Resolution of Enzymes Catalyzing Energy-linked Transhydrogenation

III. PREPARATION AND PROPERTIES OF RHODOSPIRILLUM RUBRUM TRANSHYDROGENASE FACTOR*

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SUMMARY

1. A highly purified transhydrogenase factor (THI) has been isolated from Rhodospirillum rubrum chromatophores by washing the membranes first with buffer containing 10μM NADP+ under conditions in which THI remains bound, then with buffer lacking NADP+ resulting in the dissociation of THI from the membrane.

2. Purified THI stimulated the energy-linked reduction of NADP+ by NADH, the nonenergy-linked reduction of AcPyAD+ by NADPH, and the reduction of AcPyAD+ by NADH in the presence of chromatophores resolved with respect to these activities. THI did not catalyze these reactions independently of these resolved chromatophores.

3. The energy-linked reduction of NADP+ by NADH has been demonstrated to occur by the transfer of hydrogen without prior exchange to the medium from the 4A (nicotinamide) locus of NADH to the 4B (nicotinamide) locus of NADPH. This stereospecificity is identical with that found for the analogous mitochondrial reaction.

4. It is concluded that THI functions in the transfer of hydrogen between NAD(H) and NADP(H) in both the energy- and nonenergy-linked transhydrogenase reactions.

The energy-linked reduction of NADP+ by NADH catalyzed by Rhodospirillum rubrum chromatophores resembles in many respects the reaction catalyzed by submitochondrial particles (1). However, in contrast to the mitochondrial transhydrogenase system, that of R. rubrum is easily resolved into a soluble component, the transhydrogenase factor (THI), and the insoluble chromatophore membrane (2, 3). The complex formed between THI and the membrane is stabilized by the presence of low concentrations of either NADP+ or NADPH (3). This complex is dissociated in the presence of high concentrations of NADPH or NADH.

In addition to the essentially irreversible energy-linked transhydrogenation, both mitochondria (4) and chromatophores (1) catalyze a nonenergy-linked transhydrogenation which can be described by Equation 1.

NADH + NADP+ + NADf + NADPH (1)  

Kaufman and Kaplan (5) have described the solubilization of a lipoprotein by detergent treatment of ox heart mitochondria which catalyzed this reaction. Antibodies produced against the "soluble" enzyme inhibited both the energy-linked and nonenergy-linked transhydrogenase reactions of submitochondrial particles. The membrane-bound enzyme (6) as well as the solubilized enzyme (7) transfers hydrogen in a stereospecific manner from the 4A locus of NADH to the 4B locus of NADPH.

When it was discovered that crude THI was able to catalyze the reversible nonenergy-linked reduction of NADP+ and NADH analogues by NADH (2), the possibility presented itself that THI might itself be a transhydrogenase enzyme. In order to examine this possibility, it was felt necessary to prepare a highly purified THI.

It is the purpose of this paper to describe a unique method for the isolation of a purified preparation of THI from chromatophore membranes and to describe some of its properties. In addition, the stereospecificity of the energy-linked R. rubrum transhydrogenase was studied and was found to occur by direct hydrogen transfer and with the same stereospecificity as the mitochondrial reaction.

EXPERIMENTAL PROCEDURE

R. rubrum was cultured, chromatophores and C5-particles were prepared, and assays for energy-linked transhydrogenation and protein and for BChl were performed as previously described (3).

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The abbreviations used are: C5-particles, chromatophores depleted of energy-linked transhydrogenase activity; TD transhydrogenase, reduction of AcPyAD+ by NADPH; DD transhydrogenase, reduction of AcPyAD+ by NADH; BChl, bacteriochlorophyll.
**Assay for Nonenergy-linked (TD) Transhydrogenase Activity—** This reaction was assayed at 23° in a medium (3 ml) containing 44 mM Tris-HCl buffer, pH 8, 125 mM sucrose, 0.134 mM AcPyAD+, 3.3 mM glucose-6-P, 0.067 mM NADPH, 1 unit of glucose 6-phosphate dehydrogenase, and 0.008 mM rotenone (added to inhibit chromatophore NADH oxidase (1)). AcPyAD+ was omitted from the blank. The rate of AcPyAD+ reduction was calculated from the increase in absorption at 375 nm assuming a millimolar extinction coefficient of 5.1 (8). The reaction was initiated by the addition of chromatophores or C₃-particles containing about 20 µg of BChl.

