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MODIFICATION OF BOVINE HEART MITOCHONDRIAL TRANSHYDROGENASE WITH TETRANITROMETHANE

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Modification of pyridine dinucleotide transhydrogenase with tetranitromethane resulted in inhibition of its activity. Development of a membrane potential in submitochondrial particles during the reduction of 3-acetylpyridine adenine dinucleotide ($AcPyAD^+$) by NADPH decreased to nearly the same extent as the transhydrogenase rate on tetranitromethane treatment of the membrane. Kinetics of the inactivation of homogeneous transhydrogenase and the enzyme reconstituted into phosphatidylcholine liposomes indicate that a single essential residue was modified per active monomer. $NADP^+$, NADPH and NADH gave substantial protection against tetranitromethane inactivation of both the nonenergy-linked and energy-linked transhydrogenase reactions of submitochondrial particles and the NADPH → $AcPyAD^+$ reaction of reconstituted enzyme. NAD^+ had no effect on inactivation. Tetranitromethane modification of reconstituted transhydrogenase resulted in a decrease in the rate of coupled H^+ translocation that was comparable to the decrease in the rate of NADPH → $AcPyAD^+$ transhydrogenation. It is concluded that tetranitromethane modification controls the H^+ translocation process solely through its effect on catalytic activity, rather than through alteration of a separate H^+-binding domain. Nitrotyrosine was not found in tetranitromethane-treated transhydrogenase. Both 5,5′-dithiobis(2-nitrobenzoate)-accessible and buried sulfhydryl groups were modified with tetranitromethane. NADH and NADPH prevented sulfhydryl reactivity toward tetranitromethane. These data indicate that the inhibition seen with tetranitromethane results from the modification of a cysteine residue.

Introduction

Mitochondrial pyridine dinucleotide transhydrogenase couples directly the translocation of H^+ across the inner membrane to H^- transfer between either NADPH and NAD^+ or NADH and $NADP^+$ [1–4]. The active site is composed of domains specific for the binding of NAD and NADP substrates [5] giving H^- transfer stereospecific for the 4A hydrogen of NADH and the 4B hydrogen of NADPH [6]. Bovine heart transhydrogenase is a dimer composed of presumably identical subunits having a molecular weight of approx. $1-1.2 \cdot 10^5$ [3,7]. On reconstitution of homogeneous transhydrogenase into phosphatidylcholine liposomes, the NADPH → NAD^+ reaction is coupled to the uptake of approximately one H^+ for each H^- transferred between the substrates [8]. Two types of mechanisms have been

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Abbreviations: $AcPyAD^+$, oxidized 3-acetylpyridine adenine dinucleotide; thio-$NADP^+$, oxidized thionicotinamide adenine dinucleotide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ANS, 8-anilino-1-naphthalenesulfonic acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); Tricine, *N*-tris(hydroxymethyl)methylglycine; DCCD, *N*,*N*′-dicyclohexylcarbodiimide.

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proposed for transhydrogenase-dependent H^+ translocation. Loop mechanisms employ a reduced-enzyme intermediate located on the side of the membrane opposite from the active site [9]. Recent evidence indicates that the transhydrogenase reaction mechanism incorporates a reduced-enzyme intermediate in that the reconstituted bovine heart [10] and *Rhodospirillum rubrum* chromatophore [11] enzymes catalyze an exchange reaction between NADH and NAD^+, involving only the 4A locus of NADH. However, the identity and localization in the membrane of the proposed intermediate remain unknown. Alternatively, pump mechanisms are envisioned to utilize H^+-binding domains separate from the active sites that are accessible to either side of the membrane depending on which substrates occupy the active site [12–15]. Recently, it has been reported that DCCD, a carboxyl group reagent, which inhibits H^+ translocation by mitochondrial ATPase [16] and cytochrome *c* oxidase [17], covalently modifies submitochondrial particle and reconstituted transhydrogenase [18,19]. In the latter preparation, H^+ translocation activity was inhibited to a coniderably greater extent than was catalytic activity, suggesting that modification was occurring at an H^+-binding domain [19].

