomes.

Results

Sulfhydryl Quantitation of Purified and Reconstituted Transhydrogenase. Titration of homogeneous transhydrogenase with DTNB demonstrated the presence of two exposed sulfhydryl groups. Inclusion of 0.2% SDS to denature the enzyme exposed four additional buried sulfhydryl groups per monomer (Table I). The effect of substrates on the number of accessible sulfhydryl groups was investigated by including 1 mM of each substrate during DTNB titration. In the presence of $NADP^+$, 1.2 exposed and 4.0 buried cysteines were found, while with NADPH, 3.8 exposed and 2.0 buried cysteines were detected. A high percent of SDS (0.4%) was needed for DTNB titration in the presence of $NADP^+$, to allow quantitation of all buried sulfhydryl groups. NAD^+ and NADH gave essentially the same number of exposed residues as without substrates. The differences in the number of exposed sulfhydryl groups strongly support the notion of conformational changes accompanying the binding of either $NADP^+$ or NADPH.

As reconstitution of transhydrogenase into phosphatidylcholine liposomes yields unidirectionally incorporated enzyme with the NAD- and NADP-binding sites exposed to the external media (Fisher & Earle, 1982), titration with DTNB

[1] Abbreviations: SMP, submitochondrial particles; $AcPyAD^+$, oxidized 3-acetylpyridine adenine dinucleotide; $Cu(OP)_2$, copper–(*o*-phenanthroline)$_2$; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; IAA, iodoacetamide; DDT, dithiothreitol; FCCP, carbonyl cyanide (*p*-trifluoromethoxy)phenylhydrazone; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

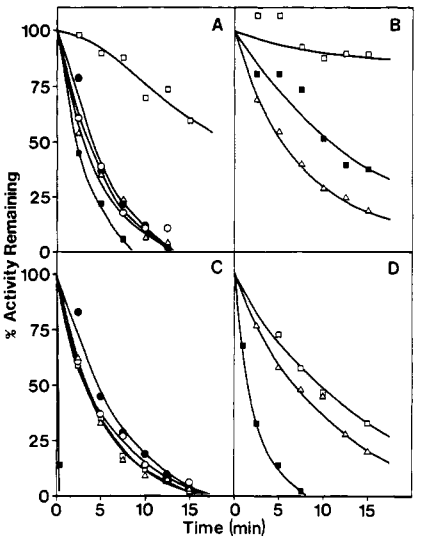

FIGURE 1: Substrate effects on the rate of NEM and DTNB inactivation in purified and reconstituted transhydrogenase. Soluble transhydrogenase was incubated at 50 μg/mL of protein in 100 mM sodium phosphate/0.05% sodium cholate, pH 7.5 at 24 °C, with either 25 μM DTNB (A) or 500 μM NEM (C) in the presence of 1 mM substrates where indicated. Reconstituted transhydrogenase was incubated at 30 μg/mL of protein in 1 mM Tricine–sodium hydroxide/100 mM choline chloride, pH 7.2 at 24 °C, with either 25 μM DTNB (B) or 500 μM NEM (D) in the presence of 1 mM substrates where indicated. At the specified times, aliquots were removed and assayed for transhydrogenase activity. [Absence of substrate (△), NAD^+ (○), NADH (●), $NADP^+$ (□), and NaDPH (■).]

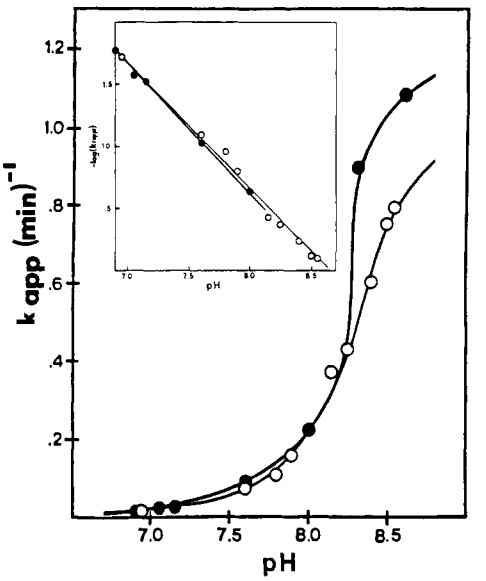

FIGURE 2: pH dependency of DTNB and NEM inactivation. Soluble transhydrogenase was incubated with either 25 μM DTNB (○) or 500 μM NEM (●) at a concentration of 50 μg/mL of protein in 100 mM sodium phosphate/0.05% sodium cholate at various pH values at 24 °C. At appropriate times, aliquots were removed and assayed. Plots of log of percent activity remaining against incubation time were linear, and the first-order rate constants (k_{app}) were determined for each pH indicated. k_{app} or $-\log k_{app}$ was plotted versus each pH value. The apparent pK_a determined for both reactions is 8.6.

