Chemical Modification of Mitochondrial Transhydrogenase: Evidence for Two Classes of Sulfhydryl Groups†

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ABSTRACT: Chemical-modification studies on submitochondrial particle pyridine dinucleotide transhydrogenase (EC 1.6.1.1) demonstrate the presence of one class of sulfhydryl group in the nicotinamide adenine dinucleotide phosphate (NADP) site and another peripheral to the active site. Reaction of the peripheral sulfhydryl group with *N*-ethylmaleimide, or both classes with 5,5′-dithiobis(2-nitrobenzoic acid), completely inactivated transhydrogenase. $NADP^+$ or NADPH nearly completely protected against 5,5′-dithiobis(2-nitrobenzoic acid) inactivation and modification of both classes of sulfhydryl groups, while $NADP^+$ only partially protected against and NADPH substantially stimulated *N*-ethylmaleimide inactivation. Methyl methanethiolsulfonate treatment resulted in methanethiolation at both classes of sulfhydryl groups, and either $NADP^+$ or NADPH protected only the NADP site group. *S*-Methanethio and *S*-cyano transhydrogenases were active derivatives with pH optima shifted about 1 unit lower than that of the native enzyme. These experiments indicate that neither class of sulfhydryl group is essential for transhydrogenation. Lack of involvement of either sulfhydryl group in energy coupling to transhydrogenation is suggested by the observations that *S*-methanethio transhydrogenase is functional in (a) energy-linked transhydrogenation promoted by phenazine methosulfate mediated ascorbate oxidation and (b) the generation of a membrane potential during the reduction of NAD^+ by reduced nicotinamide adenine dinucleotide phosphate (NADPH).

Pyridine dinucleotide transhydrogenase, an integral protein of the inner mitochondrial membrane, catalyzes a reversible and direct transfer of a hydride ion equivalent between oxidized and reduced forms of matrix NAD and NADP (Lee and Ernster, 1966; Rydström, 1977). Transhydrogenation between NADPH and NAD^+ is coupled to the (a) generation of a membrane potential having the same polarity as that observed upon the hydrolysis of ATP or the oxidation of respiratory substrates (Dontsov et al., 1972), (b) uptake of protons by submitochondrial particles (Mitchell and Moyle, 1965), and (c) synthesis of ATP from ADP and P_i (van de Stadt et al., 1971). Respiration or ATP hydrolysis provides the energy for reversal of transhydrogenation, stimulating the rate and extent of $NADP^+$ reduction by NADH (Danielson and Ernster, 1963; Lee and Ernster, 1964).

Based on these observations, and by analogy to other respiratory chain energy-conserving sites, transhydrogenase has been proposed to function as a reversible proton pump. Several mechanisms have been proposed for energy coupling at this site, including a redox loop incorporating a reduced enzyme intermediate (Mitchell, 1966) and substrate-dependent conformational changes in the enzyme that reorient a proton-binding domain, either on transhydrogenase itself or on an associated coupling protein, from one side of the membrane to the other (Skulachev, 1974; Blazyk et al., 1976; Rydström, 1977).

Selective chemical modification of specific amino acid residues in the catalytic and putative proton-binding domains is the approach used here to evaluate the relationship between transhydrogenation and energy coupling. Sulfhydryl groups are essential for transhydrogenase activity (Humphrey, 1957; Kaufman and Kaplan, 1961; Kurup and Sanadi, 1968). A sulfhydryl group in the NADP binding site of the bovine heart enzyme has been identified by chemical modification with Nbs_2,[1] during which inhibition of non-energy-linked transhydrogenase was prevented by the presence of $NADP^+$ or NADPH, while NAD^+ and NADH had no effect (O'Neal and Fisher, 1977). $NADP^+$ or NADPH nearly completely protected the ability of the enzyme to couple transhydrogenation to membrane energization, indicating that a Nbs_2 reactive sulfhydryl group outside the catalytic site is not involved in energy conservation. In this paper, chemical modifications with several sulfhydryl reagents provide evidence for a second class of sulfhydryl groups outside of the active site of transhydrogenase and that this class of sulfhydryl groups is also not involved directly in energy coupling.

Materials and Methods

Phosphorylating submitochondrial particles (Löw and Vallin, 1963) were prepared from bovine heart mitochondria (Sanadi et al., 1967) and stored at −70 °C at 17 mg of protein/mL in 10 mM Tris-HCl buffer (pH 8.0) containing 250 mM sucrose. Protein was analyzed using the Biuret procedure (Jacobs et al., 1956), with bovine serum albumin as a standard.

Chemical modifications of submitochondrial particle transhydrogenase with NEM or Nbs_2 were performed at 23 °C in a medium (0.1 mL) containing 0.2–0.5 mg of submitochondrial particle protein, 23 mM Tris–acetate buffer (pH 7.5), 50 mM sucrose, and other additions as indicated. Chemical modifications with MMTS were performed in an

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[1] Abbreviations used: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); $AcPyAD^+$, oxidized 3-acetylpyridine adenine dinuclotide; AcPyADH, reduced 3-acetylpyridine adenine dinucleotide; DPCC, diphenylcarbamyl chloride; MMTS, methyl methanethiolsulfonate; NEM, *N*-ethylmaleimide; thio-NAD^+, oxidized thionicotinamide adenine dinucleotide; thio-$NADP^+$, oxidized thionicotinamide adenine dinucleotide phosphate; thio-NADPH, reduced thionicotinamide adenine dinucleotide phosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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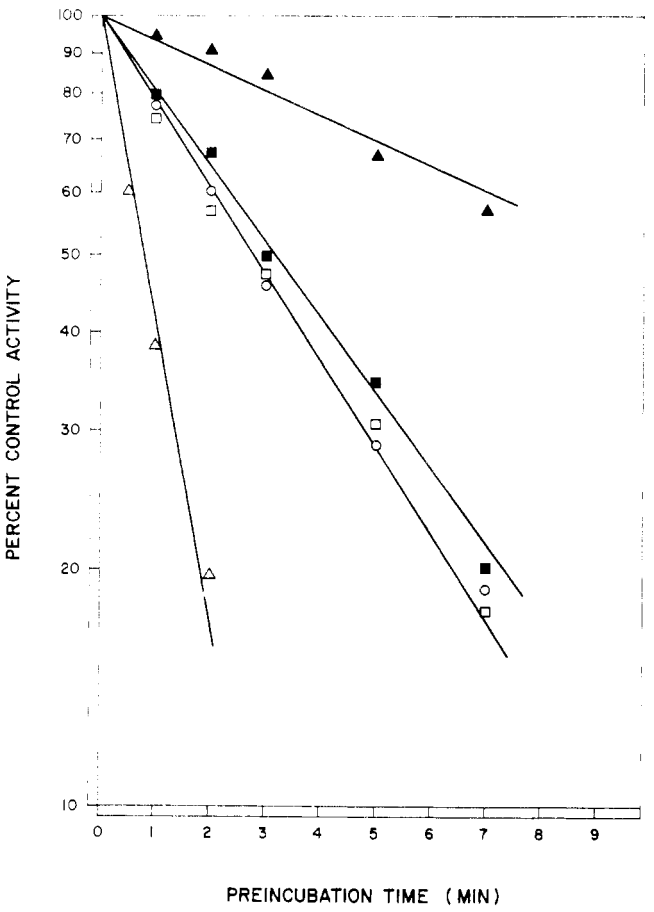

