Resolution and Reconstitution of *Rhodospirillum rubrum* Pyridine Dinucleotide Transhydrogenase: Chemical Modification with *N*-Ethylmaleimide and 2,4-Pentanedione†

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ABSTRACT: *Rhodospirillum rubrum* chromatophore pyridine dinucleotide transhydrogenase complex, which catalyzes the reversible transfer of a hydride ion equivalent between the 4A locus of NAD and the 4B locus of NADP, consists of an integral membrane-bound component and a soluble peripheral protein factor. *N*-Ethylmaleimide inactivated both separated transhydrogenase components. A NADP-binding site on both components [McFadden, B. J., & Fisher, R. R. (1978) *Arch. Biochem. Biophys. 190*, 820–828] is confirmed by $NADP^+$ protection against inactivation. NADPH and NAD^+ did not affect *N*-ethylmaleimide modification of either component. Modification of the intact transhydrogenase complex was promoted by NADPH, partially prevented by $NADP^+$, and unaffected by NAD^+. NADPH selectively enhanced the modification of the complexed soluble factor. 2,4-Pentanedione inactivation of the transhydrogenase complex and membrane component was partially prevented by $NADP^+$ or NADPH but not affected by NAD^+. Substrates did not influence the inactivation of the soluble factor. Hydroxylamine treatment reversed inactivation of the separated components, suggesting that inactivation results from modification of lysyl residues. Thermal inactivation studies indicate that modification of a lysyl residue in the NADP-binding site prevents substrate interaction with the membrane component. However, membranes modified with *N*-ethylmaleimide or 2,4-pentanedione retained their full capacity to form a stable complex with the soluble factor dependent on the presence of $NADP^+$, demonstrating that $NADP^+$ binding to the soluble factor promotes formation of the transhydrogenase complex. The addition of purified soluble factor to factor-depleted membranes reconstitutes the reduction of the 3-acetylpyridine analogue of NAD^+ by NADH as well as by NADPH [Fisher, R. R., & Guillory, R. J. (1971a) *J. Biol. Chem. 246*, 4678–4686]. 2,4-Pentanedione modification of membranes prevented reconstitution. Either $NADP^+$ or NADPH, but not NAD^+, protected against inactivation. The reduction by NADH occurs by direct transfer of hydrogen from the 4A locus of NADH to the 4A locus of 3-acetylpyridine adenine dinucleotide. These data indicate that the reduction of NAD^+ by NADH is a partial reaction catalyzed by the transhydrogenase complex and that the reaction mechanism incorporates a reduced enzyme intermediate.

Rhodospirillum rubrum chromatophore membrane-bound transhydrogenase (EC 1.6.1.1) catalyzes the reversible transfer of a hydride ion equivalent between oxidized and reduced forms of NAD and NADP (Keister & Yike, 1966, 1967).

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH$$

In contrast to an analogous enzyme found in the inner mitochondrial membrane, *R. rubrum* transhydrogenase is comprised of two components, a membrane-bound integral protein component and a soluble peripheral protein factor (Fisher & Guillory, 1971a). A reduced enzyme intermediate has been postulated in the reaction mechanism because addition of purified soluble factor to factor-depleted membranes reconstituted transhydrogenation between NADH and $AcPyAD^+$[1] (DD-transhydrogenation) as well as between NADPH and $AcPyAD^+$ (TD-transhydrogenation) (Fisher & Guillory, 1971b). Proteolytic and thermal inactivation studies demonstrated a NADP-binding site on the membrane component (Fisher et al., 1975). Substrate protection against butanedione modification and retention of the protein on a NAD^+–agarose affinity column with selective elution by NAD^+ demonstrated separate NAD- and NADP-binding sites on the soluble factor (McFadden & Fisher, 1978).

The transhydrogenase reaction constitutes an energy-coupling site in chromatophores and submitochondrial particles (Skulachev, 1971, 1972; Skulachev et al., 1972). Recent experiments reconstituting homogeneous bovine heart mitochondrial transhydrogenase into liposomes demonstrate that the enzyme links transhydrogenation to the formation of a membrane electrochemical potential (Höjeberg & Rydström, 1977) and a pH gradient (Anderson & Fisher, 1978; Earle et al., 1978a) in the absence of additional coupling factors. It has been proposed, in accordance with the chemiosmotic hypothesis, that transhydrogenase functions directly to translocate protons by a redox loop mechanism incorporating a reduced enzyme intermediate (Mitchell, 1972; Moyle & Mitchell, 1973). Alternatively, proton translocation may be linked to a conformational change in transhydrogenase, occurring concomitantly with hydride ion transfer, that shifts a proton-binding domain from one side of the membrane to the other (Blazyk et al., 1976; Rydström, 1977).

The present investigation reports chemical modification studies which establish the presence of a NADP-binding site on both the soluble factor and membrane-bound components. Evidence that a reduced enzyme intermediate functions in the *R. rubrum* transhydrogenase reaction mechanism is provided.

Materials and Methods

Materials. *R. rubrum*, strain S1, was grown photosynthetically on the medium of Ormerod et al. (1961) supplemented with 0.1% yeast extract. Chromatophores, membranes depleted of soluble factor and devoid of transhydrogenase activity (C_T-particles), crude soluble factor (type II), and

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[1] Abbreviations and symbols used: $AcPyAD^+$, oxidized 3-acetylpyridine adenine dinucleotide; C_T-particles, chromatophores depleted of soluble transhydrogenase factor and devoid of transhydrogenase activity; BChl, bacteriochlorophyll; MalNEt, *N*-ethylmaleimide; DD-transhydrogenation, reduction of $AcPyAD^+$ by NADH; TD-transhydrogenation, reduction of $AcPyAD^+$ by NADPH.

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purified soluble factor were prepared as described previously (Fisher & Guillory, 1971a,b). Membrane component was solubilized with lysolecithin by the procedure of Jacobs et al. (1977). Soluble factor was stored at −10 °C. QAE-cellulose (Cellex-QAE) (Bio-Rad Laboratories) was regenerated following manufacturer's instructions. [4A-^{3}H]NADH was prepared as previously described (Fisher & Guillory, 1971b) from [4-^{3}H]NAD^+ (Amersham Corp.). $AcPyAD^+$ was synthesized according to Kaplan & Ciotti (1956). Sources for other compounds were as follows: *N*-ethylmaleimide, lactate dehydrogenase, glutamate dehydrogenase, and lysolecithin, Sigma Chemical Co.; NAD^+, $NADP^+$, and NADPH, P-L Biochemicals, Inc.; 2,4-pentanedione (99+%), Aldrich Chemical Co.; and DEAE-Sephadex A-25, Pharmacia Fine Chemicals, Inc.