could distinguish between sulfhydryl groups embedded within or on either side of the lipid bilayer. The reconstituted enzyme, however, could only be titrated in the absence of SDS, which caused irregular absorbance changes due to solubilization of the liposomes. As shown in Table I, in the absence of substrates, 2.1 cysteines are exposed. In the presence of $NADP^+$, none are titrated, and with NADPH, 3.8 cysteines are counted. In the absence of substrates then, both the NADP-binding site and the peripheral sulfhydryl groups are on the outer face of the liposomes and can be titrated. Binding of NADPH apparently causes two additional sulfhydryl groups to be exposed and modified, while binding of $NADP^+$ shields both sulfhydryl groups from titration.

DTNB Modification of Purified and Reconstituted Transhydrogenase. In order to ascertain the site of DTNB modificaton, the effects of substrates on the inhibition of transhydrogenase activity by DTNB were determined. As can be seen in Figure 1A, only $NADP^+$ partially protects the soluble purified enzyme from inactivation while NADPH slightly stimulates inhibition by DTNB. Figure 1B shows that, in the reconstituted system, $NADP^+$ almost completely protects whereas NADPH slightly protects inhibition by DTNB. These results indicate that DTNB does not react at the NAD-binding site since neither NAD^+ nor NADH provided protection and only $NADP^+$ is effective in protecting against DTNB inactivation. In contrast, O'Neal and Fisher (1977) found both $NADP^+$ and NADPH to protect the enzyme from DTNB inactivation in SMP. Figure 2 shows the pH dependency for DTNB inactivation (open circles). The pK_a for the reactive sulfhydryl group of the purified enzyme was determined to be ~8.6. This differs markedly from the previously reported pK_a for SMP transhydrogenase of 7.4 (O'Neal & Fisher, 1977). The enzyme as purified in this work is stable up to pH 8.6 with less than 10% of the initial activity lost in 15 min. Studies at pHs higher than 8.6 were unsuccessful due to denaturation of the enzyme.

NEM Modification of Purified and Reconstituted Transhydrogenase. In SMP, Earle et al. (1978) found the presence of substrates influenced the rate of NEM inactivation of transhydrogenase. In their studies, $NADP^+$ protected against and NADPH stimulated inactivation while NAD^+ and NADH had no effect. Our studies with the purified enzyme show that NADPH greatly stimulated the rate of inactivation while NAD^+, NADH, and $NADP^+$ had no effect (Figure 1C). The effects of substrates on the rate of inactivation on soluble transhydrogenase by 50 mM IAA (data not shown) were essentially identical. Similarly, NADPH significantly stimulated NEM inactivation of the reconstituted enzyme (Figure 1D). The differing effects of $NADP^+$ and NADPH on the rate of NEM inactivation are likley a result of conformational changes as in the case of DTNB inactivation. In the presence of NADPH, NEM may modify one or more sulfhydryl groups outside the NADP-binding site to enhance inactivation since NADPH does expose additional sulfhydryl groups (cf. Table I). The reactive sulfhydryl group in the absence of substrates was reported to have a pK_a of 9.1 in SMP (Earle et al., 1978). However, the pH dependency of NEM inactivation of the purified enzyme displayed a pK_a of ~8.6 (Figure 2, closed circles), identical with that determined for DTNB inactivation.