FIGURE 1: Effect of substrates on NEM inactivation of transhydrogenase. Submitochondrial particles (0.28 mg of protein) were preincubated at 23 °C in the presence of 700 μM NEM for the indicated times and assayed for reverse transhydrogenase activity as described under Materials and Methods. Substrates were present at 300 μM: no addition (○), $NADP^+$ (▲), NADPH (△), NAD^+ (■), NADH (□). Potassium cyanide (2 mM) was added to reaction mixtures containing NADH to inhibit NADH oxidase. The control rate without NEM was 232 nmol of AcPyADH·min^{-1}·(mg of protein)$^{-1}$.

identical manner, except that 14 mM Tris-acetate (pH 7.5) was present as buffer. Modification reactions were quenched by the addition of the incubation medium to the transhydrogenase assay mixture.

S-Methanethio transhydrogenase was prepared by incubating 5.0–7.5 mg of submitochondrial particle protein with 250 μM MMTS in a medium (2 mL) containing 14 mM Tris-acetate buffer (pH 7.5) and 50 mM sucrose. After 10 min, 3 mL of ice-cold 10 mM Tris-HCl buffer (pH 8.0) containing 250 mM sucrose was added, and the particles were sedimented at 105 000*g* for 30 min. The pellet was washed once in 5 mL of 10 mM Tris-HCl buffer (pH 8.0) containing 250 mM sucrose and resuspended in the same buffer to approximately 13 mg of protein/mL.

S-Thionitrobenzoate transhydrogenase was prepared by incubating 5.5–8.6 mg of submitochondrial particle protein with 70 μM Nbs_2 at 23 °C in a medium (1 mL) containing 200 mM sucrose, 15 mM Tris-HCl buffer (pH 8.0), and other additions as indicated. After 15 min, the reaction was quenched by the addition of 4 mL of ice-cold 10 mM Tris-HCl buffer (pH 8.0) containing 250 mM sucrose. The particles were sedimented, washed, and resuspended exactly as described for *S*-methanethio transhydrogenase.

The *S*-cyano derivative of submitochondrial particle transhydrogenase was prepared from the thionitrobenzoate derivative as described by O'Neal and Fisher (1977).

Proteolytic inactivation of transhydrogenase was performed at 23 °C in a medium (0.1 mL) containing 23 mM Tris-acetate buffer (pH 7.5), 0.25 mg of submitochondrial particle protein, 7.5 μg of trypsin, and other additions as indicated. After 30 s, 40 μg of trypsin inhibitor was added, and the incubation medium was immediately diluted into the assay mixture.

All transhydrogenase assays were performed at 23 °C without the use of regenerating systems for substrates. Reverse non-energy-linked transhydrogenase was assayed using the 3-acetylpyridine analogue of NAD^+ ($AcPyAD^+$), as described by Blazyk and Fisher (1975). Forward non-energy-linked transhydrogenase was assayed by following the reduction of thio-$NADP^+$ by NADH at 395 nm, assuming a millimolar extinction coefficient of 11.3 (Fisher and Kaplan, 1973). The 2.8-mL assay medium contained 0.27 mg of submitochondrial particle protein, 100 mM Tris-acetate buffer, 140 mM sucrose, 23 μM rotenone, 0.45 μg of antimycin A, 3 μg of oligomycin, 175 μM NADH, and 117 μM thio-$NAPD^+$. Energy-linked transhydrogenase, driven by electron transport through the terminal coupling site of the electron transport chain, was assayed in the same medium after the addition of 5 mM ascorbate plus 1.1 μM phenazine methosulfate (final volume 3.0 mL).

Membrane potential generated during transhydrogenase-catalyzed reduction of NAD^+ by NADPH was monitored by the fluorescence response of 8-anilino-1-naphthalenesulfonate (Donstov et al., 1972) at 480 nm after excitation at 405 nm. The assay medium (1.2 mL) contained 2.2 mg of submitochondrial particle protein, 186 mM sucrose, 25 mM Tris-acetate buffer (pH 7.5), 8.3 mM pyruvate, 0.4 mM glucose 6-phosphate, 4.2 mM KCN, 0.6 mM rotenone, 8 μM 8-anilino-1-naphthalenesulfonate, 30 μg of glucose-6-phosphate dehydrogenase (11 units), 10 μg of lactate dehydrogenase (9.7 units), 40 μM NAD^+, and 40 μM NADPH.

Cytochrome *c* oxidase activity of submitochondrial particles was measured using a Clark electrode at 30 °C in a medium (1.60 mL) containing 19 mM Tris-acetate buffer (pH 7.5), 130 mM sucrose, 3 μg of oligomycin, 0.45 μg of antimycin A, 0.26 mg of submitochondrial particle protein, 3.3 μM phenazine methosulfate, and 15 mM ascorbate.

NADPH, $NADP^+$, thio-$NADP^+$, NADH, and NAD^+ were products of P-L Biochemicals, Inc. $AcPyAD^+$ was prepared as described by Kaplan and Ciotti (1954). Trypsin (DPCC treated), trypsin inhibitor, and all other materials were products of Sigma Chemical Co. Thio-NAD^+ and MMTS were generous gifts from Dr. R. B. Dunlap. Dephospho-CoA was a gift from Boehringer Mannheim Biochemicals.