Assays. Transhydrogenase activity was reconstituted by mixing C_T-particles containing 30–50 μg of bacteriochlorophyll (BChl) with sufficient soluble factor to give maximal activity. The reaction mixture (3 mL), at 23 °C, contained 88 mM potassium phosphate (pH 6.8), 0.19 mM $AcPyAD^+$, and either 0.15 mM NADPH for TD-transhydrogenation or 0.17 mM NADH for DD-transhydrogenation. The rate of reduction of $AcPyAD^+$ was calculated from the increase in absorption at 375 nm, assuming an extinction coefficient of 5.1×10^3 M^{-1} cm^{-1} (Stein et al., 1959). A unit of activity is defined as 1 μmol of $AcPyAD^+$ reduced/min. Transhydrogenase activity of chromatophores was assayed in a similar manner, except soluble factor was omitted. BChl was determined by assuming an extinction coefficient at 880 nm of 1.4×10^5 M^{-1} cm^{-1} (Clayton, 1963). Protein was estimated by the procedure of Sedmak & Grossberg (1977).

N-Ethylmaleimide Modification. Membrane preparations, containing 500 μg of BChl or 1–9 mg of soluble factor (type II) protein, were incubated at 22.5 °C in 1 mL of 10 mM MalNEt, 90 mM Tris-HCl (pH 7.5), 260 mM sucrose, 8.5% ethanol, and substrates where indicated. To analyze for residual TD-transhydrogenase activity, we removed 0.1-mL aliquots from the incubation mixture at various times. Dithiothreitol (25 mM) was added to terminate the reaction, and the samples were assayed immediately. When separated components were modified, a saturating quantity of native second component, as determined by a separate titration, was added to the assay mixture to reconstitute TD-transhydrogenation.

2,4-Pentanedione Modification. Membrane preparations, containing 350 μg of BChl or 1.6 mg of soluble factor (type II) protein, were incubated with 156 mM pentanedione at 30 °C in 1 mL of 84 mM Tris-HCl (pH 8.0), 250 mM sucrose, and substrates where indicated. At various times, 0.1-mL aliquots were removed from the incubation mixture and added directly to the assay mixture for determination of residual TD- or DD-transhydrogenase activity as described for MalNEt modification.

Binding Experiments. To determine the extent of soluble factor binding to modified C_T-particles depleted of soluble factor, we treated membranes with either MalNEt or pentanedione for 30 min as described above. The reaction mixture (5 mL) was diluted with 25 mL of ice-cold 0.1 M Tris-HCl (pH 8.0) and 290 mM sucrose, and the membranes were sedimented by centrifugation at 160000*g* for 30 min. Modified particles containing 350 μg of BChl and sufficient soluble factor to saturate 35% of the membrane-bound component, as determined by titrating native particles with soluble factor and assaying for TD-transhydrogenase activity, were incubated in 6 mL containing 0.1 M Tris-HCl (pH 8) and 290 mM sucrose at room temperature (21–24 °C) for 15 min and then sedimented at 160000*g* for 30 min. Where indicated, 25 μM $NADP^+$ was added to induce soluble factor binding to the membrane-bound component (Fisher & Guillory, 1971a). The amount of soluble factor not complexing with membrane component was determined by assaying the supernatant solution for transhydrogenase activity reconstituted with native C_T-particles containing 30 μg of BChl as previously described (Fisher et al., 1975). Soluble factor, in the absence of membranes, was not cleared from solution by centrifugation.

Stereospecificity of DD-Transhydrogenation. Purified soluble factor (0.28 mg) and solubilized membrane component (0.03 mg) were incubated in the DD-transhydrogenase reaction mixture containing 3 μmol of [4A-^{3}H]NADH (specific activity 19 μCi/μmol) and 3 μmol of $AcPyAD^+$. When the reaction reached equilibrium, as determined by monitoring the increase in absorption at 363 nm, the incubation mixture was diluted 100-fold with deionized H_2O and applied to a QAE-cellulose column (16 × 1.7 cm) at a flow rate of 40 mL/h. Elution of the pyridine dinucleotides was effected by a linear gradient in ammonium bicarbonate, (0–0.1 M) (pH 8.2) over a period of 16 h. Fractions of 2 mL were collected, and elution of the nucleotides was followed by monitoring the absorption at 260, 340, and 360 nm. Only AcPyADH and NADH absorb at 340 and 360 nm with the A_{360}/A_{340} ratio being approximately 1.4 and 0.7 for the compounds, respectively. AcPyADH-containing fractions were pooled and oxidized with either glutamate or lactate dehydrogenase. Oxidized products were separated from the mixture by passage through DEAE-Sephadex A-25 (Fisher & Guillory, 1971b). Since the QAE-cellulose column did not completely resolve NADH from AcPyADH, samples of the oxidized products from both the lactate and glutamate dehydrogenase reactions were lyophilized, redissolved in a minimal amount of deionized H_2O, applied to a strip of Whatman no. 1 paper, and developed by descending chromatography with 95% ethanol–1.0 M ammonium formate (pH 7.5) (7:3) for 16 h. The $AcPyAD^+$ and NAD^+ spots were identified by UV irradiation, and radioactivity was localized with a Packard, Model 7220/21, radiochromatogram scanner.