**Assay for Nonenergy-linked (DD) Transhydrogenase Activity—** This reaction was assayed at 23° in a medium (3 ml) containing 44 mM Tris-HCl buffer, pH 8, 125 mM sucrose, 0.13 mM AcPyAD+, 0.134 mM NADH, and 0.0085 mM rotenone. The rate of AcPyAD+ reduction was measured as outlined above following the addition of chromatophores or C₃-particles.

**Assay for Tritium Incorporation into [4B-3H]-NADPH—** The concentration and specific activity of [4B-3H]-NADPH was determined after oxidation with glutathione and glutathione reductase according to the procedure of Lee et al. (6). In this method, hydrogen from the 4B locus of NADPH exchanges with the medium water. The quantity of tritium recovered in the medium water following treatment of the sample with glutathione reductase minus the quantity of tritium in the water prior to the oxidation of NADPH was taken as a measure of the amount of tritium in [4B-3H]-NADPH. Water was isolated from reaction mixtures by the following method. Of the reaction mixture, 0.4 ml was transferred to the side bulb of a Thunberg tube and frozen in a methyl Cellosolve bath cooled with Dry Ice. The tube was evacuated and sealed, and its body was immersed in the Dry Ice bath. When the water had distilled from the side bulb into the body of the tube its tritium content was determined (0.3 ml samples) by liquid scintillation counting.

**Preparation of [4A-3H]-NADH—** Of the 4A-3H-labeled NAD+ (specific activity 1.24 Ci per mmole), 5.36 µg were reduced in a medium (1.28 ml) containing 31 mM Tris-HCl buffer, pH 8, 88 mM sucrose, 7.8 mM unlabeled NAD+, 47 mM L-sodium glutamate, 280 µM hydrazine hydrate, and 1 mg of glutamate dehydrogenase (ammonium sulfate free). The reaction mixture was incubated at 20° for 25 min and the reaction terminated by heating for 3 min in a boiling water bath. Distilled water was added to bring the volume to 5 ml and the mixture was centrifuged to remove denatured protein. The specific activity of the [4A-3H]-NADH was evaluated by liquid scintillation counting after separation from unreacted [4-3H]-NAD+ by DEAE-Sephadex column chromatography (see below).

**Preparation of [4B-3H]-NADH—** Of the 4B-3H-labeled NAD+ (specific activity 5.1 Ci per mmole), 2.86 µg were reduced in a medium (2.5 ml) containing 8 mM Tris-HCl buffer, pH 8, 23 mM sucrose, 2 mM unlabeled NAD+, 96 mM hydrazine hydrate, 65 µM ethanol, and 0.2 mg of yeast alcohol dehydrogenase. The reaction mixture was incubated at 21° for 15 min and the reaction terminated by heating for 3 min in a boiling water bath. The specific activity of [4B-3H]-NADH was evaluated as described for [4A-3H]-NADH.

**Separation of Pyridine Nucleotides—** A neutralized solution containing a mixture of pyridine nucleotides (12 µmoles total) was placed on a column (0.65 × 10 cm) of DEAE-Sephadex A-25. The column was washed with 50 ml of distilled water and NAD+, NADH, NADP+, and NADPH were eluted stepwise with 0.04 M, 0.09 M, 0.13 M, and 0.25 M Tris-HCl buffer, pH 7, respectively (9).

**Liquid Scintillation Counting—** This was done with a Nuclear Chicago Mark I counter. Samples of 0.1 to 0.3 ml were added to 10 ml of scintillation fluid prepared by adding 5 g of 2,5-diphenyloxazole (PPO), 0.2 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl-POPOP), and 100 g of naphthalene to 1 liter of dioxane. The counting efficiency was estimated to be 35%.

**Definition of Units—** A unit of TH₁ activity is defined as the amount of protein required to stimulate the ATP-dependent transhydrogenase reaction rate in C₃-particles by 1 µmole of NADP⁺ reduced per mg of BChl per min. Specific activity is expressed as units per mg of protein.