Intramolecular Cross-Linking of Purified Transhydrogenase. Kobashi has introduced the copper–(*o*-phenanthroline)$_2$ complex [$Cu(OP)_2$] as a reagent for the specific cross-linking of sulfhydryl groups (Kobashi, 1968). Actions of this reagent on proteins result in enhanced air oxidation of cysteine to cystine residues. $Cu(OP)_2$ was found to inhibit activity by formation of an intramolecular cross-link. Figure 3 shows the nonreducing SDS–polyacrylamide gel electrophoretic pattern obtained from $Cu(OP)_2$ inactivation of the purified enzyme (lane 12) as well as that obtained in the presence of NAD^+ (lane 13) and $NADP^+$ (lane 14). A control sample without any reagent was run beforehand to ensure the enzyme's homogeneity (data not shown). Only the area of interest was shown; no other bands were present on the rest of the gel. The effect of substrates on $Cu(OP)_2$ in-

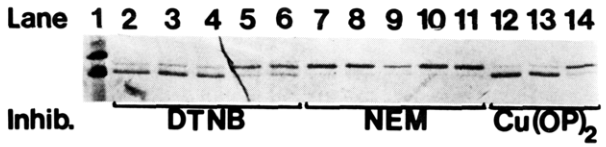

FIGURE 3: Substrate effects on the electrophoretic mobility of DTNB-, NEM-, and $Cu(OP)_2$-inactivated soluble transhydrogenase. Soluble transhydrogenase was incubated at 50 μg/mL of protein with either 25 μM DTNB, 500 μM NEM, or 100 μM $Cu(OP)_2$ in 100 mM sodium phosphate/0.05% sodium cholate, pH 7.5. After 15 min, 2.5 μg was removed, incubated with sample buffer (nonreducing) for 40 min at 25 °C, and electrophoresed as described under Experimental Procedures. Lane 1, M_r markers phosphorylase *b*, 97.4 kDa (lower band), and β-galatosidase, 116 kDa; lanes 2–6, DTNB with no substrates and with NAD^+, NADH, $NADP^+$, and NADPH present, respectively; lanes 7–11, NEM with no substrates and with NAD^+, NADH, $NADP^+$, and NADPH present, respectively; lanes 12–14, $Cu(OP)_2$ with no substrates and with NAD^+ and $NADP^+$ present, respectively.

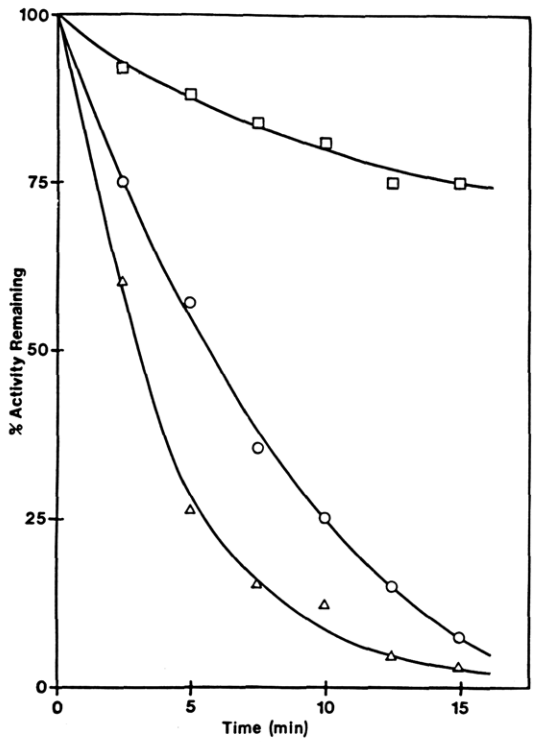

FIGURE 4: Substrate effects on the rate of $Cu(OP)_2$ inactivation in purified transhydrogenase. Soluble transhydrogenase was incubated at 50 μg/mL of protein in 100 mM sodium phosphate/0.05% sodium cholate, pH 7.5 at 24 °C, with 100 μM $Cu(OP)_2$ in the absence (△) or presence of 1 mM NAD^+ (○) or $NADP^+$ (□). At the indicated times, aliquots were removed and assayed for transhydrogenase activity.

activation of transhydrogenase is illustrated in Figure 4. As can be seen, the extent of cross-linking paralleled the extent of inactivation. $NADP^+$ protects the enzyme from inactivation and cross-linking, while NAD^+ provides no effect on either. The effect of the reduced substrates NADH and NADPH cannot be tested due to their oxidation by $Cu(OP)_2$. Presumably, cross-linking inactivates the enzyme either by destroying the NADP-binding site or by causing a loss of the flexibility of the enzyme, thus preventing conformational changes normally seen after substrate binding.