Results

Effect of Substrates on MalNEt Modification. *N*-Ethylmaleimide has been employed to localize sulfhydryl groups in both the NAD- and NADP-binding sites of *Escherichia coli* transhydrogenase (Houghton et al., 1976). However, the active-site sulfhydryl group in bovine heart transhydrogenase (O'Neal & Fisher, 1977) is not susceptible to MalNEt modification (Earle et al., 1978b). Inactivation with *p*-chloromercuribenzoate demonstrated the presence of an essential sulfhydryl group on the *R. rubrum* transhydrogenase soluble factor (Koenings & Guillory, 1973). Figure 1 shows the effect of $NADP^+$ and NADPH on MalNEt inactivation of the transhydrogenase complex of *R. rubrum*. NADPH potentiated inactivation at low concentrations, and $NADP^+$ partially protected the complex. Half-maximal effects were observed at about 15 μM NADPH and 1 mM $NADP^+$. The rate of inactivation of the complex was first order during the incubation period (data not shown). Although $NADP^+$ protection suggests the presence of a sulfhydryl group in the NADP site, stimulation of inactivation by NADPH indicates an induced conformational change that enhances the reactivity of a sulfhydryl group outside the NADP site. NAD^+ did not affect MalNEt modification. NADH was not studied because of an active rotenone and cyanide-insensitive NADH oxidase in the preparation.

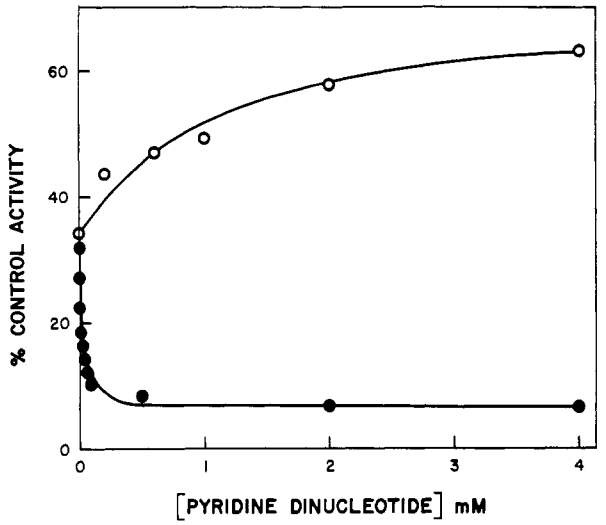

FIGURE 1: Influence of substrates on MalNEt inactivation of the transhydrogenase complex. Chromatophores (500 μg of BChl) were incubated for 30 min with 10 mM MalNEt, as described under Materials and Methods, in the presence of various concentrations of $NADP^+$ (○) or NADPH (●). Data are expressed as the percentage of zero-time controls containing the indicated concentrations of substrate.

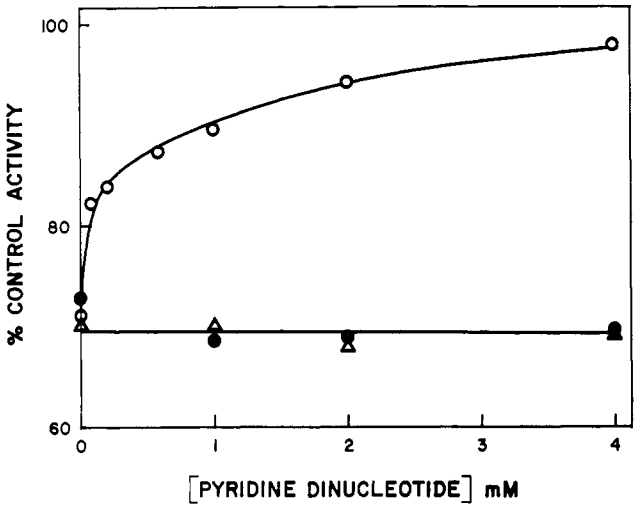

FIGURE 2: Influence of substrates on MalNEt inactivation of the membrane component. C_T-particles (500 μg BChl) were incubated for 30 min with 10 mM MalNEt in the presence of various concentrations of $NADP^+$ (○), NADPH (●), or NAD^+ (△) as described under Materials and Methods. Reconstituted transhydrogenase activity was determined in the presence of saturating titers of soluble factor. Data are expressed as the percentage of zero-time controls containing the indicated concentrations of substrate.

It was of interest to localize the reactive group activated in the presence of NADPH on either the membrane component or the soluble factor. In an attempt to determine this, C_T-particles and soluble factor were preincubated separately with MalNEt in the presence or absence of $NADP^+$ or NADPH. As shown in Figures 2 and 3, $NADP^+$ protected both components against MalNEt inactivation, but neither NADPH nor NAD^+ affected the extent of inactivation of either component. These data provide evidence for a NADP-binding site on both components and indicate that $NADP^+$ possibly induces a protein conformational change to limit accessibility or alter the reactivity of an essential group with MalNEt in the separated components as well as in the active transhydrogenase complex. It is unlikely that the reactive group is in the respective NADP-binding sites because NADPH also does not offer protection. Previous studies have

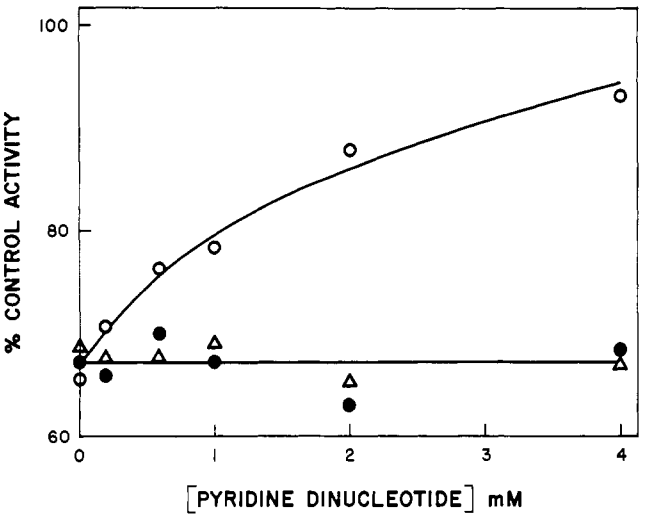

FIGURE 3: Influence of substrates on MalNEt inactivation of the soluble factor. Soluble factor (type II, 2.6 mg) was incubated for 30 min with 10 mM MalNEt in the presence of various concentrations of $NADP^+$ (○), NADPH (●), or NAD^+ (△) as described under Materials and Methods. Saturating amounts of C_T-particles (30 μg of BChl) were added to the assay mixture to reconstitute transhydrogenation. Data are expressed as the percentage of zero-time controls containing the indicated concentration of substrate.