**Reagents—** Sources of reagents not given previously (3) were retenone, Sigma Chemical Company; dithiothreitol and L-glutamic acid, Calbiochem; hydrazine hydrate, Matheson, Coleman and Bell Company, Inc.; 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene and 2,5 diphenyloxazole, Packard Instrument Company; 3-acetylpyridine-NAD⁺, Pabst Laboratories; DEAE-Sephadex A-25, Pharmacia Fine Chemicals Inc.; [4-3H]-NAD⁺ (99% radiochemically pure), New England Nuclear; and glutamate dehydrogenase (No. 15140), Boehringer Mannheim Corporation.

**RESULTS**

**Preparation of Purified TH₁ from Chromatophores**

The finding that NADP⁺ promoted the binding of TH₁ to C₃-particles and stabilized the TH₁ membrane complex of chromatophores during washing (3) led to the development of a procedure for the preparation of TH₁ of high purity. This method involves (a) removal of loosely bound membrane proteins excluding TH₁, by washing chromatophores with buffer containing NADP⁺, (b) solubilization of TH₁ by washing with buffer lacking NADP⁺, and (c) concentration of TH₁ by precipitation with ammonium sulfate. All steps were carried out at 0–4°.

**Step 1: Disruption of Cells—** R. rubrum cells (40 g, wet wt) were suspended in 115 ml of 0.1 M Tris-HCl buffer, pH 8, 10% sucrose containing 10 µM NADP⁺, and 2 mM MgCl₂, and transferred to a 200-ml stainless steel beaker chilled in ice. The cells were sonicated for 1 min with a Bronson sonic oscillator (J-32 probe) and the sonic extract was centrifuged for 10 min at 18,000 × g. The supernatant solution was decanted and centrifuged a second time for 30 min at 150,000 × g. The chromatophore pellets from this second centrifugation were suspended by homogenization in 15 ml of the above buffer and the supernatant solution was saved for the preparation of crude TH₁ (see below).

**Step 2: Pyridine Nucleotide Wash—** The 15 ml of suspended chromatophores (Step 1) were diluted to 165 ml with the same buffer, slowly stirred for 5 min, and then sedimented by centrifugation at 150,000 × g for 30 min. The supernatant solution was discarded.

**Step 3: Solubilization of TH₁—** The pellets (washed chromatophores) obtained from Step 2 were suspended in 95 ml of 0.1 M Tris-HCl buffer, pH 8, 10% sucrose containing 0.001 M dithiothreitol and stirred for 5 min prior to sedimentation by centrifugation at 150,000 × g for 20 min. The pellets were suspended again in 95 ml of the above buffer to solubilize the remaining membrane-bound TH₁. After repeating the stirring and centrifugation procedure, the supernatant solutions from the first and second washes were combined and treated as described in Step 4.

**Step 4: Concentration of Solubilized TH₁ by Ammonium Sul-
fate Precipitation.—To the combined wash supernatant solutions, solid ammonium sulfate was added with stirring to a final concentration of 70% of saturation. Stirring was continued for 30 min after the salt completely dissolved. The precipitate was sedimented by centrifugation for 15 min at 150,000 × g. The film-like precipitate was dissolved in 3 ml of 0.01 M Tris-HCl buffer, pH 8, 1% sucrose containing 0.15 mg of dithiothreitol per ml. This solution was centrifuged for 60 min at 150,000 × g. The colorless supernatant solution containing purified TH₁ was stable for at least a week when stored at 0-4°.

Notes on Preparation of Purified TH₁

During the development of the above procedure, it was found that TH₁ was very unstable in dilute protein solutions. Dithiothreitol was found to stabilize the factor and for this reason it is included in the wash medium of Step 3. However, dithiothreitol was not able to reactivate TH₁ which had been inactivated by dilution. Bovine serum albumin (at 0.5 mg per ml) has been successfully employed in place of dithiothreitol; however, its use prevents the accurate determination of TH₁ specific activity.