In attempts to elucidate amino acid residues in the active site and the putative H^+-binding domain of transhydrogenase, our laboratory and others have employed specific chemical modification reagents. In studies with a variety of sulfhydryl reagents, Earle et al. [20] concluded that transhydrogenase contains two classes of exposed sulfhydryl groups, one in the NADP site and the other peripheral to the active site. However, methanethiolation of neither class of sulfhydryl group resulted in inactivation of transhydrogenation or membrane potential formation in submitochondrial particles. The presence of arginine in the active site is suggested by the observation that either NAD^+ or $NADP^+$ partially protects and together the substrates completely protect against inactivation by butanedione [21]. Inhibition by diethylpyrocarbonate, a histidine reagent, was not affected by substrates [18].

In this paper, transhydrogenase was modified with tetranitromethane to further characterize the catalytic residues of the enzyme.

Experimental Procedure

Methods

Submitochondrial particles were prepared from bovine heart mitochondria [22] and stored at −70°C. Pyridine dinucleotide transhydrogenase was purified to homogeneity from bovine heart mitochondria as described by Anderson and Fisher [23]. Reconstitution of transhydrogenase into phosphatidylcholine liposomes was performed according to the method of Earle and co-workers [8,24]. Transhydrogenase was assayed for reverse NADPH → $AcPyAD^+$ activity by the procedure of Blazyk and Fisher [25], and forward energy-linked NADH → thio-$NADP^+$ activity was assayed after the addition of 5 mM ascorbate plus 1.1 μM phenazine methosulfate [20]. Generation of a membrane potential during NADPH → NAD^+ transhydrogenation was monitored in submitocondrial particles by enhancement of ANS fluorescence according to the procedure of O'Neal and Fisher [26]. pH electrode measurements of H^+ uptake by proteoliposomes were performed as described in Ref. 8 using a combination pH glass electrode (Beckman 39505) and a Corning Model 12 pH meter together with a Fisher Recordall recorder operated at 0.1 pH unit full scale. The electrode was calibrated by addition of standardized HCl in each experiment. The protein concentration of submitochondrial particles was estimated using the biuret procedure [27] and that of purified transhydrogenase was analyzed by the procedure of Sedmak and Grossberg [28]. Procedures for tetranitromethane modification of submitochondrial particles, soluble or reconstituted transhydrogenase, are described in the figure and table legends. The reaction of native and tetranitromethane-modified transhydrogenase with DTNB was followed spectrophotometrically at 412 nm [29]. Protein samples were hydrolyzed at 110°C with 6 M HCl for 22 h and the hydrolysates were analyzed on a Beckman 119C amino acid analyzer equipped with an automatic integrator. SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn [30]. Samples were boiled for 3 min in 10 mM sodium phosphate, pH 7.0, containing 1% SDS, 10% glycerol and 1% β-mercaptoethanol before loading onto the gel.

Materials

All pyridine dinucleotide substrates were obtained from P-L Biochemicals, Inc. Trizma base, Tricine, tetranitromethane, DTNB, egg yolk phosphatidylcholine (type III-E), valinomycin, phenazine methosulfate, choline chloride, L-ascorbic acid, cholic acid, ANS and rotenone were from Sigma Chemical Co. FCCP was a product of Pierce Chemical Co. Cholic acid was recrystallized from 70% ethanol after decolorizing with activated charcoal.

Results

Inhibition of submitochondrial particle transhydrogenase by tetranitromethane

Tetranitromethane, a reagent considered to be specific for the nitration of tyrosine residues at pH 8 [31,32], was used to provide further insights into the mechanism of transhydrogenase-catalyzed reactions. As shown in Fig. 1, low tetranitromethane concentrations inhibited submitochondrial NADPH → AcPyAD$^+$ transhydrogenase activity. The extent of inhibition after a 30 min incubation at 25°C was dependent on tetranitromethane concentration, giving 50% inhibition at approx. 20 μM and nearly complete inhibition at 40 μM. Enhancement of ANS fluorescence has been used previously to monitor the development of a membrane potential across submitochondrial particle membranes (positive inside) during NADPH → AcPyAD$^+$ transhydrogenation [26]. Tetranitromethane modification of submitochondrial particles resulted in a decrease in the extent of ANS fluorescence enhancement that was comparable to the loss of transhydrogenase activity.