Table I: Localization of the NADPH-Induced MalNEt Reactive Group in Chromatophores[a]

membrane prepn	preincubn addn	% act. remaining after modification	% reconstituted transhydrogenase
expt 1			
chromatophores	none	108	100
chromatophores	MalNEt	27	53
chromatophores	MalNEt, $NADP^+$	56	80
chromatophores	MalNEt, NADPH	11	59
expt 2			
C_T-particles	none	95	100
C_T-particles	MalNEt	62	64
C_T-particles	MalNEt, $NADP^+$	91	90
C_T-particles	MalNEt, NADPH	61	64

[a] Chromatophores or C_T-particles (1.45 mg of BChl) were incubated with 10 mM MalNEt for 30 min in the presence or absence of $NADP^+$ or NADPH (2 mM) as described under Materials and Methods, and the residual transhydrogenase activity was determined. The reaction mixture was diluted from 3 to 30 mL with 0.1 M Tris-HCl (pH 8.0) and 290 mM sucrose. The membranes were sedimented at 160000*g* for 30 min and resuspended in 1 mL of 0.1 M Tris-HCl (pH 8.0) and 290 mM sucrose prior to reconstitution of transhydrogenase by the addition of saturating titers of fresh soluble factor (type II). Data are expressed as the percentage of the zero-time control. Maximum reconstituted transhydrogenase activity was 0.57 and 0.62 nmol of AcPyADH/(min μg of BChl) for experiments 1 and 2, respectively.

shown that NADPH binds to both separated components (Fisher et al., 1975; Jacobs et al., 1977; McFadden & Fisher, 1978). Furthermore, the binding of NADPH to either separate component does not expose or otherwise enhance the reactivity of an essential group. It is apparent that an intact transhydrogenase complex is required to observe NADPH-stimulated MalNEt inactivation, but the above data do not allow the determination of which component is being modified.

Localization of the NADPH-Induced MalNEt Reactive Group. That the NADPH-induced MalNEt reactive group is located on the soluble factor is demonstrated by the experiment shown in Table I. Chromatophores were inactivated

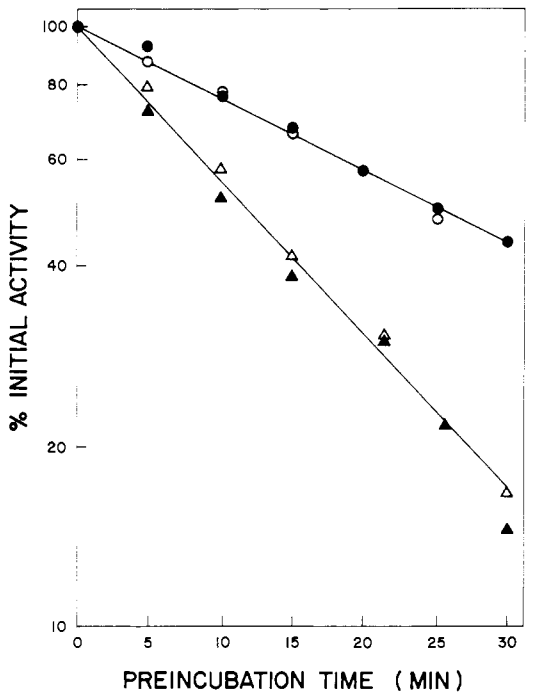

FIGURE 4: Pentanedione modification of the transhydrogenase complex. Chromatophores (350 μg of BChl) were incubated with 156 mM pentanedione, as described under Materials and Methods, in the absence (▲) or presence of 1 mM NAD^+ (△), $NADP^+$ (○), or NADPH (●).

by MalNEt either alone or in the presence of $NADP^+$ or NADPH (2 mM). The membranes were subsequently washed to remove unreacted MalNEt and to dissociate the soluble factor. The resulting C_T-particles were then combined with optimal titers of native soluble factor to reconstitute TD-transhydrogenase activity. As can be seen from Table I (expt 1), MalNEt inactivated the membrane component of chromatophores since derived C_T-particles were optimally reconstituted with native soluble factor to only ~53% of control untreated membranes. MalNEt inactivation of the intact complex was greater than that of the derived C_T-particles because the former is a sum of the inactivation of both transhydrogenase components. $NADP^+$ protected the complex and the membrane component against inactivation. However, NADPH promoted inactivation of the complex but had no effect on membrane component inactivation. From parallel experiments with C_T-particles (Table I, expt 2) it can be seen that MalNEt inactivation, in the presence or absence of $NADP^+$ or NADPH, of the membrane component of either chromatophores or C_T-particles was comparable. The lack of NADPH-stimulated inactivation of the membrane component of transhydrogenase complex strongly indicates that NADPH selectively alters the extent of inactivation of the complexed soluble factor.

Effect of Substrates on 2,4-Pentanedione Modification. Gilbert and O'Leary (1975) showed that 2,4-pentanedione is a reversible chemical modification reagent for lysyl residues, but that it irreversibly modifies arginine residues. Both of these moieties have been characterized in the coenzyme-binding sites of various pyridine dinucleotide linked dehydrogenases (Blumenthal & Smith, 1973; Jörnvall et al., 1977; Suhadolnik et al., 1977). Pseudo-first-order kinetics are observed for pentanedione modification of the chromatophore transhydrogenase complex in the absence or presence of protecting concentrations (1 mM) of $NADP^+$ and NADPH (Figure 4). No protection was seen with NAD^+. NADPH was slightly more effective at protecting chromatophores from inactivation

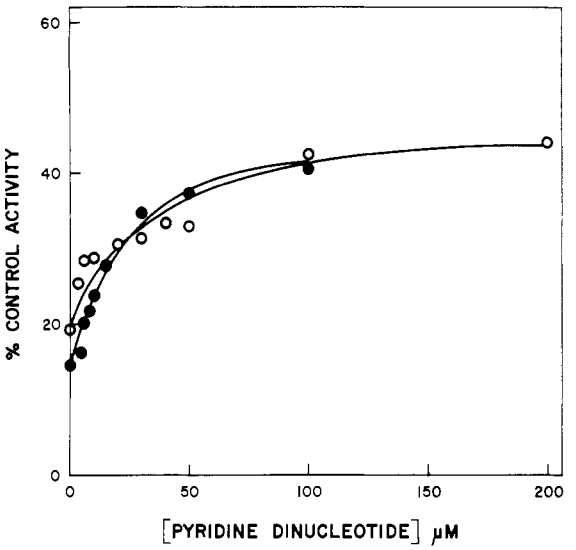

FIGURE 5: Effect of substrates on pentanedione modification of the transhydrogenase complex. Chromatophores (350 μg of BChl) were incubated with 156 mM pentanedione for 30 min, as described under Materials and Methods, with varying concentrations of $NADP^+$ (○) or NADPH (●). The data are expressed as the percentage of zero-time activity in the presence of the indicated substrate concentrations.