The ease with which TH₁ is dissociated from the membrane in the pyridine nucleotide wash step. When NADP⁺ concentrations in excess of 10 μM were used in Step 2, solubilization of the factor during Step 3 required as many as five or six washings to effect complete solubilization. Thus, the procedure became unduly laborious and the subsequent precipitation of TH₁ by ammonium sulfate in Step 4 was hampered. If less than 10 μM NADP⁺ was used in Step 2, significant amounts of membrane-bound TH₁ could be solubilized prior to Step 3.

Preparation of Crude TH₁.—The supernatant solution from Step 1 was diluted with an equal volume of 0.1 M Tris-HCl buffer, pH 8, 1% sucrose containing 0.001 M dithiothreitol and centrifuged at 150,000 × g for 60 min. TH₁ was precipitated by bringing the high speed supernatant solution to 70% saturation with solid ammonium sulfate. The solution was stirred for an additional 30 min after the salt had dissolved and then centrifuged at 18,800 × g for 10 min to sediment the TH₁. The supernatant solution was decanted and the interior of the centrifuge tube wiped free of excess ammonium sulfate solution. The pellets were then suspended in a minimal volume of the above buffer and centrifuged at 150,000 × g for 60 min. The deep orange supernatant solution is termed crude TH₁.

It was shown in the previous paper (8) that TH₁ solubilized directly from unwashed chromatophore membranes had a specific activity only 5-fold greater than that of TH₁ prepared from the cell-free extract. The relative specific activities of the crude TH₁ and the purified TH₁ prepared as described above, were determined by their ability to stimulate the ATP-dependent transhydrogenase reaction in C₇-particles. As can be seen in Table I, the specific activity of purified TH₁ was over 1200 times that found in the crude supernatant fraction. Precipitation of TH₁ from the crude supernatant fraction with ammonium sulfate resulted in a preparation with a specific activity 7-fold greater than that of the crude supernatant fraction. The observed increase in total units obtained by this treatment was as great as the increase in specific activity. This activation can be explained if the extract, unlike the ammonium sulfate fraction, contained a component which inhibits the interaction of TH₁ with the chromatophore membrane.

It is obvious that the washing procedure which allows for the isolation of TH₁, directly from washed chromatophores resulted in a factor preparation of considerable purity. Two preliminary experiments with polyacrylamide disc gel electrophoresis showed the purified TH₁ to be composed of components giving four protein bands.

**Table I**

| Different factor preparation | Total protein | Specific activity | Relative specific activity | Units |
|------------------------------|---------------|------------------|---------------------------|-------|
| 1. Crude supernatant fraction| 1136 mg       | 0.056            | 1.0                       | 63.5  |
| 2. Crude TH₁                 | 1260 mg       | 0.408            | 7.2                       | 457.0 |
| 3. Purified TH₁              | 3.7 mg        | 68.0             | 1215.0                    | 255.3 |

**Fig. 1. Reconstitution of energy-linked transhydrogenation with purified TH₁.** The light- (●—●) and ATP- (○—○) dependent transhydrogenase reactions were assayed in the presence of C₇-particles (22 μg of BChl) as described previously (3).

**Reconstitution of Energy-linked Transhydrogenase Activity with Purified TH₁**

Purified TH₁ reconstituted the light-dependent as well as the ATP-dependent transhydrogenase reactions in C₇-particles (Fig. 1). Both reactions were stimulated to approximately the same percentage of their maximal rate at each titer of TH₁. Unlike reconstitution with less pure factor preparations (2, 3, 10), the maximal rate obtainable with the light-dependent reaction was identical with that of the ATP-dependent reaction. The relative abilities of purified and crude TH₁ to restore ATP- and light-dependent transhydrogenase activity in C₇-particles is shown in Table II. Saturating titers of these factor preparations stimu-
Reconstitution of energy-linked transhydrogenation in C7-particles with crude and purified TH1

Transhydrogenation was assayed at 23°C as described under Table I.

| Additions           | Transhydrogenase energy source | μmoles NADPH/mg BChl/hr |
|---------------------|--------------------------------|-------------------------|
| None                | ATP                            | 1.2                     |
|                    | Light                          | 1.0                     |
| Crude TH1, 2.90 mg  | ATP                            | 33.2                    |
|                    | Light                          | 52.1                    |
| Purified TH1, 0.022 mg | ATP                            | 33.2                    |
|                    | Light                          | 32.4                    |