Results

NEM Modification of Transhydrogenase. The rate of inhibition of submitochondrial particle transhydrogenase by NEM was pseudo first order. The effects of transhydrogenase substrates on inactivation by NEM are shown in Figure 1. The presence of NADH or NAD^+ had no significant effect. However, both $NADP^+$ and NADPH influenced the rate of inactivation of transhydrogenase by NEM. The enzyme was partially protected from inactivation by $NADP^+$, while NADPH potentiated the inactivation of the enzyme. By comparison, Nbs_2 inactivation of transhydrogenase was nearly completely prevented by either $NADP^+$ or NADPH, while NAD^+ and NADH were without effect (O'Neal and Fisher, 1977). These latter results were interpreted to indicate that a class of sulfhydryl groups exists at, or is controlled by, the NADP binding site. The opposite effects induced by NADPH on the inactivation of transhydrogenase by NEM and Nbs_2

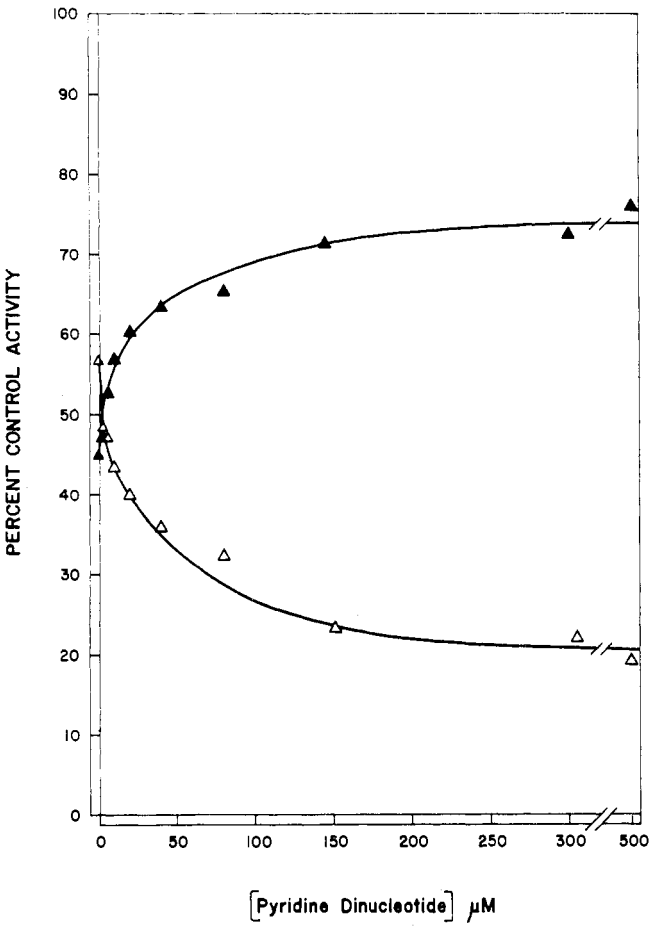

FIGURE 2: Influence of $NADP^+$ and NADPH concentration on NEM inactivation of transhydrogenase. Submitochondrial particles (0.28 mg of protein) were preincubated with 700 μM NEM at 23 °C for 2 min in the presence of $NADP^+$ (▲) or NADPH (△) and immediately assayed for reverse transhydrogenase activity as described under Materials and Methods. The control rate in the absence of NEM was 259 nmol of AcPyADH·min^{-1}·(mg of protein)$^{-1}$.

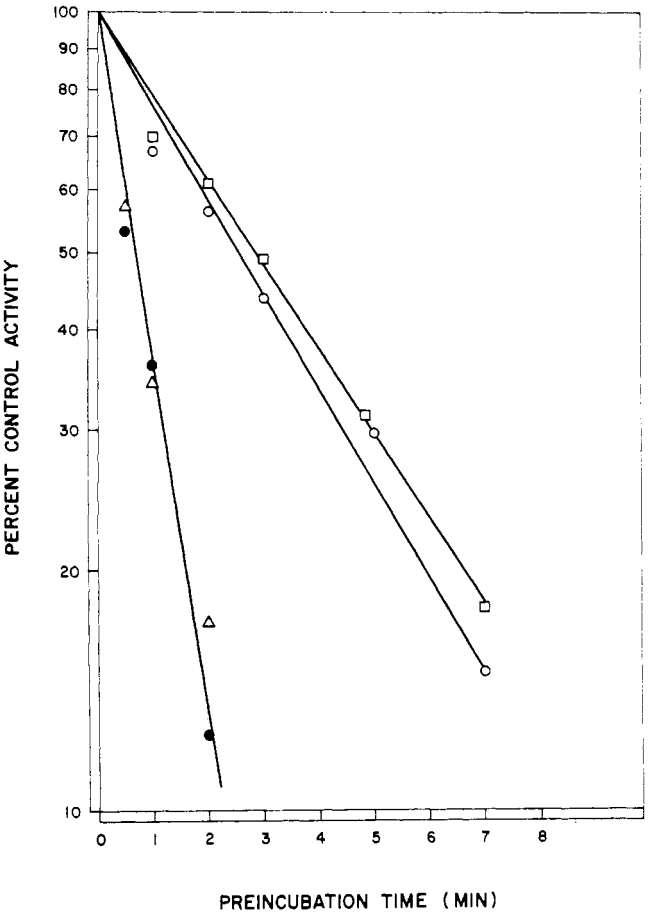

FIGURE 3: Effect of NADH on NADPH-induced NEM inactivation of transhydrogenase. Submitochondrial particles (0.28 mg of protein) were preincubated at 23 °C with 700 μM NEM in the absence of substrate (○) or in the presence of 300 μM NADH (□), NADPH (△), or NADH + NADPH (●) for the indicated times and then immediately assayed for reverse transhydrogenase activity as described under Materials and Methods. All preincubation mixtures contained 2 mM KCN. The control rate in the absence of NEM was 236 nmol of AcPyADN·min^{-1}·(mg of protein)$^{-1}$.

suggest the existence of a second class of peripheral sulfhydryl groups outside the NADP binding site. In the presence of NADPH, the peripheral sulfhydryl groups apparently react with NEM but not with Nbs_2. The influence of $NADP^+$ and NADPH concentration on NEM inactivation of transhydrogenase is shown in Figure 2. $NADP^+$ protects against, and NADPH stimulates, NEM inactivation maximally at about 300 μM, with each substrate providing half-maximal effect at a concentration of about 25 μM. The latter values are consistent with the reported Michaelis constants for these substrates (Teixeira Da Cruz et al., 1971).