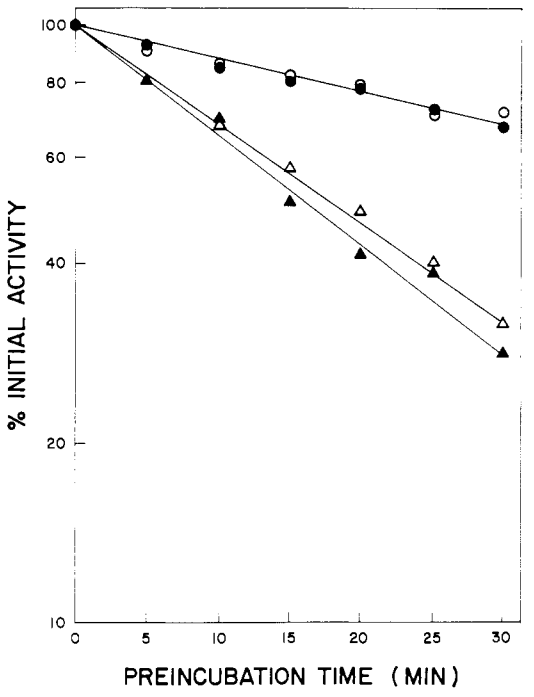

FIGURE 6: Pentanedione modification of the membrane component. C_T-particles (350 μg of BChl) were incubated with 156 mM pentanedione, as described under Materials and Methods, in the absence (▲) or presence of 1 mM NAD^+ (△), $NADP^+$ (○), or NADPH (●). Reconstituted transhydrogenase activity was determined in the presence of saturating amounts of soluble factor (type II).

than $NADP^+$; concentrations of the dinucleotides giving half-maximal protection were about 15 and 30 μM, respectively (Figure 5). Pseudo-first-order inactivation of C_T-particles depleted of soluble factor was effected by pentanedione (Figure 6). NADPH was 10-fold more effective than $NADP^+$ at protecting C_T-particles against pentanedione modification (Figure 7). NAD^+ afforded no protection. Inactivation of C_T-particles is reversed completely upon treatment with 0.5 M NH_2OH in 0.1 M Tris-HCl (pH 6.5) and 10% sucrose for 90 min at 22 °C, indicating that essential lysyl residues are being modified specifically. Even though

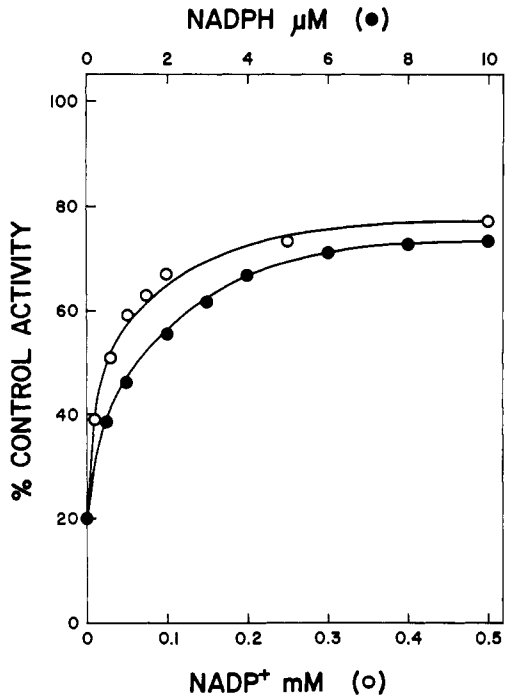

FIGURE 7: Effect of substrates on pentanedione modification of the membrane component. C_T-particles (350 μg of BChl) were incubated with 156 mM pentanedione for 30 min, as described under Materials and Methods, with varying concentrations of $NADP^+$ (○) or NADPH (●). Reconstitutable transhydrogenase activity was determined in the presence of saturating amounts of soluble factor (type II). The data are expressed as the percentage of zero-time activity in the presence of the indicated substrate concentrations.

$NADP^+$ and NADPH protect against pentanedione inactivation of C_T-particles, modification need not be occurring at a NADP-binding site. Therefore, the binding of substrates to pentanedione-modified C_T-particles was assessed by thermal inactivation studies. The binding of $NADP^+$ or NADPH to the membrane component substantially increases its thermostability by an induced conformational change (Fisher et al., 1975; Jacobs et al., 1977). Neither $NADP^+$ nor NADPH was found to protect pentanedione-modified C_T-particles against thermal inactivation, even though native membranes were protected by $NADP^+$ and NADPH (Table II). These data suggest that modification of a lysine residue in the NADP-binding site of the membrane component prevents NADP binding. A comparison of Figures 5 and 7 illustrates that the extent of $NADP^+$ and NADPH protection of the membrane component of C_T-particles was substantially greater than that of the chromatophore transhydrogenase complex. An explanation for the difference is provided by the fact that pentanedione inactivation of the soluble factor is not prevented by $NADP^+$ or NADPH (data not shown). Thus, protection of chromatophores from inactivation by substrates affects only the membrane component of the enzyme. Although NH_2OH treatment partially inactivated the soluble factor, it was completely effective at reversing pentanedione inactivation of the factor to control activities.

Soluble Factor Binding to 2,4-Pentanedione- and MalNEt-Modified Membranes. The inactivation of the membrane component after chemical modification may result from the inability of modified particles to bind either the soluble factor or NADP substrates. The capacity of C_T-particles resolved of soluble factor to bind soluble factor, before and after chemical modification, was investigated. C_T-particles, native or modified with either 2,4-pentanedione or MalNEt, were incubated with soluble factor in the absence or presence of 25 μM $NADP^+$ to induce the soluble factor to complex with the membrane component (Fisher & Guillory, 1971a). Membranes were then sedimented, and the quantity of soluble factor remaining in the supernatant solution was evaluated by reconstitution of TD-transhydrogenase activity with native C_T-particles (Fisher et al., 1975). As shown in Table III, $NADP^+$ induced the same amount of soluble factor binding to unmodified C_T-particles and to particles inactivated by 77% with MalNEt or by 86% with pentanedione.