Inhibition by DTNB is generally caused by formation of a thionitrobenzoate adduct. However, upon electrophoresis, it was discovered that DTNB also caused the formation of an intramolecular cross-link (Figure 3, lanes 2–6). The change in mobility for both DTNB- and $Cu(OP)_2$-inactivated transhydrogenase is ~9000 Da. The identity of sulfhydryl groups involved in the formation of the disulfide cross-link was analyzed by DTNB titrations. Sulfhydryl quantitation of $Cu(OP)_2$-inactivated enzyme (Table II) showed that, with 20% of the activity remaining after $Cu(OP)_2$ inhibition, only 0.6 sulfhydryl group was titrated by DTNB before SDS addition while all 4.0 buried groups were reactive to DTNB after SDS addition. In contrast, when the enzyme was treated with $Cu(OP)_2$ in the presence of $NADP^+$ followed by titration with DTNB after removal of $NADP^+$, approximately two exposed sulfhydryl groups were observed. This indicates that the two DTNB-reactive sulfhydryl groups can form a disulfide bond upon reaction with $Cu(OP)_2$. Hence, the disulfide bond formed is between the NADP-binding site and the peripheral sulfhydryl groups. Cross-linking of these residues prevented complete unfolding of the protein during SDS gel electrophoresis and thereby altered its mobility. The mechanism by which DTNB inactivates and cross-links transhydrogenase is proposed to result from two sequential disulfide exchanges taking place at the NADP-binding site. Initially, one sulfhydryl group at the NADP-binding site becomes modified to form a transhydrogenase–thionitrobenzoate adduct. The impending displacement exerted by a nearby peripheral sulfhydryl group causes the formation of a intramolecular disulfide bond. Therefore, the binding site and the peripheral sulfhydryl groups are vicinal in the tertiary structure. It is likely then, because of the apparent close proximity and the similarity of the pK_a's, these two exposed sulfhydryl groups are both in the NADP-binding domain of the active site. NEM does not cross-link the enzyme (Figure 3, lanes 7–11) due to the irreversible nature of covalent modification.

Table II: Sulfhydryl Quantitation of $Cu(OP)_2$-Modified Soluble Transhydrogenase

addition	% control activity	no. of sulfhydryl groups/monomer −SDS	no. of sulfhydryl groups/monomer +SDS
	100	2.0	3.7
$Cu(OP)_2$[b]	20	0.6	4.0
$Cu(OP)_2$, dialyzed[c]	10	0.5	3.6
$Cu(OP)_2$ + $NADP^+$, dialyzed[d]	100	1.8	3.5

[a] Sulfhydryl quantitation was performed as in Table I. [b] Transhydrogenase was incubated at 101 μg/mL of protein in 100 mM sodium phosphate/0.05% sodium cholate, pH 8.0, with 50 μM $Cu(OP)_2$ for 20 min at 26 °C. The reaction was stopped with EDTA (5 mM final concentration) and immediately used for sulfhydryl quantitation. [c] After the incubation, the stopped reaction mixture was dialyzed against 1 L of 100 mM sodium phosphate/0.05% sodium cholate, pH 8.0, with one change of buffer after 4 h. [d] The incubation was performed in the presence of 1 mM $NADP^+$ and then dialyzed as in footnote *c* to remove $NADP^+$.

Reversal of Cross-Linking by DTT. Since the presumed cause of inactivation by either $Cu(OP)_2$ or DTNB is the formation of a disulfide bond, activity should be restored by sulfhydryl reducing agents provided the enzyme did not denature while being modified. Figure 5 shows that DTT (5 mM) restores 78% and 86% of the original activity to DTNB- and $Cu(OP)_2$-inactivated enzyme, respectively. This result provides further evidence that oxidation of two sulfhydryl groups is indeed involved during cross-linking reactions.