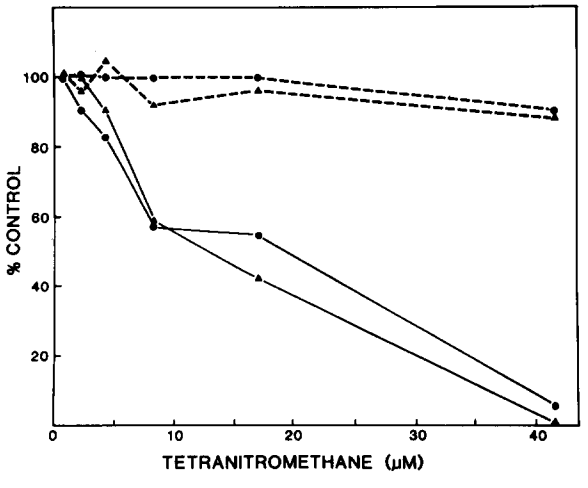

Fig. 1. Effect of tetranitromethane modification on submitochondrial NADPH → AcPyAD$^+$ transhydrogenase activity and on developing membrane potential. Submitochondrial particles (5 mg protein) were preincubated with various tetranitromethane concentrations ranging from 8.3 to 41.6 μM in a medium (6 ml) containing 40 mM Tris-HCl (pH 8.0) for 30 min at 25°C, then placed in ice. The entire reaction mixture was then centrifuged at 105000× *g* for 30 min at 4°C and the pellet was resuspended by homogenization in 0.3 ml of 0.25 M sucrose and 10 mM Tris-HCl, pH 8.0. NADPH–AcPyAD$^+$ transhydrogenase activity (●———●) was assayed at 25°C using 15 μl of the resuspended membranes in a medium (3 ml) containing 80 mM potassium phosphate (pH 6.3), 180 μM AcPyAD$^+$, 180 μM NADPH and 0.5 μM rotenone. The enhancement of ANS fluorescence (▲———▲) was monitored according to the procedure of O'Neal and Fisher [26] using 100 μl of the resuspended membranes. Similar modification experiments were conducted in the presence of 300 μM NADPH (- - - - -).

In order to investigate the site of tetranitromethane modification of submitochondrial particle transhydrogenase, the effects of substrates on the inactivation were determined. Energization of the submitochondrial particle membrane by ATP hydrolysis or by oxidation of respiratory substrates promotes by several-fold the rate of NADP$^+$ reduction by NADH [33]. The K_{eq} for this energy-linked reaction approaches 500, whereas that of the nonenergy-linked NADPH → NAD$^+$ reaction is approx. 1 [33]. During the energy-linked reaction, the membrane potential (positive inside) promotes NADH → NADP$^+$ transhydrogenation by coupling efflux of H$^+$ from the vesicle interior to H$^-$ transfer. Table I shows the effect of 350 μM substrates on tetranitromethane inactivation for nonenergy-linked NADPH → AcPyAD$^+$ transhydrogenation and for energy-linked NADH → NADP$^+$ transhydrogenation employing ascorbate and phenazine methosulfate as an energy source. As can be seen, NAD$^+$ protected neither transhydrogenase reaction, while NADH, NADP$^+$ and NADPH provided substantial protection for both reactions. These data suggest that tetranitromethane modification occurs at or near the NADP-binding domain of the active site. Protection by NADH, but not by NAD$^+$, indicates that tetranitromethane does not react at the NAD-binding domain. Rather, the protection seen with NADH may reflect a substrate-induced