Fig. 2. Reconstitution of the nonenergy-linked (TD) and energy-linked transhydrogenation with purified TH1. The nonenergy-linked transhydrogenase rate with increasing concentrations of TH1 in the presence of 18.2 μg of BChl of C7-particles (O—O) is compared with the rate of the reaction in the absence of C7-particles (●—●) and the rate of the reconstituted ATP-dependent transhydrogenation (Δ—Δ). The maximal rate of the nonenergy-linked reaction was 17.7 μmole of AcPyADH reduced per min, while that of the reconstituted ATP-dependent transhydrogenase was 9.5 μmole of NADP+ reduced per min.

Reconstitution of Nonenergy-linked Transhydrogenase Activity with Purified TH1

As can be seen from Fig. 2, the ATP-dependent reduction of NADP+ by NADH and the nonenergy-linked reduction of AcPyADH by NADPH were stimulated in C7-particles to an identical percentage of their respective maximal rates at each concentration of purified TH1. Unlike crude factor preparations (2), purified TH1 did not catalyze a nonenergy-linked transhydrogenation in the absence of C7-particles. Attempts to elicit transhydrogenase activity in purified TH1 by variation of pH or addition of bovine serum albumin or soya bean phospholipid micelles were unsuccessful.

Since NADP+ was known to prevent the loss of TH1 from chromatophore membranes during washing (3), it was of interest to compare the relative levels of the nonenergy- and energy-linked transhydrogenase activities during washing. Therefore, chromatophores containing 7.5 mg of BChl were washed three times with 90 ml of either 0.1 M Tris-HCl buffer, pH 8, 10% sucrose or with 0.1 M Tris-HCl buffer, pH 8, 10% sucrose containing 10 μM NADP+ and 2 mM Mg2+. Following each washing, the chromatophore pellets were sedimented by centrifugation at 150,000 × g for 30 min and resuspended in 3 ml of Tris-HCl buffer. The rates of ATP-dependent and nonenergy-linked (TD) transhydrogenases were determined following each wash. It is apparent from Fig. 3 that the presence of NADP+ in the wash buffer resulted in the retention of both transhydrogenase activities to the same extent, while washing in the absence of NADP+ led to depletion of both activities.

Stimulation of DD Transhydrogenation in C7-particles by Purified TH1

Enzymes which catalyze transhydrogenation between NADPH and NAD+ (TD transhydrogenase) have been isolated from a variety of sources, and a number have been reported to catalyze at least a trace of transhydrogenation between NADH and NAD+ analogues (UD transhydrogenase) (8, 11, 12). Since many dehydrogenases are known to catalyze DD transhydrogenation (13), it is difficult to conclude whether the DD activity represents an inherent property of the TD transhydrogenase enzyme or whether it represents a contaminating dehydrogenase activity. A homogeneous flavoprotein pyridine nucleotide transhydrogenase from Pseudomonas aeruginosa has been found to catalyze both DD and TD transhydrogenase reactions (12).
Fig. 4. Effect of purified TH1 on CT-particle DD and TD transhydrogenase reactions. DD transhydrogenase in the presence (A—A) and absence (Δ—Δ) of CT-particles, with 18 μg of BChl. TD transhydrogenase in the presence (○—○) and absence (O—O) of CT-particles.

Purified TH1 was tested for DD transhydrogenase activity (reduction of AcPyAD+ by NADH) in the presence and absence of CT-particles. Fig. 4 shows that TH1 did not manifest DD transhydrogenase activity in the absence of CT-particles. However, the factor stimulated the endogenous DD transhydrogenase of CT-particles maximally about 3-fold at the same titers which maximally stimulated the nonenergy-linked TD transhydrogenase more than 8-fold. These observations suggest that TH1 is required for both kinds of nonenergy-linked transhydrogenase reactions of chromatophores.

Stereopecificity of Hydrogen Transfer during Transhydrogenation

The energy linked reduction of NADP+ by NADH catalyzed by R. rubrum chromatophores possesses properties similar to the mitochondrial reaction (1). On the other hand, the mode of hydrogen transfer for the chromatophore reaction has not been investigated. It was felt necessary to determine whether the over-all process occurs by a direct hydrogen transfer mechanism similar to the energy-linked transhydrogenases of mitochondria (7, 9) and of Rhodospirillum spheroides (14).