Attempts were made to assess further the location of the peripheral sulfhydryl groups. The peripheral sulfhydryl could form part of the NAD binding domain and be exposed for reaction only after NADPH binding. Houghton et al. (1976) have reported evidence for an essential sulfhydryl group in both the NADP and NAD binding sites of *Escherichia coli* transhydrogenase. Alternatively, the peripheral sulfhydryl groups could be located outside of the active site. Submitochondrial particle transhydrogenase is particularly susceptible to tryptic inactivation (Juntti et al., 1970). It has been reported that NAD^+ and NADH protect transhydrogenase from inactivation by trypsin (O'Neal and Fisher, 1977) and that NAD^+ protects the enzyme from inactivation by butanedione (Djavadi-Ohaniance and Hatefi, 1975), indicating that the enzyme possesses a functional NAD binding site in the absence of the NADP-site substrate. Further support for this notion derives from kinetic studies which show that the binding of substrates during transhydrogenation is ordered, with the NAD-site substrate being bound first (Rydström, 1972). As indicated in Figure 1, it is apparent that, since NAD^+ and NADH do not protect against inactivation, in the absence of NADPH there is no peripheral NEM reactive sulfhydryl group in the NAD binding site. In order to determine whether a peripheral sulfhydryl group is exposed in the NAD binding site as a result of a NADPH-induced transhydrogenase conformational change, submitochondrial particles were incubated with NEM and NADPH in the presence and absence of NADH. As can be seen in Figure 3, NADH had no effect on the rate of NADPH-induced inactivation. In similar studies, substitution of the NAD-site-specific inhibitor, dephospho-CoA (Rydström, 1972) or the NAD^+ analogue thio-NAD^+ (Anderson and Kaplan, 1959), for NADH had no effect on NADPH potentiated inactivation by NEM. It is concluded from these results that the peripheral sulfhydryl groups are outside both the NAD and NADP binding sites.

NEM Modification of S-Thionitrobenzoate Transhydrogenase. To define the reactivity of the two classes of sulfhydryl groups with Nbs_2, submitochondrial particle transhydrogenase was treated with the sulfhydryl reagent in the presence and absence of substrates. The resulting thionitrobenzoate transhydrogenase derivative was isolated and tested to determine

TABLE I: NEM Modification of *S*-Thionitrobenzoate Transhydrogenase.

addition to modif membr	initial preincubation additions[a]					
	none	Nbs_2	Nbs_2 + NAD^+	Nbs_2 + NADH	Nbs_2 + $NADP^+$	Nbs_2 + NADPH
expt 1						
none	200[e]	6.1	4.2	4.1		
NEM[b]	23.6	6.1	4.2	4.1		
DTT[c]	25.8	192	194	182		
expt 2						
none	323	6.9			265	202
NEM[d]	101				66.6	60.8
NEM + $NADP^+$[d]	216				163	137
NEM + NADPH[d]	33.6				23.5	15.7

[a] *S*-Thionitrobenzoate transhydrogenase was prepared as described under Materials and Methods, by incubating submitochondrial particles (8.6 mg of protein, expt 1; 7.5 mg of protein, expt 2) with 70 μM Nbs_2. Where indicated, 1 mM substrate was present, and the preincubation mixture containing NADH also contained 35 μM rotenone. After sedimentation, the particles were resuspended in 0.5 mL of 20 mM Tris-acetate buffer (pH 7.5) (expt 1) or 0.45 mL of 10 mM Tris-HCl buffer (pH 8) containing 250 mM sucrose (expt 2) and assayed for reverse transhydrogenase activity. [b] Resuspended *S*-thionitrobenzoate transhydrogenase (8.6 mg of protein) was preincubated a second time in a medium (2.8 mL) containing 20 mM Tris-acetate buffer (pH 7.5) and 1 mM NEM. After 15 min, the particles were diluted twofold with 10 mM Tris-HCl buffer (pH 8) containing 250 mM sucrose and centrifuged at 105 000*g* for 30 min. The pellet was resuspended in the dilution buffer (0.45 mL) and assayed for reverse transhydrogenase activity. [c] NEM-treated *S*-thionitrobenzoate transhydrogenase (0.38 mg of protein) was preincubated a third time at 23 °C in a medium (0.62 mL) containing 20 mM Tris-acetate buffer (pH 7.5) and 10 mM dithiothreitol. After 15 min, the incubation medium was added to the reverse transhydrogenase assay mixture. [d] Resuspended *S*-thionitrobenzoate transhydrogenase (0.33 mg of protein) was preincubated a second time at 23 °C in a medium (0.1 mL) containing 20 mM Tris-acetate (pH 7.5) and 1 mM NEM. After 2.5 min, the incubation medium was added to the reverse transhydrogenase assay mixture. Where indicated, $NADP^+$ or NADPH was present at 300 μM. [e] Table entries indicate transhydrogenase rate in (nmol of AcPyADH formed)·$(min)^{-1}$·$(mg\ of\ protein)^{-1}$.

if it remained sensitive to inhibition by NEM. From Table I (expt 1) it can be seen that NADH and NAD^+ had no effect on the extent of Nbs_2 inactivation. The resulting membranes containing *S*-thionitrobenzoate transhydrogenase were sedimented and preincubated a second time with sufficient NEM to give 85–90% inactivation of transhydrogenase in native submitochondrial particles. Subsequently, the membranes were treated with dithiothreitol under conditions which nearly completely reversed Nbs_2 inactivation when compared to control preparations not reacted with NEM. Membranes treated with Nbs_2 alone, and with Nbs_2 followed by NEM, gave essentially identical transhydrogenase activities after dithiothreitol reactivation, indicating that all NEM-reactive sulfhydryl groups had been protected by formation of thionitrobenzoate derivatives. These data confirm that there are no NEM-reactive essential sulfhydryl groups in the NAD binding site. In Table I (expt 2), membranes were preincubated with Nbs_2 in the presence and absence of either NADPH or $NADP^+$. Both $NADP^+$ and NADPH substantially protected transhydrogenase against Nbs_2 inactivation. After sedimentation of the membranes to remove excess Nbs_2, the membranes were resuspended and treated with NEM in the presence or absence of NADPH or $NADP^+$. It can be seen that the enzyme protected from Nbs_2 inactivation by $NADP^+$ or NADPH is affected in the same manner as native submitochondrial particle transhydrogenase by NEM; i.e., $NADP^+$ protects enzyme activity and NADPH potentiates inactivation. Taken together, these experiments suggest that when no substrate is present, or when NAD^+ or NADH is present, Nbs_2 reacts with both classes of sulfhydryl groups, while when $NADP^+$ or NADPH are present both classes of sulfhydryl groups are protected from reaction with Nbs_2, and transhydrogenase subsequently reacts normally with NEM. Further evidence that Nbs_2 reacts with both classes of sulfhydryl groups in the absence of substrates is demonstrated by studies on NEM modification of *S*-cyano transhydrogenase.

NEM Modification of S-Cyano Transhydrogenase. O'Neal and Fisher (1977) have reported that *S*-thionitrobenzoate transhydrogenase is converted into *S*-cyano transhydrogenase by reaction with cyanide ion. *S*-Cyano transhydrogenase has unaltered Michaelis constants for $NADP^+$ and NADPH (O'Neal, 1977) but differs from native enzyme in that the pH–rate profile is altered (cf. Figure 4). If Nbs_2 reacts with both the NADP site and peripheral classes of sulfhydryl groups, *S*-cyano transhydrogenase would be expected to be derivatized at both classes of sulfhydryl groups. To test this possibility, *S*-cyano transhydrogenase was treated with NEM alone and with NEM in the presence of $NADP^+$ or NADPH (Table II). Unlike native submitochondrial particle transhydrogenase, *S*-cyano transhydrogenase was only slightly inhibited by NEM. Furthermore, $NADP^+$ and NADPH had no effect on NEM inactivation. Higher preincubation concentrations of NEM did not give greater inhibition of *S*-cyano transhydrogenase, indicating that the slight inactivation does not result from the modification of essential sulfhydryl groups. Hence, it appears that NADPH binding to transhydrogenase does not expose a buried sulfhydryl group for selective reaction with NEM.