TABLE I

EFFECT OF SUBSTRATES ON TETRANITROMETHANE INACTIVATION OF NONENERGY- AND ENERGY-LINKED TRANSHYDROGENATION

Submitochondrial particles (0.5 mg protein) were preincubated for 30 min at 25°C in a medium (0.2 ml) containing 50 mM Tris-HCl, pH 8.0, 12 μM tetranitromethane, 14 μM rotenone and 350 μM pyridine dinucleotide where indicated. No tetranitromethane was added to the control. Aliquots (0.1 ml) were removed and assayed for nonenergy-linked NADPH→AcPyAD$^+$ transhydrogenase activity as described in Fig. 1. The forward energy-linked transhydrogenase (NADH→thio-NADP$^+$) assay mixture contained 50 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 140 mM sucrose, 3 μg oligomycin, 175 μM NADH, 117 μM thio-NADP$^+$, 5 mM ascorbate, 1.1 μM phenazine methosulfate and 0.1 ml of the preincubation mixture.

Substrate additions	Nonenergy-linked transhydrogenation (nmol AcPyADH/min per mg protein)	% control	Energy-linked (nmol AcPyADH/min per mg protein)	% control
Control	140	100	37.6	100
None	53.2	38	15.1	40
NAD$^+$	57.4	41	14.3	38
NADH	130.2	93	30.1	80
NADP$^+$	81.2	58	30.5	81
NADPH	129	92	35.3	92

conformational change that reduces the reactivity of the modified group either by steric hindrance or through an alteration in its environment. In addition to protecting transhydrogenase almost totally against inactivation at various tetranitromethane concentrations, NADPH protects to the same extent the ability of the enzyme to generate a membrane potential during NADPH → AcPyAD$^+$ transhydrogenation (Fig. 1).

Inhibition of purified and reconstituted transhydrogenase by tetranitromethane

In order to determine the order of the reaction between tetranitromethane and transhydrogenase, as well as the number of essential residues modified on each active monomer, both purified and reconstituted transhydrogenase were reacted with the reagent. Homogeneous transhydrogenase was preincubated for various times with a series of tetranitromethane concentrations ranging from 9.4 to 75 μM. As shown in Fig. 2, the inactivation rate followed pseudo-first-order kinetics. These data can be analyzed to estimate the number of essential residues modified per active monomer by utilizing the equation $k = M^n$, where k is the rate constant of inactivation, M the molar concentration of tetranitromethane, and n the number of sites reacted [34]. A plot of the log of of the rate

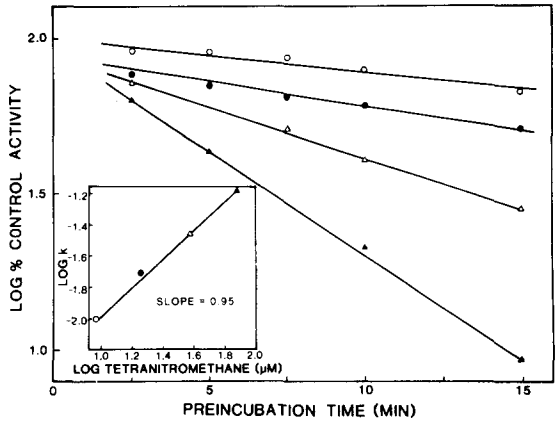

Fig. 2. Kinetics of inhibition of soluble transhydrogenase by tetranitromethane. Homogeneous transhydrogenase (53 μg/0.3 ml) was dialyzed against 250 ml of 100 mM sodium phosphate (pH 7.5) containing 0.05% sodium cholate with three changes of buffer at 3-h intervals to remove dithiothreitol. The enzyme (11 μg) was then preincubated in a medium (0.8 ml) containing 0.012% sodium cholate, 0.77 mM Tricine-NaOH, 77 mM choline chloride (pH 8.0) and various concentrations of tetranitromethane, i.e., 9.4 (○), 18.8 (●), 37.6 (△) and 75.2 μM (▲), for 15 min at room temperature. 0.1 ml of the reaction mixture was removed at indicated intervals for measurement of NADPH→AcPyAD$^+$ transhydrogenase activity. The inset shows the log-log plot of the rate constant of inactivation (k) against tetranitromethane concentration. The slope of the line represents the number of essential residues reacted during tetranitromethane inactivation.