Table II: Thermal Inactivation of Membrane Component[a]

membrane prepn	heat treatment addn	% control act.[b]
native C_T-particles	none	50
native C_T-particles	$NADP^+$	79
native C_T-particles	NADPH	100
modified C_T-particles	none	52
modified C_T-particles	$NADP^+$	55
modified C_T-particles	NADPH	62

[a] C_T-particles were modified with 156 mM pentanedione for 40 min as described under Materials and Methods. After sedimentation, depleted membranes containing 300 μg of BChl were resuspended in 0.4 mL containing 60 mM Tris-HCl (pH 8), 175 mM sucrose, and 250 mM substrate where indicated. The sample was preincubated for 3 min at 48 °C and then cooled on ice. Heat-treated particles containing 150 μg of BChl were incubated with 0.5 M NH_2OH in 1 mL of 0.1 M Tris-HCl (pH 6.5) and 290 mM sucrose for 30 min at room temperature to reverse the pentanedione modification. Aliquots (0.2 mL) containing 30 μg of BChl were assayed, as described under Materials and Methods, for reconstituted transhydrogenation with 3.4 mg of soluble factor protein (type II). [b] Activity remaining after NH_2OH reactivation. Control activities, obtained by treating unheated membranes with hydroxylamine, were 0.31 and 0.28 nmol of AcPyADH/(min μg of BChl) for native and modified C_T-particles, respectively.

Table III: Effect of Chemical Modification on Binding of Soluble Factor to Membrane Component[a]

preincubn addn	% sol factor bound: MalNEt	% sol factor bound: pentanedione
soluble factor, C_T-particles (native)	0	0
soluble factor, C_T-particles (native), $NADP^+$	63	44
soluble factor, C_T-particles (modified)	0	7
soluble factor, C_T-particles (modified), $NADP^+$	64	41

[a] The ability of C_T-particles modified with either MalNEt or 2,4-pentanedione, as described under Materials and Methods, to bind soluble factor was compared to that of native membranes as described under Materials and Methods.

DD-Transhydrogenation. A reduced enzyme intermediate has been postulated in the *R. rubrum* transhydrogenase reaction mechanism since addition of purified soluble factor to C_T-particles reconstitutes the reduction of $AcPyAD^+$ by either NADH (DD-transhydrogenation) or NADPH (TD-transhydrogenation) (Fisher & Guillory, 1971b; Table IV). If the mechanism of TD-transhydrogenation involves the transfer of a hydride ion equivalent from NADPH to an intermediate in the transhydrogenase reaction mechanism (eq 1) prior to reduction of NAD^+ (eq 3), then formation of the reduced enzyme intermediate from NADH (eq 2) would result in DD-transhydrogenation. Since TD-transhydrogenation occurs

$$NADPH + Enz \rightleftharpoons NADP^+ + Enz\text{–}H \quad (1)$$

$$NADH + Enz \rightleftharpoons NAD^+ + Enz\text{–}H \quad (2)$$

$$AcPyAD^+ + Enz\text{–}H \rightleftharpoons AcPyADH + Enz \quad (3)$$

Table IV: Reconstitution of *R. rubrum* Transhydrogenase Reactions[a]

addn	transhydrogenation:	nmol of AcPyADH/min TD	DD
C_T-particles		0.06	0.10
soluble factor		0.00	0.18
C_T-particles and soluble factor		5.18	2.76

[a] Reduction of $AcPyAD^+$ by NADPH (TD-transhydrogenation) or by NADH (DD-transhydrogenation) was assayed as described under Materials and Methods. Purified soluble factor (0.14 mg) and C_T-particles (15 μg of BChl) were added where indicated.

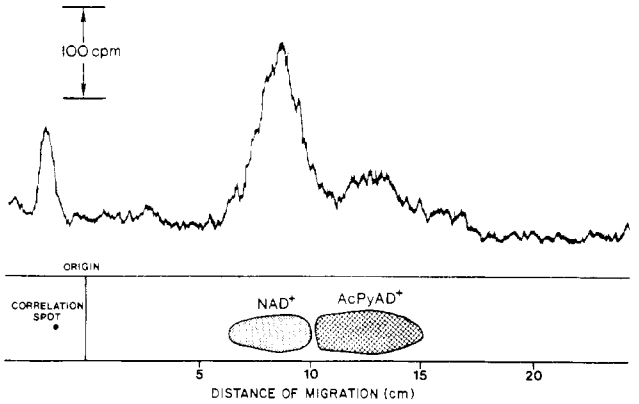

FIGURE 8: Paper chromatographic separation of ^3H-labeled DD-transhydrogenase products. AcPyADH-containing fractions from the QAE-cellulose column were pooled, treated with glutamate dehydrogenase, and fractionated on DEAE Sephadex A-25, and the oxidized reaction products were separated by paper chromatography as described under Materials and Methods. $AcPyAD^+$ and NAD^+ spots were identified by UV irradiation. Radioactivity was localized by utilizing a Packard radiochromatogram scanner.

by transfer of hydrogen from the 4A (nicotinamide) locus of NADH to the 4B (nicotinamide) locus of NADPH (Fisher & Guillory, 1971b), it would be predicted that if DD-transhydrogenation is catalyzed by transhydrogenase and if $AcPyAD^+$ binds to the NAD catalytic site, the stereospecificity of the exchange would be from the 4A locus of NADH to the 4A locus of AcPyADH. To determine the stereospecificity of DD-transhydrogenation, we incubated [4A-^3H]NADH and $AcPyAD^+$ with purified soluble factor and solubilized membrane component. Solubilized membrane component was employed because unlike C_T-particles it demonstrated no intrinsic DD-transhydrogenase activity. Reaction products were separated on a QAE-cellulose column. The AcPyADH-containing fractions were oxidized with either lactate dehydrogenase to remove the 4A hydrogen or glutamate dehydrogenase to remove the 4B hydrogen. Because the AcPyADH fractions from the QAE-cellulose column were contaminated with trace amounts of NADH, the oxidized products were separated by paper chromatography. The $AcPyAD^+$ and NAD^+ spots were identified by their characteristic absorption of UV light, and radioactivity was localized by using a paper strip scanner. Figure 8 illustrates the radioactive profile of the glutamate dehydrogenase oxidation products. $AcPyAD^+$ and NAD^+ remain labeled after removal of the 4B hydrogen. On the other hand, lactate dehydrogenase removed all of the ^3H label from the pyridine dinucleotides. It is therefore concluded that DD-transhydrogenation occurs by direct transfer of hydrogen from the 4A locus of NADH to the 4A locus of AcPyADH.