Methanethiolation of Transhydrogenase. Kenyon and coworkers (Smith et al., 1975) have introduced MMTS as a reversible blocking reagent specific for sulfhydryl groups. Methanethiolation of creatine kinase (Smith et al., 1975) and lactate dehydrogenase (Bloxham and Wilton, 1976) results in only partial inactivation, whereas modification of these two enzymes with larger sulfhydryl reagents causes complete inactivation. Incubation of submitochondrial particles with MMTS at 4 °C resulted in an instantaneous partial (~50%) inhibition of transhydrogenase when assayed at pH 6.8. The extent of inhibition was independent of MMTS concentration in the range of 0.05 to 2 mM. The pH–rate profiles of reverse non-energy-linked native and *S*-methanethio transhydrogenases are shown in Figure 4. As can be seen, the pH optimum for the derivatized enzyme is lowered by at least 1 pH unit. A similar shift in the pH–rate profile for *S*-cyano transhydrogenase was observed (Figure 4).

Reaction of S-Methanethio Transhydrogenase with NEM and Nbs_2. It was of interest to determine whether MMTS reacts with the same sulfhydryl groups as Nbs_2 and NEM. As

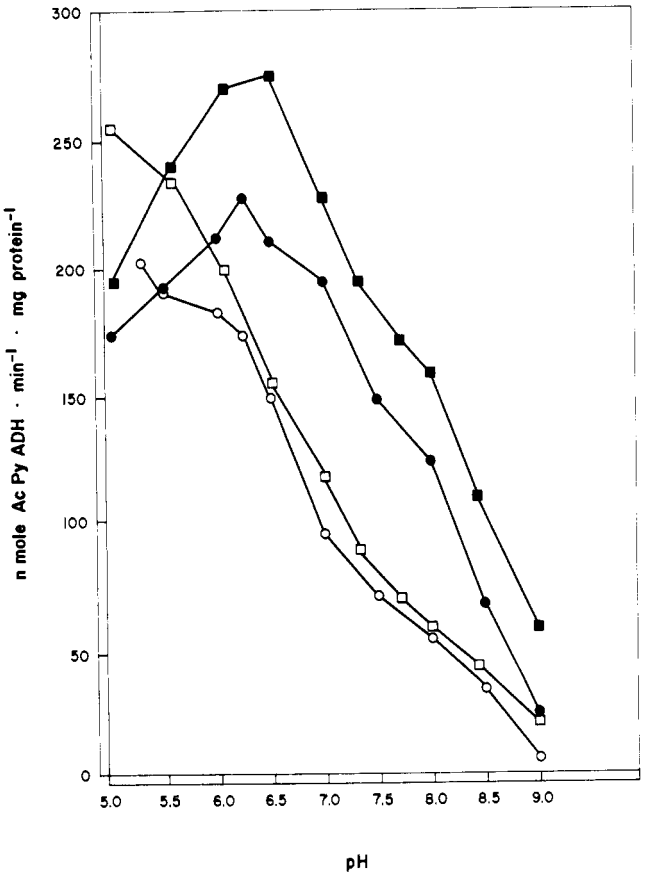

FIGURE 4: pH-rate profiles for reverse transhydrogenation catalyzed by native, *S*-methanethio, and *S*-cyano transhydrogenases. Native (●) and methanethiolated (○) submitochondrial particles (0.47 mg of protein) were assayed at 23 °C for reverse transhydrogenase activity as described under Materials and Methods, except that 0.1 M Tris-acetate buffer was substituted for phosphate buffer. In a separate experiment, native (■) and *S*-cyano derivatized (□) submitochondrial particles (0.50 mg of protein) were assayed as described above.

TABLE II: NEM Inhibition of *S*-Cyano Transhydrogenase.[a]

addition	native transhydrogenase act.[b]	native transhydrogenase % control	*S*-cyano transhydrogenase act.[b]	*S*-cyano transhydrogenase % control
none	175	100	82.5	100
NEM	72.5	40.7	69.7	84.5
NEM + NADPH	18.0	10.1	69.7	84.5
NEM + $NADP^+$	118	66.7	67.6	81.9

[a] Native and *S*-cyano transhydrogenases (0.37 mg of protein) were preincubated with NEM (1 mM for 2.5 min) alone or with NEM in the presence of 300 μM NADPH or $NADP^+$ and assayed immediately for reverse transhydrogenase activity. [b] Activity indicates transhydrogenase rate in (nmol of AcPyADH formed)·$(min)^{-1}$·$(mg\ of\ protein)^{-1}$.

TABLE III: Reaction of *S*-Methanethio Transhydrogenase with Nbs_2 or NEM.

addition to modif membr	initial preincubation addition[a] none	MMTS	MMTS + NADPH	MMTS + $NADP^+$
expt 1[b]				
none	279	131	122	123
Nbs_2	0	112	44.4	51.9
Nbs_2 + NADPH	151	112	106	95.7
Nbs_2 + $NADP^+$	156	110	97.0	93.1
expt 2[c]				
NEM	98.5	129	117	115
NEM + NADPH	24.5	129	122	121
NEM + $NADP^+$	184	129	119	112

[a] Methanethiolated submitochondrial particles were prepared and assayed at pH 6.8 for reverse transhydrogenase activity as described under Materials and Methods. The preincubation mixture contained 1 mM NADPH or $NADP^+$ where indicated. [b] Methanethiolated submitochondrial particles (0.27 mg of protein) and control particles (0.27 mg of protein) were preincubated with 100 μM Nbs_2 for 5 min and assayed for reverse transhydrogenase activity as described under Materials and Methods. Where indicated, NADPH or $NADP^+$ was present at 300 μM. [c] Methanethiolated submitochondrial particles (0.27 mg of protein) and control particles (0.27 mg of protein) were preincubated with 1 mM NEM for 2 min and assayed for reverse transhydrogenase activity as described under Materials and Methods. Where indicated, NADPH or $NADP^+$ was present at 300 μM. [d] Table entries indicate transhydrogenase rates in (nmol of AcPyADH formed)·$(min)^{-1}$·$(mg\ of\ protein)^{-1}$.