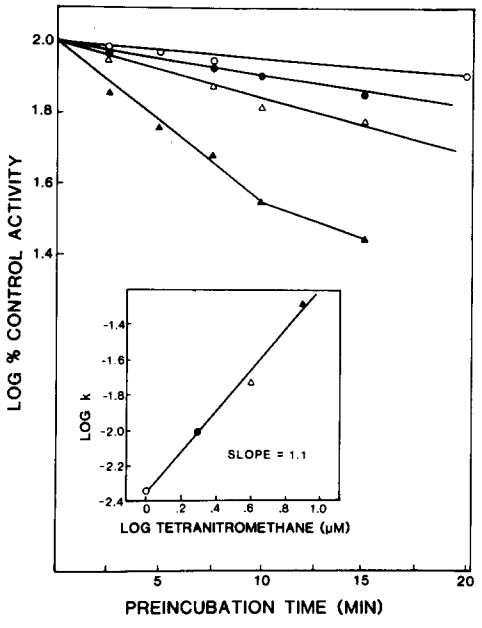

Fig. 3. Inhibition of reconstituted transhydrogenase by tetranitromethane. K^+-loaded proteoliposomes were prepared as described by Earle and Fisher [8] as follows: 4.4 mg egg yolk phosphatidylcholine in hexane were dried under N_2. 0.06 ml of 10% sodium cholate and 0.06 ml of 3 mM Tricine-NaOH containing 100 mM K_2SO_4 (pH 7.2) were added and the mixture was sonicated for 10 min. Transhydrogenase (15 μg), 0.19 ml of the buffer, and water were added to the sonicated mixture to a final volume of 0.5 ml. The mixture was dialyzed against 65 ml of 1.5 mM Tricine-NaOH, pH 7.2, containing 50 mM K_2SO_4 and 0.2 mM EDTA for 20 h with one change of buffer after 4 h. The proteoliposomes were further dialyzed against 100 ml of 1 mM Tricine-NaOH containing 100 mM choline chloride (pH 8.0) with three buffer changes at 3-h intervals to remove external K_2SO_4. Proteoliposomes containing 4.2 μg of transhydrogenase were preincubated at 25°C in a medium (0.8 ml) containing 1 mM Tricine, 100 mM choline chloride (pH 8.0), and various concentrations of tetranitromethane, i.e., 1 (○), 2 (●), 4 (△) and 8 μM (▲). Aliquots of the reaction mixture (0.1 ml) were removed at times indicated for measurement of NADPH→AcPyAD$^+$ transhydrogenase activity. The inset shows the log-log plot of inactivation similar to that described in Fig. 2.

constant for inactivation against log[tetranitromethane] gave a straight line with a slope of 0.95 as shown in Fig. 2 (inset). It would appear that tetranitromethane modifies only one essential residue per 120000 Da subunit. A similar experiment performed on transhydrogenase reconstituted into phosphatidylcholine liposomes is presented in Fig. 3. At lower concentrations of tetranitromethane (1–4 μM) the rate of inhibition was pseudo first order, while at higher tetranitromethane concentrations (8 μM) the inactivation became nonlinear after 10 min. The number of essential residues modified by tetranitromethane was about 1.1 per active monomer (Fig. 3, inset).

The effect of substrates on tetranitromethane inactivation of reconstituted transhydrogenase was studied by including 340 μM of each substrate in the preincubation mixture containing a 35-fold molar excess of tetranitromethane. After 30 min incubation at room temperature, the remaining transhydrogenase activity was determined. As with the submitochondrial particle enzyme, NADH and NADPH protected completely and NADP$^+$ substantially protected against tetranitromethane inactivation (Table II). NAD$^+$ afforded no protection.