Intramolecular Cross-Linking of Submitochondrial Particle Transhydrogenase. To investigate whether or not the phenomenon of intramolecular cross-linking by $Cu(OP)_2$ and DTNB occurs in native SMP transhydrogenase, incubation of SMP with either reagent was performed and then the reaction mixture was subjected to SDS gel electrophoresis and immunoblot analysis. As shown in Figure 6A, the mobility of SMP transhydrogenase analyzed by SDS–polyacrylamide

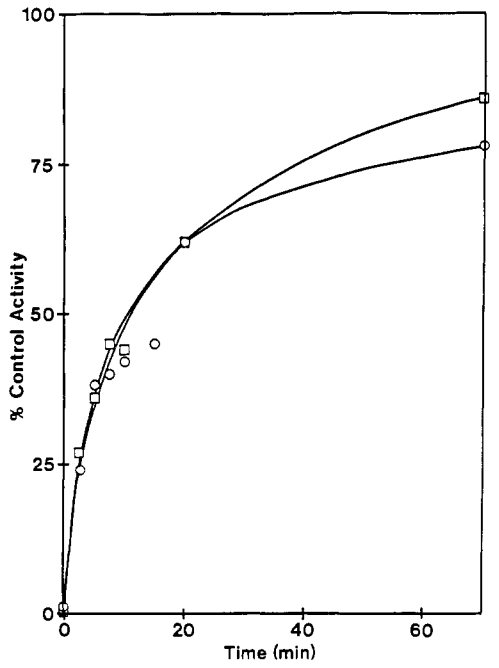

FIGURE 5: DTT reactivation of DTNB- and $Cu(OP)_2$-inactivated soluble transhydrogenase. Soluble transhydrogenase (50 μg/mL) was preincubated with either 25 μM DTNB (○) or 100 μM $Cu(OP)_2$ (□) for 15 min in 100 mM sodium phosphate/0.05% sodium cholate, pH 7.5 at 24 °C. The reaction mixtures were both made to 10 mM EDTA and 5 mM DTT and incubated further. At the indicated times, aliquots were removed and assayed for activity with zero time being the time at which DTT was added. The regained transhydrogenase activity was expressed as the percent of the control activity present before inactivation.

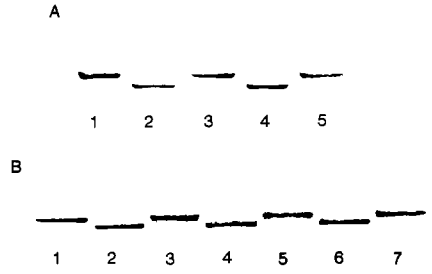

FIGURE 6: Cross-linking of submitochondrial particle transhydrogenase with $Cu(OP)_2$ and DTNB. (A) $Cu(OP)_2$ cross-linking of SMP. Submitochondrial particles were preincubated at 25 °C for 5 min in a medium containing 8 mg/mL SMP protein, 50 mM sucrose, 50 mM HEPES (pH 8.0), 10.5 μM rotenone, and 5 μg/mL antimycin A. The inactivation was initiated by addition of 400 μM $Cu(OP)_2$ and 1 mM NAD^+ or $NADP^+$ as indicated and the mixture incubated at 25 °C for 6 min. EDTA was added to 40 mM to stop the reaction, and then half of the reaction volume of three times concentrated Laemmli sample buffer containing 15 mM NEM (no β-mercaptoethanol) was added and incubated for 40 min at 25 °C. The reaction mixture (100 μg of SMP protein) was electrophoresed on 10% SDS–polyacrylamide gels and analyzed by Western blot immunoanalysis (Wu et al., 1985). Lane 1, untreated SMP; lane 2, $Cu(OP)_2$; lane 3, $Cu(OP)_2$ + $NADP^+$; lane 4, $Cu(OP)_2$ + NAD^+; lane 5, $Cu(OP)_4$ + EDTA. (B) DTNB cross-linking of SMP. Submitochondrial particles were preincubated at 4 °C for 5 min in a medium containing 4 mg/mL SMP protein, 50 mM sucrose, 50 mM HEPES (pH 8.0), 5 μg/mL antimycin A, and 10.5 μM rotenone. The inactivation was initiated with addition of 50 μM DTNB and 1 mM substrates where indicated, and the mixture was incubated at 4 °C for 10 min. The reaction mixture (100 μg of SMP protein) was subjeted to SDS–polyacrylamide gel electrophoresis and then analyzed by Western blot immunoanalysis. Lane 1, untreated SMP; lane 2, DTNB; lane 3, DTNB + $NADP^+$; lane 4, DTNB + NAD^+; lane 5, DTNB + NADPH; lane 6, DTNB + NADH; lane 7, DTNB, zero time.