Pentanedione modification of C_T-particles inhibited the reconstitution of DD-transhydrogenation, as well as TD-transhydrogenation (Table V). The inactivation of the membrane component functional in DD-transhydrogenation followed pseudo-first-order kinetics and approximated the rate of inactivation of the membrane component functional in TD-transhydrogenation. The effects of substrates on pentanedione inactivation of the membrane were identical for both transhydrogenase reactions in that $NADP^+$ and NADPH protected whereas NAD^+ was without effect. These data indicate that the same membrane component is involved in both TD- and DD-transhydrogenation.

Table V: Influence of Substrates on Pentanedione Modification of Membrane Component[a]

preincubn addn	transhydrogenation:	% control act. TD	DD
pentanedione		4	13
pentanedione, NAD^+		2	13
pentanedione, $NADP^+$		78	93
pentanedione, NADPH		89	97

[a] C_T-particles (700 μg of BChl) were incubated with 156 mM pentanedione, as described under Materials and Methods, in the absence or presence of 1 mM substrate. The reaction mixture was diluted from 2 to 30 mL with 0.1 M Tris-HCl (pH 8.0) and 290 mM sucrose. The membranes were sedimented at 160000g for 30 min and resuspended in 0.5 mL of 0.1 M Tris-HCl (pH 8.0) and 290 mM sucrose. Membranes (15 μg of BChl) were assayed for reconstituted TD- and DD-transhydrogenation as described for Table IV in the presence of saturating amounts of purified soluble factor. The data are presented as the percentage of control transhydrogenase activity of membranes incubated in the absence of pentanedione.

Discussion

Chemical modification of *R. rubrum* transhydrogenase has been employed to study the location of NADP-binding sites on the membrane and soluble factor components of the complex. Protection against MalNEt inactivation of both separated components by $NADP^+$, but not by NAD^+, indicates the presence of a NADP-binding site on each component. However, since NADPH does not afford protection against MalNEt inactivation, the reactive essential residue apparently does not reside in the NADP-binding domain. Rather, $NADP^+$ binding may alter the MalNEt accessibility or reactivity of an essential residue, outside the NADP site, by an induced conformational change.

Although NADPH did not affect the rate of MalNEt inactivation of separated components, it substantially enhanced inactivation of the intact transhydrogenase complex by specifically promoting modification of the soluble factor (Table I). These data do not allow a conclusion as to whether potentiation of inactivation results from a NADPH-induced conformational change in the membrane component that, in turn, is coupled to a conformational change in the soluble factor or if NADPH binding to the complexed soluble factor directly alters its conformation. These observations are consistent with the recent demonstration that $NADP^+$ prevents, whereas NADPH promotes, MalNEt modification of a peripheral sulfhydryl group outside the NADP-binding site of bovine heart mitochondrial transhydrogenase (Earle et al., 1978b). Similar results have been observed for MalNEt inactivation of *E. coli* transhydrogenase (Houghton et al., 1976). These data imply that there is considerable homology between these enzymes in that similar structural alterations in the environment of the reactive sulfhydryl residue are induced by NADPH.

Reversible inactivation of the membrane component and soluble factor by 2,4-pentanedione indicates the presence of

essential lysyl residues on both components. Protection of the membrane component by $NADP^+$ or NADPH, but not by NAD^+, suggests that a lysyl residue is present in the NADP site, a conclusion which is supported by thermal inactivation studies which demonstrate that $NADP^+$ or NADPH cannot bind to the pentanedione-modified membrane component (Table II). The lack of effect of substrates on inactivation of the soluble factor shows that a reactive lysyl residue is not present in a dinucleotide-binding site of this component.

Fisher & Guillory (1971a) previously proposed the existence of two NADP-binding sites differing in affinity for substrates with the high-affinity NADP site functioning only in complex formation and the low-affinity NADP site forming part of the active site. Stimulation of tryptic inactivation of the membrane component by $NADP^+$ and NADPH demonstrates that these substrates promote a conformational change in the component and led to the suggestion that this structural rearrangement was necessary for the formation of a stable complex with the soluble factor (Fisher et al., 1975). However, the elucidation of a NADP site on the soluble factor raises the following questions: (a) which NADP binding domain is catalytic, and (b) are both NADP sites functional in regulating the formation of active transhydrogenase complex? Substrate protection against butanedione modification of the separated components indicates that the high-affinity NADP site resides on the membrane component, whereas the soluble factor contains the low-affinity NADP site (McFadden & Fisher, 1978). These findings support the concept that the soluble factor is an inactive transhydrogenase activated by complexing with the membrane component. McFadden & Fisher (1978) further showed that butanedione modification of membrane component arginyl residues inhibited $NADP^+$-dependent binding of the soluble factor to membranes. Butanedione inhibition of the soluble factor binding was prevented in the presence of $NADP^+$. These results suggested that NADP-site arginyl residues are modified and are consistent with the requirement of NADP binding to the membrane component for complex formation. On the other hand, pentanedione modification of the membrane component inhibited NADP binding to the component (Table II) but did not prevent $NADP^+$-dependent binding of the soluble factor to the membranes (Table IV). Thus, NADP interaction with the soluble factor apparently promotes formation of a stable, but nonfunctional, transhydrogenase complex. Furthermore, since modification of a membrane component NADP-site lysyl residue did not hinder complex formation, while inhibiting substrate binding, the arginyl residue modified by butanedione must reside outside the NADP-binding domain. It is therefore concluded that (a) arginyl moieties on the membrane component function in the recognition and binding of the soluble factor and (b) NADP binding to the membrane component promotes a conformational change which protects these residues from butanedione modification. The current results suggest that besides representing part of the active site, the low-affinity NADP site on the soluble factor, as well as the high-affinity NADP site on the membrane component, can promote the formation of the transhydrogenase complex.