TABLE II

EFFECT OF SUBSTRATES ON TETRANITROMETHANE INACTIVATION OF RECONSTITUTED TRANSHYDROGENASE

Proteoliposomes were prepared according to the method of Earle et al. [24]; 4.4 mg of egg yolk phosphatidylcholine in hexane were dried under nitrogen, 0.06 ml of 10% sodium cholate and 0.06 ml of 10 mM Tricine-NaOH (pH 8.3) containing 4 mM dithiothreitol, 0.2 mM EDTA and 50 mM sucrose were added, and the mixture was sonicated for 10 min. Transhydrogenase (15 μg) and cold water were added to 0.5 ml final volume. The entire mixture was dialyzed against 65 ml of 10 mM Tricine-NaOH (pH 8) containing 0.2 mM EDTA and 10% methanol for 20 h with one change of buffer after 4 h. Transhydrogenase-containing proteoliposomes (0.76 μg protein) were incubated with 2.2 μM tetranitromethane, except in control incubation, in a medium (0.1 ml) containing 35 mM Tris-HCl (pH 8.0) and 340 μM of the substrate indicated for 30 min at 25°C. Subsequently, NADPH→AcPyAD$^+$ transhydrogenase activity was measured with the addition of 1.7 μM FCCP [38] in a medium (3 ml) containing 80 mM potassium phosphate, pH 6.3, 180 μM AcPyAD$^+$ and 180 μM NADPH.

Substrate additions	Transhydrogenation rate (μmol AcPyADH/min per mg protein)	% control
Control	11.9	100
None	5.8	48.7
NAD$^+$	5.2	43.9
NADH	11.9	100
NADP$^+$	9.2	77
NADPH	12.8	108

Loss of transhydrogenase activity may have been related to the formation of high molecular weight cross-linked products after tetranitromethane treatment [35]. However, samples of tetranitromethane-treated transhydrogenase revealed no higher molecular weight species (data not shown).

Effect of tetranitromethane modification on the stoichiometry of H^+ translocation by reconstituted transhydrogenase

In an attempt to ascertain if transhydrogenase acts to translocate H^+ by a pump or a loop mechanism, the effect of tetranitromethane modification on H^+ translocation, as well as the NADPH → $AcPyAD^+$ activity of reconstituted transhydrogenase, was investigated. If transhydrogenase possesses an H^+-binding domain separate from the active site, as postulated by a pump mechanism, it might be demonstrated by a selective inhibition of H^+ translocation. Loop mechanisms would appear to require a parallel inhibition of both functions [19].

Earle and Fisher [8] described previously conditions that allow the direct measurement of H^+ uptake into K^+-loaded transhydrogenase containing proteoliposomes during NADPH → $AcPyAD^+$ transhydrogenation in the presence of valinomycin. In the absence of valinomycin, no H^+ uptake was observed. Reconstituted transhydrogenase was incubated at room temperature for 45 min in the presence of various concentrations of tetranitromethane. The reaction was quenched by placing the reaction mixtures in ice, and transhydrogenase activity and H^+ uptake into the vesicles were determined. Table III compares the rates of H^+ uptake and AcPyADH formation by the modified reconstituted transhydrogenase. The quotient of H^+ uptake and the AcPyADH synthesis rates represents the $H^+ : H^-$ ratio of the reaction. Although tetranitromethane modification decreased both rates, the $H^+ : H^-$ ratio remained unchanged. Consistent with the protective effect of NADPH on transhydrogenase-dependent membrane energization (Fig. 1), NADPH protected not only the activity of reconstituted enzyme, but also nearly completely protected its ability to translocate H^+.

The nature of tetranitromethane reactivity

Although tetranitromethane has been widely used in chemical modification of tyrosine residues, it also oxidizes cysteine side chains forming disulfide or sulfinate derivatives [36]. To determine the identity of the essential residue modified by tetranitromethane, the modified enzyme was analyzed for nitrotyrosine and sulfhydryl groups. 1.2 nmol of inactivated transhydrogenase were hydrolyzed in 6 M HCl for 22 h and subjected to amino acid analysis. No nitrotyrosine was detected. Native transhydrogenase contains a total of

TABLE III

EFFECT OF TETRANITROMETHANE MODIFICATION ON H^+ TRANSLOCATION BY RECONSTITUTED TRANSHYDROGENASE

K^+-loaded proteoliposomes (6 μg protein) prepared as described in Fig. 3 in 0.2 ml of 1 mM Tricine-NaOH/100 mM choline chloride (pH 8.0) were incubated with various concentrations of tetranitromethane at room temperature for 45 min. NADPH (300 μM) was added where indicated. After the preincubation, aliquots (0.1 ml) were removed and assayed in the presence of valinomycin for NADPH → $AcPyAD^+$ transhydrogenation and the rate of H^+ uptake into the proteoliposomes exactly as described by Earle and Fisher [8]. The $H^+ : H^-$ ratio represents the stoichiometry of H^+ uptake to H^- transferred between the substrates.