gel electrophoresis is substantially increased upon $Cu(OP)_2$ treatment, and confirmation that the protein of higher mobility is transhydrogenase (lane 2) was provided by Western blot immunoanalysis. $NADP^+$ protected completely against cross-linking (lane 3), and NAD^+ was without effect (lane 4). DTNB modification of SMP transhydrogenase also leads to cross-linking of the vicinal cysteines (Figure 6B, lane 2). $NADP^+$ (lane 3) and NADPH (lane 5) completely protected SMP transhydrogenase from cross-linking by DTNB, which further supports previous reported results (O'Neal & Fisher, 1977) that both substrates protected against inactivation. NAD^+ (lane 4) and NADH (lane 6) did not affect cross-linking.

Table III: Sulfhydryl Quantitation of Modified Soluble Transhydrogenase

modification reagent	substrate present[b]	no. of sulfhydryl groups/monomer[a] -SDS	+SDS
		1.9	4.0
NEM[c]		0.9	3.5
NEM[c]	NADPH	0.9	1.0
NEM/IAA[d]		0.1	3.9
IAA[e]		0.2	4.2
IAA[e]	NADPH	0.8	0.8

[a] Sulfhydryl quantitation was performed as in Table I. [b] Where noted, NADPH was present at a final concentration of 1 mM during NEM or IAA inactivation but absent during quantitation. [c] Transhydrogenase was inactivated in 100 mM sodium phosphate/0.05% sodium cholate, pH 7.5, with 500 μM NEM for 15 min and then dialyzed against 1 L of 100 mM sodium phosphate/0.05% sodium cholate, pH 7.5, with one change after 4 h. [d] Following inactivation as described in footnote *c*, 50 mM IAA was added and the mixture was incubated for an additional 30 min before dialysis. [e] Transhydrogenase was inactivated in 100 mM sodium phosphate/0.05% sodium cholate, pH 7.5, with 50 mM IAA for 30 min before dialysis against 1 L of 100 mM sodium phosphate/0.05% sodium cholate, pH 7.5, with one change after 4 h.

DTNB Titrations of Chemically Modified Transhydrogenase. To determine the number of sulfhydryl groups modified by NEM or IAA in the presence or absence of NADPH, the modified enzyme was further titrated with DTNB. Sulfhydryl quantitation of NEM-modified transhydrogenase gives one exposed sulfhydryl group prior to SDS addition and about four sulfhydryl groups after SDS addition (Table III). This confirms previous SMP inactivation studies that NEM can only react with one cysteine in the NADP site (Earle et al., 1978). Inactivation with IAA caused loss of two DTNB-reactive sulfhydryl groups. The NEM-modified enzyme was further reacted with IAA, which resulted in modification of both exposed sulfhydryl groups because none were titrated with DTNB in the absence of SDS. These results show that NEM can modify only one while IAA and DTNB can modify two surface sulfhydryl groups in the absence of substrates and NEM does not sterically hinder the other surface sulfhydryl groups. NEM and IAA modified, in the presence of NADPH, approximately four sulfhydryl groups, one exposed and three buried, in both cases. These data indicate then that three additional sulfhydryl groups are exposed to the medium in the NADPH–enzyme complex.

Discussion

In this report conformational features of bovine heart mitochondrial transhydrogenase have been evaluated with respect to sulfhydryl accessibility. Both soluble and reconstituted transhydrogenases contain two cysteine residues at the NADP-binding site that are reactive to DTNB. Binding of $NADP^+$ resulted in DTNB titration of one less cysteine before SDS addition in the soluble enzyme and two less cysteines in the reconstituted system. This discrepancy between the two systems suggests that possibly $NADP^+$ can protect the binding

site better in the reconstituted system as a result of tighter binding to shield two cysteines from contact with DTNB while selectively protecting one in the purified enzyme.