illustrated in Table III (expt 1), under conditions where Nbs_2 inhibited native submitochondrial transhydrogenase completely, *S*-methanethio transhydrogenase was inhibited by Nbs_2 only slightly, suggesting that both sulfhydryl reagents react with the same classes of sulfhydryl groups. From these results, it was predicted that the NADP-site sulfhydryl groups should be protected from methanethiolation by the presence of $NADP^+$ or NADPH. However, no significant protection of transhydrogenase activity, when assayed at pH 6.8, by these substrates from MMTS inactivation was found, indicating that at least one of the two classes of sulfhydryl groups was accessible to MMTS in the presence of these substrates. To test the possibility that $NADP^+$ and NADPH protect the NADP site, but not the peripheral sulfhydryl group from methanethiolation, transhydrogenase methanethiolated in the presence of $NADP^+$ or NADPH was subsequently incubated with sufficient Nbs_2 to completely inactivate native transhydrogenase. It can be seen (Table III, expt 1) that *S*-methanethio transhydrogenase prepared in the presence of $NADP^+$ or NADPH is further inhibited by Nbs_2 treatment and that inhibition was prevented by including $NADP^+$ or NADPH in the Nbs_2 reaction mixture. However, as shown in Table III (expt 2), NEM did not inhibit transhydrogenase methanethiolated in the presence of $NADP^+$ or NADPH, and neither $NADP^+$ nor NADPH influenced NEM reactivity with the enzyme. It is concluded that $NADP^+$ and NADPH partially protect the NADP-site sulfhydryl group, but not the peripheral sulfhydryl group, from reaction with MMTS and that NEM reacts exclusively with the peripheral sulfhydryl groups. Incomplete inactivation of *S*-methanethio transhydrogenase prepared in the presence of $NADP^+$ or NADPH by Nbs_2 probably indicates failure of these substrates to completely protect the NADP-site sulfhydryl group from methanethiolation. The fact that $NADP^+$ and NADPH protect the NADP-site sulfhydryl group, but do not protect the enzyme activity against partial MMTS inhibition, indicates that MMTS affects transhydrogenase activity by modifying a peripheral sulfhydryl group and not by reaction of the NADP-site sulfhydryl group.

Binding of Substrates to S-Methanethio Transhydrogenase. It was conceivable that NADPH did not stimulate NEM inactivation of *S*-methanethio transhydrogenase because derivatization of the enzyme prevented a conformational change exposing or increasing the reactivity of the peripheral sulfhydryl group upon the binding of NADPH. It has been previously shown that substrate-induced conformational changes in native rat liver (Blazyk et al., 1976) and both native and *S*-cyano

TABLE IV: Proteolytic Inactivation of Native and *S*-Methanethio Transhydrogenases[a]

addition	native transhydrogenase act.[b]	native transhydrogenase % control	*S*-methanethio transhydrogenase act.[b]	*S*-methanethio transhydrogenase % control
none	305	100	138	100
trypsin	90.4	29.6	42.3	30.7
trypsin + NADPH	27.1	8.9	16.9	12.2
trypsin + NAD^+	191	62.5	79.1	57.3

[a] Native and *S*-methanethio transhydrogenases were prepared, assayed for reverse transhydrogenase activity, and treated with trypsin as described under Materials and Methods. NADPH or NAD^+ was present at 300 μM where indicated. [b] Activity indicates transhydrogenase rates in (nmol of AcPyADH formed)·(min)$^{-1}$·(mg of protein)$^{-1}$.

bovine heart (O'Neal and Fisher, 1977) transhydrogenases can be measured as changes in the trypsin susceptibility of the enzymes. Table IV reports trypsinolysis studies in which NAD^+ protects against and NADPH promotes proteolytic inactivation of native and *S*-methanethio transhydrogenases to similar extents. Hence, by the criterion of trypsin susceptibility, and considering that *S*-methanethio transhydrogenase is active, induced conformational changes resulting from substrate binding are not appreciably altered in *S*-methanethio transhydrogenase.

Apparent pK_a of NEM-Reactive Sulfhydryl Group. Previous experiments by O'Neal and Fisher (1977) indicated that Nbs_2 inactivation of transhydrogenase resulted from modification of a sulfhydryl group having an apparent pK_a of 7.4. However, NEM inactivation resulted from a modification of a sulfhydryl group displaying a pK_a of about 9.1 (Figure 5). The differences in apparent pK_a values determined with Nbs_2 and NEM are consistent with the conclusion that the reagents are titrating two different classes of essential sulfhydryl groups. Since Nbs_2 has been shown above (Table I) to react with both classes of sulfhydryl groups and NEM apparently reacts only with a peripheral class of sulfhydryl groups, the pK_a previously determined with Nbs_2 (O'Neal and Fisher, 1977) probably reflects only that of the NADP-site sulfhydryl group.

Effect of Methanethiolation on Energy-Linked Transhydrogenase. Respiration or ATP hydrolysis by submitochondrial particles promotes the rate of reduction of $NADP^+$ by NADH several fold and increases the apparent equilibrium constant from 1 to values as high as 500 (Danielson and Ernster, 1963; Lee and Ernster, 1964). Transhydrogenase has been proposed to function as a reversible proton pump (Mitchell, 1966; Skulachev, 1974; Blazyk et al., 1976; Rydström, 1977) that responds to a pH gradient across the submitochondrial membrane, as shown in eq 1:

$$NADP^+ + NADH + nH^+_{in} \rightleftharpoons NADPH + NAD^+ + nH^+_{out} \quad (1)$$

Blazyk et al. (1976) and Rydström (1977) suggest that a basic group on transhydrogenase, having access to both sides of the membrane, may function to translocate protons concomitant with transhydrogenation. O'Neal and Fisher (1977) concluded that a Nbs_2-reactive transhydrogenase mercaptide ion probably is not involved in proton translocation. This conclusion was based on (a) the assumption that a sulfhydryl group in a proton-translocating domain outside the active site might potentially participate in energy coupling and (b) that submitochondrial particle transhydrogenase protected against Nbs_2 inactivation by $NADP^+$ or NADPH retained the capacity to energize the membrane during reduction of NAD^+ by NADPH. However, the present study demonstrates that in the presence of $NADP^+$ or NADPH, Nbs_2 reacts with neither class of sulfhydryl groups.