We have proposed previously that the mechanism of *R. rubrum* transhydrogenase incorporates a reduced enzyme intermediate (Fisher & Guillory, 1971a). This notion was based on the observation that purified soluble factor reconstitutes, when added to soluble factor depleted membranes, transhydrogenation between NADH and $AcPyAD^+$, as well as between NADPH and $AcPyAD^+$. In the present study it is shown that DD-transhydrogenation is catalyzed by transhydrogenase in that (a) pentanedione modification of C_T-particles inhibited soluble factor dependent reconstitution of both TD- and DD-transhydrogenation and (b) the membrane component for both transhydrogenase reactions is protected against inactivation by $NADP^+$ and NADPH but not by NAD^+. It is possible that the two transhydrogenase reactions require different membrane components. However, protection by NADP of the membrane component involved in DD-transhydrogenation would be predicted only if this reaction was catalyzed by the component functional in TD-transhydrogenation. Furthermore, it is demonstrated that reconstituted DD-transhydrogenation occurs by the transfer of a hydride ion equivalent from the 4A locus of NADH to the 4A locus of AcPyADH (Figure 8). This stereospecificity indicates that the mechanism of DD-transhydrogenation involves NADH binding to transhydrogenase, followed by the dissociation of NAD^+ and then the binding of $AcPyAD^+$ which is subsequently reduced. An alternate mechanism for DD-transhydrogenation, not involving a reduced enzyme intermediate, would invoke the formation of a substrate–enzyme ternary complex in which $AcPyAD^+$ bound to the NADP site is reduced by NADH bound to the NAD site or $AcPyAD^+$ bound at the NAD site is reduced by NADH bound at the NADP site. In the first instance [4B-^{3}H]AcPyADH would be formed, whereas in the other case no label would be transferred. The validity of determining stereospecificity utilizing AcPyAD has been demonstrated by results with a number of pyridine dinucleotide analogues which show that the substituent at carbon 3 of the pyridine ring does not affect the stereospecificity of hydride ion transfer (You et al., 1978). In addition, the stereospecificity of hydride ion exchange is typically the same, whether NAD or NADP is substrate, with enzymes which employ either coenzyme (You et al., 1978).

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Covalent Cross-Linking of Transfer Ribonucleic Acid to the Ribosomal P Site. Mechanism and Site of Reaction in Transfer Ribonucleic Acid†

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ABSTRACT: The covalent cross-linking of unmodified *Escherichia coli N*-acetylvalyl-tRNA to the 16S RNA of *Escherichia coli* ribosomes upon near-UV irradiation previously reported by us [Schwartz, I., & Ofengand, J. (1978) *Biochemistry 17*, 2524–2530] has been studied further. Up to 70% of the unmodified tRNA, nonenzymatically bound to tight-couple ribosomes at 7 mM Mg^{2+}, could be cross-linked by 310–335-nm light. Covalent attachment was solely to the 16S RNA. It was dependent upon both irradiation and the presence of mRNA but was unaffected by the presence or absence of 4-thiouridine in the tRNA. The kinetics of cross-linking showed single-hit behavior. Twofold more cross-linking was obtained with tight-couple ribosomes than with salt-washed particles. Puromycin treatment after irradiation released the bound *N*-acetyl[^{3}H]valine, demonstrating that the tRNA was covalently bound at the P site and that irradiation and covalent linking did not affect the peptidyl transferase reaction. Cross-linking was unaffected by the presence of O_2, argon, ascorbate (1 mM), or mercaptoethanol (10 mM). Prephotolysis of a mixture of tRNA and ribosomes in the absence of poly(U_2,G) did not block subsequent cross-linking in its presence nor did it generate any long-lived chemically reactive species. There was a strong tRNA specificity. *E. coli* $tRNA_1^{Val}$ and $tRNA_1^{Ser}$ and *Bacillus subtilis* $tRNA^{Val}$ and $tRNA^{Thr}$ could be cross-linked, but *E. coli* $tRNA_2^{Val}$, 5-fluorouracil-substituted $tRNA_1^{Val}$, $tRNA^{Phe}$, or $tRNA_f^{Met}$ could not. By sequence comparison of the reactive and nonreactive tRNAs, the site of attachment in the tRNA was deduced to be the 5′-anticodon base, cmo^5U, or mo^5U in all of the reactive tRNAs. The attachment site in 16S RNA is described in the accompanying paper [Zimmerman, R. A., Gates, S. M., Schwartz, I., & Ofengand, J. (1979) *Biochemistry* (following paper in this issue)]. The link between tRNA and 16S RNA is either direct or involves mRNA bases at most two nucleotides apart since use of the trinucleotide GpUpU in place of poly(U_2,G) to direct the binding and cross-linking of *N*-acetylvalyl-tRNA to the P site did not affect either the rate or yield of cross-linking. Both *B. subtilis* $tRNA^{Val}$ (mo^5U) and *E. coli* $tRNA_1^{Val}$ (cmo^5U) gave the same rate and yield of cross-linking when directed by the trinucleotide GpUpU. Therefore, the presence of the charged carboxyl group in the cmo^5U-containing tRNA apparently does not markedly perturb the orientation of this base with respect to its reaction partner in the 16S RNA. The cross-linking of AcVal-tRNA only takes place from the P site. At 75 mM KCl and 75 mM NH_4Cl, <0.4% cross-linking was found at the A site, while 55.5% was obtained at the P site. However, when the salt concentration was lowered to 50 mM NH_4Cl, 5% cross-linking to the A site was detected, compared to 49% at the P site. Thus, a simple change in the ionic strength of the incubation mixture was able to alter the affinity labeling pattern of the ribosome.

Protein biosynthesis demands the close functional cooperation of a remarkable variety of macromolecules and macromolecular assemblies, including mRNA, tRNA, a number of soluble protein factors, and the 70S ribosome (Weissbach & Pestka, 1977). Physical coordination of this process is accomplished by the ribosomal particle which provides specific sites of association for the nonribosomal components and thereby assures their correct mutual orientation during initiation, elongation, and termination of the polypeptide chain. A clear understanding of ribosome function requires that the position and topography of these sites be delineated as fully as possible. One means of elucidating such features entails the covalent attachment of normal or chemically modified

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