In the presence of NADPH, at last two additonal sulfhydryl groups were exposed under the conditions examined in both systems giving four DTNB-reactive sulfhydryl residues before SDS addition. It seems very likely there are three additional sulfhydryl groups exposed in the presenceof NADPH since NEM inactivation in the presence of NADPH results in the modification of a total of four sulfhydryl groups. Of these, one is the peripheral sulfhydryl group in the NADP-binding domain that is normally exposed and reactive toward NEM while the three others are normally buried and not accessible toward NEM. It is also likely that one of these buried sulfhydryl groups, when exposed to NEM modification in the presence of NADPH, is responsible for the rapid inactivation seen with NEM. The fact that in the presence of NADPH four sulfhydryl groups were reactive to DTNB even in reconstituted transhydrogenase, a system that would more environmentally resemble the native membrane enzyme, supports the idea of substrate-induced conformational changes. Hence, it is concluded that exposure of more sulfhydryl groups by NADPH reflects its ability to alter transhydrogenase conformation upon NADPH binding.

One feature of the NADP-binding-site sulfhydryl groups confirmed here was their close proximity to one another. $Cu(OP)_2$, a vicinal sulfhydryl-specific reagent, and DTNB caused the formation of an intramolecular cross-link between the two exposed cysteines resulting in a change in mobility of ~9000 Da during SDS–polyacrylamide gel electrophoresis. This decrease in apparent molecular weight during electrophoresis is probably caused by the more globular nature of the cross-linked enzyme induced by the newly formed disulfide bridge, thus preventing the complete denaturation of the enzyme by SDS. The native submitochondrial particle transhydrogenase was shown also to have an altered electrophoretic mobility upon reaction with $Cu(OP)_2$ and DTNB. Our results show that $NADP^+$ and NADPH are both effective in completely protecting against inactivation and cross-linking by DTNB in SMP. The mechanism by which DTNB cross-links is thought to involve two sequential disulfide-exchange reactions. Note that this mechanism results in the release of two thionitrobenzoate anions. Formation of a disulfide cross-link would therefore be indistinguishable during a sulfhyryl quantitation from two separate attacks by DTNB.

If DTNB inactivates the enzyme by formation of a disulfide, those substrates that protect against inhibition should protect against cross-linking, while those that stimulate inhibition should also stimulate cross-linking. This idea held well for all conditions tested except DTNB inactivation in the presence of NADPH (Figure 3, lane 6). The purified enzyme was inhibited by DTNB slighty more rapidly in the presence than in the absence of NADPH (Figure 1A); however, cross-linking did not increase proportionally. In fact, in the presence of NADPH, the DTNB-modified product was largely not cross-linked as evidenced by the intensity of the upper major band in Figure 3 (lane 6). This contradiction suggests that NADPH protects DTNB cross-linking to some extent by entering into the NADP-binding site. Also, because IAA modifies only one NADP-site sulfhydryl group in the presence of NADPH (Table III), it would seem the other sulfhydryl group is shielded from contact with the medium. However, it is possible that those two NADP-site sulfhydryl groups are still within the necessary distance to allow some cross-linking by DTNB even in the presence of NADPH. Note that the prerequisite for cross-linking is displacement of one thionitrobenzoate anion by a nearby sulfhydryl group on the enzyme. Thus, DTNB appears to cross-link both NADP-site sulfhydryl groups and modify two buried sulfhydryl groups to some exent as demonstrated by the appearance of the faster migrating band in SDS gels. More likely though, NADPH could remain bound to protect one of the NADP-site sulfhydryl groups while exposing the peripheral and three other buried sulfhydryl groups to DTNB, in a manner similar to IAA modification in the presence of NADPH. Thus, in the presence of NADPH, it is believed, NEM, IAA, and DTNB are able to modify one binding site and three buried sulfhydryl groups. The slower rate of inactivation seen with DTNB than with NEM or IAA is possibly a result of DTNB having limited access to one of these buried sulfhydryl groups.

As mentioned previously, NEM modifies only one sulfhydryl group, in the absence of substrates. Since both NEM- and non-NEM-reactive cysteines in the NADP-binding site have the same pK_a, and modification of transhydrogenase by NEM does not sterically hinder the other cysteine from modification by either DTNB or IAA, there must be some other factor(s) influencing the reactivity of the NEM-reactive sulfhydryl group. If one of the cysteines is in a more hydrophobic environment than the other, then possibly this residue will react preferentially with a relatively hydrophobic molecule such as NEM. It is reasonable to conclude that there is some charged or polar group near the non-NEM-reactive sulfhydryl group that disturbs the overall general hydrophobicity of the NADP-binding site. Placement of the NEM-reactive sulfhydryl group would be such that $NADP^+$ cannot protect it from NEM inactivation, possibly because of an increase in local hydrophobicity after $NADP^+$ insertion into its binding pocket.