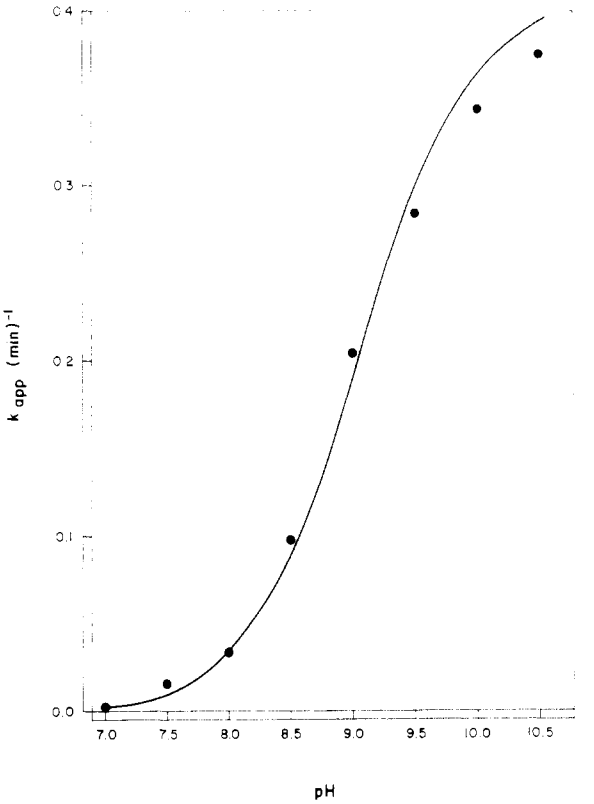

FIGURE 5: pH dependence of NEM inactivation of transhydrogenase. Submitochondrial particles (0.50 mg of protein) were incubated at 4 °C with 200 μM NEM in a medium containing 40 mM Tris–acetate buffer, and the first-order rate constants (k_{app}) for inactivation were estimated for each pH indicated. The solid line shows the calculated result for pK_a = 9.1.

To evaluate further the potential involvement of peripheral sulfhydryl groups in proton translocation, energy-linked transhydrogenation driven by electron transport through the terminal coupling site of the respiratory chain catalyzed by native and *S*-methanethio transhydrogenases was studied. As illustrated in Figure 6, the pH optimum of energy-linked transhydrogenation is shifted from 7.0–7.5 with the native enzyme to 6.5 with the *S*-methanethio transhydrogenase. The pH optimum of the forward non-energy-linked transhydrogenase was shifted from 6.0 to 5.5. The maximum velocities at optimum pH of energy-linked and non-energy-linked reactions of derivatized enzyme were only about 40% that of the native enzyme. The lower rate of energy-linked transhydrogenation catalyzed by methanethiolated submitochondrial particles did not result from either an inhibition of respiration or a dissipation of the membrane electrochemical potential, as indicated by ANS fluorescence enhancement (not shown) (Azzi et al., 1969). This experiment shows that under conditions where MMTS modifies all accessible sulfhydryl groups essential for trans hydrogenation the enzyme is still functionally linked to the energy conservation systems of the membrane. Figure 7 provides further evidence that MMTS-modified transhydrogenase retains the ability to couple transhydrogenation to the formation of a membrane potential, as monitored by uncoupler sensitive ANS fluorescence response

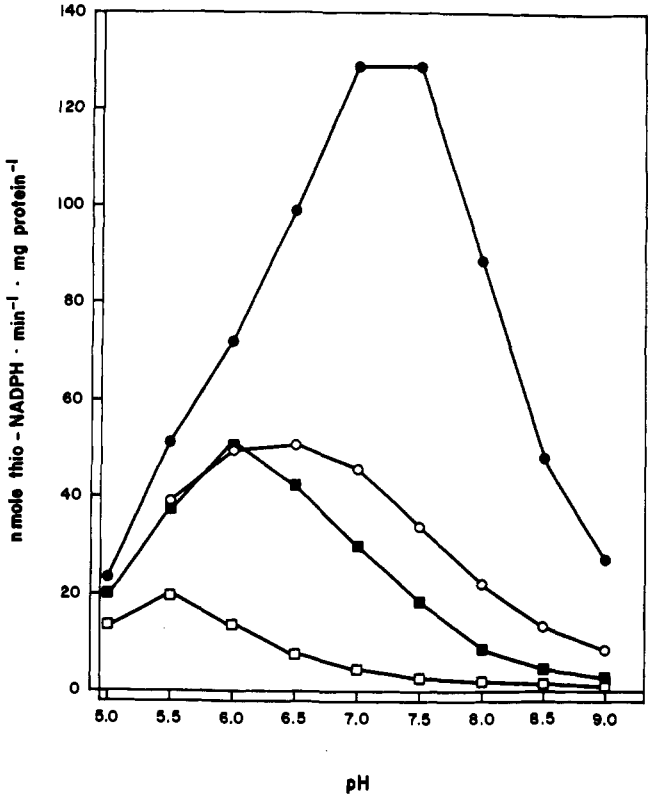

FIGURE 6: pH-rate profiles of forward non-energy-linked and energy-linked transhydrogenation catalyzed by native and *S*-methanethio transhydrogenases. Native and methanethiolated submitochondrial particles (0.26 mg of protein) were assayed at 23 °C for forward non-energy-linked transhydrogenase activity (■ and □, respectively) and energy-linked transhydrogenase activity (● and ○, respectively) as described under Materials and Methods.

during the reduction of NAD^+ by NADPH (Dontsov et al., 1972; O'Neal and Fisher, 1977). At pH 7.5, *S*-methanethio transhydrogenase retains about 50% of the activity of the native enzyme. In tightly coupled membranes, it would be predicted that the rate of ANS fluorescence response would be lower with methanethiolated membranes than with native membranes, corresponding to the relative transhydrogenase rates of the two membrane preparations. The ultimate extent of fluorescence enhancement would, however, be similar. It is, therefore, surprising that the extent of transhydrogenase-dependent membrane energization corresponds well with enzyme activity, in that ANS fluorescence response with derivatized membranes was about 40% of that observed with untreated membranes. One explanation for this observation is that the ANS response to transhydrogenation is quite small when compared to that seen during respiration supported by NADH or succinate and would, therefore, be particularly sensitive to a slow leakage of protons out of the vesicles. Assuming that the extent of steady-state membrane energization is related to the rate of transhydrogenase-dependent proton uptake compared to the rate of proton leakage from the vesicles, methanethiolated transhydrogenase would support a lower level of membrane energization than native enzyme.