Tetranitromethane (μM)	H^+ uptake (μmol/min per mg)	Transhydrogenase rate (μmol AcPyADH formed/min per mg)	$H^+ : H^-$
None	5.6	11.8	0.48
0.63	5.2	11.0	0.47
1.25	4.7	10.1	0.47
2.5	3.8	9.1	0.42
5	1.6	3.7	0.43
5 plus NADPH	4.9	10.5	0.47

TABLE IV

DTNB TITRATIONS OF TRANSHYDROGENASE BEFORE AND AFTER TETRANITROMETHANE TREATMENT

Purified transhydrogenase (12 μg) was preincubated in a medium (1.2 ml) containing 6.3 mM sodium phosphate, pH 8.0, 0.73 mM Tricine, 73.3 mM choline chloride, 0.013% sodium cholate, 0.5% methanol and the indicated concentration of tetranitromethane. NADPH→AcPyAD$^+$ transhydrogenase activity was measured by withdrawing aliquots (90 μl) after 20 min incubation at 25°C. 20 μM DTNB (in 5 μl 50% CH_3OH) was then added to titrate accessible sulfhydryl groups. Buried sulfhydryl groups (Δ + SDS) were determined on the same sample after addition of 0.2% SDS. Δ, difference in sulfhydryl groups detected ± SDS.

Tetranitromethane (μM)	% control activity remaining	Sulfhydryl groups/monomer	
		Native	Δ + SDS
0	100	2	4.1
0.76	76	1.0	4.0
1.53	44	0.2	1.7
3.8	0	0	0

10 cysteine residues [37]. As shown in Table IV, titration of native enzyme with DTNB demonstrated the presence of two accessible sulfhydryl groups. Inclusion of 0.2% SDS to denature the enzyme exposed four additional buried sulfhydryl groups per monomer. Therefore, transhydrogenase apparently contains six cysteine residues and two cystine residues. Treatment of enzyme with increasing concentrations of tetranitromethane decreased the number of DTNB-accessible and buried thiol residues. It appears that a correlation exists between oxidation of sulfhydryl groups and inhibition of transhydrogenase activity. To lend further credence to the notion that tetranitromethane inactivates transhydroganation through oxidation of essential sulfhydryl groups, the enzyme was treated with tetranitromethane in the presence and absence of NADH or NADPH and then the number of sulfhydryl groups were determined by DTNB titration. As can be seen in Table V, preincubation with tetranitromethane under conditions where all the enzyme activity was inhibited resulted in the loss of both of the DTNB-accessible sulfhydryl groups and over half of the buried sulfhydryl groups. NADH and NADPH protected not only against inhibition of enzyme activity, but also against oxidation of both types of sulfhydryl groups.

TABLE V

DTNB TITRATIONS OF TRANSHYDROGENASE MODIFIED WITH TETRANITROMETHANE IN THE PRESENCE OF SUBSTRATES

DTNB titrations were performed as described in Table IV, except the tetranitromethane concentration was fixed at 2.3 μM and the reaction time was 30 min. Where indicated, the NADH and NADPH preincubation concentrations were 500 μM. Controls contained no tetranitromethane.

Substrate additions	Control activity remaining	Sulfhydryl groups/monomer	
		Native	Δ + SDS
Control	100	2	3.7
None	0	0	1.4
Control + NADH	125	2.5	4.1
NADH	90	1.8	3.6
Control + NADPH	76	2.5	3.1
NADPH	72	1.7	3.2

Discussion

The results presented in this paper demonstrate that tetranitromethane inhibits transhydrogenase activity of submitochondrial particles, of purified transhydrogenase, and of the enzyme functionally reconstituted into phosphatidylcholine liposomes. Kinetic studies on the latter two preparations suggest that the chemical modification of one residue per active monomer results in complete inactivation.