A dithiol–disulfide interconversion has recently been suggested to be involved in the proton translocation activity of transhydrogenase (Person & Rydström, 1987; Robillard & Konings, 1982). The results presented in this paper demonstrate that the purified heart transhydrogenase possessed two exposed cysteine residues that were cross-linked to form an intramolecular disulfide bond with DTNB. Sulfhydryl group titration studies on $Cu(OP)_2$-modified enzyme confirm that the two residues reactive with DTNB are those that were cross-linked by $Cu(OP)_2$. Inhibition and cross-linking of the enzyme by DTNB and $Cu(OP)_2$ were reversible on treatment with dithiothreitol, demonstrating that modification was limited to sulfhydryl groups. Reaction of the exposed residues was presented by $NADP^+$ or NADPH, but not by NAD^+ or NADH. Hence, it is concluded that vicinal cysteines are at or near the NADP-binding site.

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Role of Peptide Conformation in the Rate and Mechanism of Deamidation of Asparaginyl Residues†

Richard Lura‡ and Verne Schirch*
Department of Biochemistry, Virginia Commonwealth University, Richmond, Virginia 23298
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ABSTRACT: The tetrapeptides Val-Asn-Gly-Ala and *N*-acetyl-Val-Asn-Gly-Ala undergo deamidation of the asparaginyl residue at pH 7.0 at similar rates. However, they form different products. The *N*-acetyl peptide gave a 3:1 ratio of *N*-acetyl-Val-isoAsp-Gly-Ala and *N*-acetyl-Val-Asp-Gly-Ala, respectively. The nonacetylated peptide gave no detectable amounts of these products but rather gave a cyclic peptide formed from the nucleophilic displacement of the asparaginyl side chain amide by the amino terminus of valine. This compound was slowly inverted at carbon 2 of the asparaginyl residue. At pH values above 7.5, the nonacetylated peptide also underwent deamidation to form Val-isoAsp-Gly-Ala and Val-Asp-Gly-Ala in the 3:1 ratio. Proton NMR spectra of the acetylated and nonacetylated tetrapeptides show that below pH 7.5 they have very different preferred conformations, and it is these different conformations which result in the different mechanisms of deamidation. Above pH 9.0, both peptides have similar conformations and deamidate by the same mechanism to give equivalent products. Neither mechanism of deamidation was subject to general base catalysis by the buffer. These results suggest that deamidation rates of the asparaginyl-glycyl sequence in proteins will vary according to the conformation of the peptide backbone of each respective protein. The results also show that asparaginyl residues which are penultimate to the amino terminus can react to form an N-terminal-blocked seven-membered ring.

Asparagine and glutamine residues in proteins have been observed to undergo nonenzymatic deamidation in neutral to mildly alkaline solutions. The rate of these reactions has been shown to be dependent on the amino acid residues near the asparagine or glutamine residue (Robinson & Rudd, 1974). The time frames of these deamidation reactions are similar to the time frames of protein turnover in the cell (Midelfort & Mehler, 1972). Robinson has proposed that these deamidation reactions serve as biological clocks in determining the function and catabolism of proteins. Recently, the deamidation of an asparagine residue has been implicated as a signal for the degradation of triosephosphate isomerase and in adrenocorticotropic hormone, being a substrate for protein carboxyl methyltransferase (Yuksel & Gracy, 1986; Murray & Clarke, 1984; Aswad, 1984).

The deamidation of asparagine residues, which occur in the sequence Asn-Gly, has been shown to be particularly susceptible to deamidation and to undergo a rearrangement to an isoaspartyl peptide bond (Sandheimer & Holley, 1954; Battersby & Robinson, 1955; Haley et al., 1966; Geiger & Clarke, 1987; Meinwald et al., 1987). Mechanism studies on model peptides have shown that an intermediate in this reaction is a succinimide compound, which hydrolyzes in approximately a 3:1 ratio to the iso and normal peptide bond, respectively. Most of the information on the mechanism and rate of deamidation of Asn-Gly sequences has been obtained from studies on short model peptides. The determination of the rate and mechanism of this reaction in proteins is complicated by

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‡ Present address: Department of Chemistry, Milligan College, Milligan College, TN 37682.

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