The chromatophore ATP-dependent NADH reduction of NADP+ was studied in the presence of NADH tritiated in position 4 of the nicotinamide ring (see “Experimental Procedure”). In order to remove soluble proteins unrelated to the transhydrogenase reaction, but which nevertheless might catalyze an exchange of tritium to the medium, chromatophores (2 mg of BChl) were first washed with 50 ml of 0.01 M Tris-HCl buffer, pH 8, 1% sucrose containing 50 μM NADP+.

![Graph showing tritiated NADH activity](http://www.jbc.org/)

![Graph showing energy-linked reduction of NADP+](http://www.jbc.org/)

**Table III**

| Tritiated NADH | Activity | NADH formed | Activity | Activity |
|----------------|----------|-------------|----------|----------|
| [4α-3H]-NADH    | 320,000  | 0.29        | 99,517   | 0.57     |
| [4β-3H]-NADH    | 392,000  | 0.33        | 171      | 1.26     |

As shown in Fig. 5, the formation of NADPH during transhydrogenation was directly proportional to the amount of tritium transferred from the 4α locus of NADH to the 4β locus of NADP+. NADP+ separated from the reaction mixture by DEAE-Sephadex chromatography (see “Experimental Procedure”), after the B-specific oxidation of NADPH by glutathione...
activated the TD transhydrogenase activity of these preparations.

The reduction of NADP⁺ by [4B-3H]-NADH during chromatophore transhydrogenation was also tested (Table III). Tritium was not incorporated into NADPH with [4B-3H]-NADH but only when [4A-3H]-NADH served as the hydrogen donor. The specific activity of the tritiated NADPH formed with [4A-3H]-NADH was less than one-third that of the initial [4A-3H]-NADH. This low specific activity is as yet unexplained, but could result from a slow exchange of tritium to the medium from a reduced reaction intermediate, from a kinetic isotope effect, or from [4B-3H]-NADPH catalyzed by an enzyme unrelated to transhydrogenation. Nevertheless, on the basis of the low specific activity of tritium in the water following the reaction (prior to oxidation of the NADPH with glutathione reductase), it is assumed that the tritium transfer between the pyridine nucleotides could not involve a prior exchange to water.

Discussion

Mode of TH₁ Action—We have previously demonstrated that TH₁ has no influence on chromatophore light-induced phosphorylation (2) or the energy-linked reduction of NAD⁺ by succinate (15). On the basis of these experiments, it is concluded that TH₁ acts neither as a coupling factor for energy conservation nor as an electron carrier in the electron transport chain. Preliminary studies with crude TH₁ suggested that it could be a soluble transhydrogenase enzyme (2), however, as documented in this paper, no such activity could be detected in the most highly purified factor preparations (Fig. 2).

The dependence of nonenergy-linked as well as energy-linked transhydrogenase reactions of C₇-particles on TH₁ indicates that the factor may act at the level of hydrogen transfer in both reactions. The view that a single factor reconstitutes both energy- and nonenergy-linked transhydrogenase reactions is supported by the following facts: (a) energy- and nonenergy-linked transhydrogenase reactions are stimulated to an identical percentage of their maximal rates at each titer of TH₁ and (b) both reactions required a factor which is membrane-bound in the presence of NADP⁺, but which is easily dissociated from the membrane in the absence of NADP⁺ (Eq. 3).

Assuming that TH₁ functions solely in hydrogen transfer, a number of possibilities for its specific mode of action can be visualized.