NADP$^+$ and NADPH, at relatively low concentrations, gave substantial protection against tetranitromethane modification of submitochondrial particles and reconstituted transhydrogenase. This suggests that modification is occurring in the in the NADP-binding domain of the active site. Alternatively, the binding of these substrates may alter the conformation of the enzyme such that an essential residue outside the active site becomes less reactive. Although NADH protected against inactivation to an extent similar to that seen with NADPH, NAD$^+$ was without effect. It is concluded, therefore, that tetranitromethane does not react with a residue in the NAD-binding domain and that NADH alters the reactivity of the essen-

tial residue through an induced conformational change in the enzyme. Previous proteolytic inactivation, thermal inactivation and chemical modification studies have shown that transhydrogenase exists in at least three different conformations represented by the unliganded enzyme, and the NADPH-enzyme and $NADP^+$-enzyme binary complexes [12,25]. NADH substantially protects submitochondrial particle transhydrogenase from tryptic inactivation, indicating that the conformation of the NADH-enzyme complex may also differ from the native enzyme [12,25].

Although these studies do not establish with certainty the identity of the essential reactive residue, typically tetranitromethane is quite specific for modification of tyrosine residues. However, sulfhydryl groups may also react with tetranitromethane [36]. Submitochondrial particle transhydrogenase has been shown in chemical modification studies to possess a DTNB-accessible sulfhydryl group in the NADP-binding domain and one peripheral to the active site [20]. Reaction of the peripheral group with *N*-ethylmaleimide, or both groups with DTNB resulted in total inactivation. $NADP^+$ or NADPH almost completely protected against DTNB inactivation and modification of both sulfhydryl groups, while $NADP^+$ only partially protected against and NADPH substantially stimulated *N*-ethylmaleimide inactivation. NAD^+ and NADH were without effect on sulfhydryl modification by either reagent. The substrate protection patterns seen with tetranitromethane inactivation are clearly different from those of *N*-ethylmaleimide and DTNB. This may suggest that tetranitromethane does not inhibit activity through sulfhydryl modification. However, no evidence was found for the formation of nitrotyrosine. Rather, it is evident that tetranitromethane is capable of oxidizing all of the sulfhydryl groups of the enzyme, both DTNB accessible and buried. Furthermore, protection by substrates against tetranitromethane inactivation is accompanied by protection against tetranitromethane modification of both types of sulfhydryl groups. Therefore, it is concluded that tetranitromethane inactivation of transhydrogenase results from the modification of a cysteine residue rather than a tyrosine residue. It is unclear from the available data whether this residue resides either in the DTNB-accessible or in the buried portion of the molecule.

Our approach to determining the mechanism of H^+ translocation by transhydrogenase is the use of chemical modification to selectively modify the putative H^+-binding domain of the enzyme, with the goal of inhibiting H^+ translocation while retaining transhydrogenase activity. Casey et al. [17] have reported the inhibition of H^+ translocation by DCCD without inhibition of the redox activity catalyzed by reconstituted cytochrome *c* oxidase. Recently, we have obtained a similar decoupling of H^+ translocation from H^- transfer with DCCD-modified reconstituted transhydrogenase [19]. The data presented do not provide evidence for a selective reaction of tetranitromethane with a H^+-binding domain separate from the active site. Firstly, tetranitromethane inhibited membrane energization of submitochondrial particles to an extent similar to inhibition of transhydrogenation and complete protection of activity in the presence of NADPH was accompanied by complete protection of membrane energization. Secondly, inhibition of energy-linked transhydrogenation corresponded to inhibition of the nonenergy-linked reaction. Finally, the stoichiometry of H^+ translocation into transhydrogenase-containing proteoliposomes to H^- transfer was unaltered under conditions where the extent of tetranitromethane inactivation ranged between 7 and 75%.

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