Discussion

Chemical modification by a variety of reagents has led to the identification of two classes of sulfhydryl groups in bovine heart mitochondrial transhydrogenase. We have previously reported (O'Neal and Fisher, 1977) that inactivation of submitochondrial particle transhydrogenase by Nbs_2 is prevented

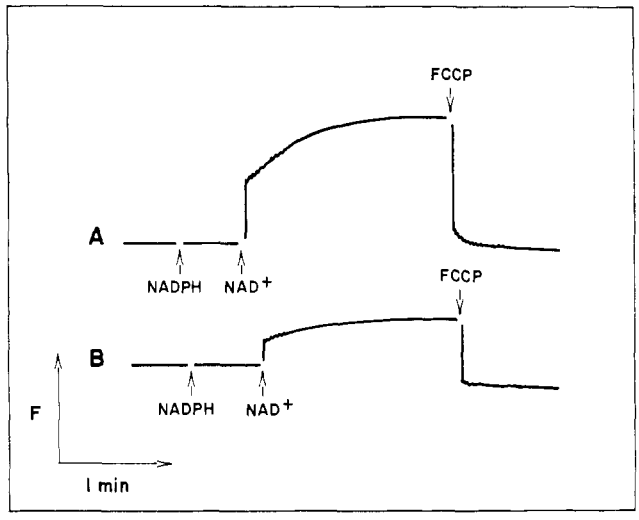

FIGURE 7: Effect of MMTS treatment on transhydrogenase-generated membrane potential. The fluorescence response of 8-anilino-1-naphthalenesulfonic acid during reverse transhydrogenation catalyzed by native (A) and methanethiolated (B) submitochondrial particles was measured as described under Materials and Methods. Oxidative phosphorylation uncoupler, FCCP (0.13 μM), was added where indicated.

by $NADP^+$ or NADPH but not by NAD^+ or NADH. The inactivation was pseudo first order and pH dependent, giving an apparent pK_a of 7.4. Treatment of inactive *S*-thionitrobenzoate transhydrogenase with cyanide resulted in the formation of a partially active *S*-cyano transhydrogenase. It was concluded that Nbs_2 modifies a sulfhydryl group at, or environmentally controlled by, the NADP catalytic binding site.

In the present study, the rate of inactivation of transhydrogenase by NEM was stimulated in the presence of NADPH but was substantially decreased by $NADP^+$, while no significant effect was seen with NAD^+ or NADH. The effects of $NADP^+$ and NADPH on thermal and proteolytic inactivation of transhydrogenase have demonstrated that the enzyme exists in at least three different conformations: the native enzyme, the $NADP^+$-enzyme complex, and the NADPH-enzyme complex (Blazyk et al., 1976; O'Neal and Fisher, 1977). The effect of substrates on NEM inactivation may reflect that the reactive sulfhydryl group is more accessible in the NADPH-enzyme complex and less accessible in the $NADP^+$-enzyme complex than in the unliganded enzyme. Clearly, in the presence of NADPH, a sulfhydryl group outside the NADP active binding site is modified by NEM.

In order to determine if NEM reacts with the NADP-site sulfhydryl group as well, the effect of NEM on *S*-methanethio transhydrogenase was investigated. Transhydrogenase is rapidly converted by MMTS to the *S*-methanethio derivative which retains 40-50% of reverse transhydrogenase activity when assayed at pH 6.8. That MMTS modifies both classes of sulfhydryl groups is shown by the lack of further transhydrogenase inactivation on incubation with either NEM, in the presence or absence of NADPH, or with Nbs_2. Lack of inhibition of methanethiolated enzyme by NEM provides evidence that it modifies a sulfhydryl group but not a lysyl residue (Blumenthal and Smith, 1973). While $NADP^+$ and NADPH do not protect against partial inactivation of transhydrogenase by MMTS, they do protect the NADP-site sulfhydryl group, since inactivation in the presence of either of these substrates results in a derivatized enzyme that can be inhibited by Nbs_2. However, NEM did not further inactivate substrate-protected enzyme, indicating that NEM inactivates through modification of a peripheral, but not a NADP-site, sulfhydryl group.

The reactivity of the two classes of sulfhydryl groups with Nbs_2 was investigated. Reactivation by dithiothreitol of inactive *S*-thionitrobenzoate transhydrogenase prepared in the absence of substrates or in the presence of NAD^+ or NADH was not affected by treatment of the modified enzyme with NEM. However, submitochondrial particle transhydrogenase incubated with Nbs_2 in the presence of $NADP^+$ or NADPH, to protect against inactivation, was inhibited by NEM to the same extent as the native enzyme. From these data, it is inferred that Nbs_2 modifies both classes of sulfhydryl groups and that $NADP^+$ and NADPH protect both classes of sulfhydryl groups from Nbs_2 modification.

It is apparent that neither class of sulfhydryl groups is essential for catalysis, since modification of these groups with MMTS does not completely inhibit the enzyme. The maximum velocity of transhydrogenation catalyzed by *S*-methanethio transhydrogenase in the reverse direction is nearly that seen with native enzyme, although the pH optimum is shifted about 1 pH unit lower. A similar result was seen with *S*-cyano transhydrogenase. An analogous shift in pH optimum was observed for the forward reaction catalyzed by *S*-methanethio transhydrogenase. That the shift in pH optima of the reactions probably results from a modification of a peripheral sulfhydryl group is indicated by the observation that partial protection of the NADP-site sulfhydryl group by $NADP^+$ (Table III) does not affect the partial inactivation (assayed at pH 6.8) of the enzyme by MMTS. Although the peripheral sulfhydryl group is not required for catalysis, it may function in the maintenance of enzyme conformation through ionic or hydrogen-bonding interactions that cannot be formed in the derivatized enzyme.

Chemical modification of both classes of transhydrogenase sulfhydryl groups by MMTS with retention of catalytic activity permitted a straightforward evaluation of the involvement of these groups in energy coupling. That *S*-methanethio transhydrogenase remained linked to the energy conservation system of the respiratory chain is demonstrated, since the enzyme functions in energy-linked transhydrogenation driven by ascorbate/phenazine methosulfate oxidation. Similar to non-energy-linked transhydrogenation, the pH optimum of energy-linked transhydrogenation in methanethiolated submitochondrial particles is about 1 pH unit lower than that of native membranes. A comparison of the pH–rate profiles of forward non-energy-linked and energy-linked transhydrogenation reveals that on membrane energization the rates of native and *S*-methanethio transhydrogenases are enhanced about twofold when assayed at optimal pH, demonstrating that the *S*-methanethio transhydrogenase is as functional in energy coupling as the native enzyme.

Since methanethiolation shifts the pH optima for non-energy-linked and energy-linked transhydrogenation to similar extents, it appears that transhydrogenation, rather than respiration, is the rate-determining step in the latter reaction. In order to explain the shift in pH optimum of forward transhydrogenase that occurs during membrane energization, it may be necessary to understand the physicochemical basis for the stimulation of forward transhydrogenation during respiration. Enhancement of transhydrogenation upon membrane energization may result from an induced conformational change and/or reflect an energy input from an influx of protons, down an electrochemical gradient, coupled to transhydrogenation. If membrane energization alters the conformation of transhydrogenase, it is not unexpected that a corresponding change in pH optimum could result.

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