1. TH₁ may be a transhydrogenase enzyme which is nonfunctional until activated by being bound to the chromatophore membrane. The term “allostery” has been used to define a change in properties, i.e. activation, inactivation, or sensitivity to inhibitors, which a number of enzymes undergo upon binding to membranes (16). This phenomenon is manifested not only by enzymes of a completely particulate nature, but also by a number of normally soluble enzymes which can be reversibly bound to membranes (17-19). In addition, such membrane-enzyme interactions may involve specific lipid and or lipoprotein components within the membrane (18, 20). By way of example, treatment of submitochondrial particles (21) or of a detergent-solubilized mitochondrial transhydrogenase enzyme (5) with phospholipase inactivated the TD transhydrogenase activity of these preparations. Such findings indicate that for the mitochondrial system transhydrogenase activity is dependent on the integrity of a lipoprotein complex. Although it has not yet been possible to stimulate nonenergy-linked transhydrogenase activity in TH₁ by incubation with artificial phospholipid micelles, the possibility that a specific lipid protein interaction is responsible for the activation and binding (3) of TH₁ to chromatophores cannot be ruled out. Alternatively, the membrane may provide a cofactor which mediates hydrogen transfer between active sites for NADH and NADP⁺ on TH₁.

2. An additional possibility is that TH₁ operates as terminal enzyme, of a multienzyme transhydrogenase system, catalyzing the dehydrogenation of either NADH or NADPH. If such is the case, a membrane component (or components) could function catalytically rather than structurally in hydrogen transfer. Griffiths and Robertson (9) have hypothesized that mitochondrial transhydrogenase may be mediated by two dehydrogenases, acting in series, one of which is A-specific for NADH and the other B-specific for NADPH. This mode of hydrogen transfer is also consistent with the mechanism for the reaction proposed by Mitchell (22).

Path of Hydrogen Transfer—The demonstration that the energy-dependent transhydrogenase reaction of R. rubrum chromatophores occurs by a direct hydrogen transfer from the 4A position of NADH to the 4B position of NADPH supports the possibility that the mechanism of this reaction is similar to the reaction found in mitochondria. Although it remains to be shown that chromatophore DD transhydrogenation also occurs by direct hydrogen transfer, the stimulation of both DD and TD transhydrogenase activities in C₇-particles by purified TH₁ suggests that the former activity may be a partial reaction of the latter activity. DD transhydrogenation would be expected to occur if the mechanism of TD transhydrogenation involves the transfer of hydrogen from NADH to an intermediate (C) in the transhydrogenase system prior to the reduction of NADP⁺ (Equation 2)

\[
\text{NADH} + \text{C} \rightleftharpoons \text{NAD⁺} + \text{CH} \quad (2)
\]

\[
\text{NADP⁺} + \text{CH} \rightleftharpoons \text{NADPH} + \text{C} \quad (3)
\]

\[
\text{AcPyAD⁺} + \text{CH} \rightleftharpoons \text{AcPyADH} + \text{C} \quad (4)
\]

The reduced intermediate could subsequently reduce NADP⁺ (Equation 3) or AcPyAD⁺ (Equation 4). The linking of Reaction 2 with Reaction 3 would catalyze a TD transhydrogenation and Reaction 2 followed by Reaction 4 would result in a DD transhydrogenation.

The possible occurrence of a reduced enzyme intermediate in transhydrogenase reactions has not been ruled out. The fact that the process in mitochondria and chromatophores takes place without exchange of hydrogen to medium water indicates that an easily accessible hydrogen acceptor is an unlikely participant in the reaction. On the other hand, a direct transhydrogenation between NADH and NADP⁺ catalyzed by an enzyme from Pseudomonas aeruginosa has been reported to incorporate a reduced flavin intermediate (12). As of yet no such cofactor requirement has been found for TH₁.

Orlando (23) has recently shown that chromatophores of Rhodopseudomonas spheroides subjected to a discontinuous sucrose gradient lose the ability to catalyze the light- and ATP-dependent transhydrogenation. The light-dependent, but not ATP-dependent, activity can be reactivated by the addition of a factor isolated from lyophilized powders of the same cells. Both the light- and ATP-dependent reactions can as well be reactivated by the dithiols, dithiothreitol, dithioerythritol, or reduced thiocic acid, in the absence of the factor. The factor from K.
spheroides does not influence the nonenergy-linked transhydrogenation which is still active in particles inactivated with respect to the energy-linked reaction.

The above characteristics represent major differences in comparison with the characteristics of the transhydrogenase factor isolated from *R. rubrum* and described in this paper. (a) The TH factor reconstitutes the light-driven, the ATP-driven, and the nonenergy-linked reaction in resolved chromatophores of *R. rubrum*, whereas the factor from *R. spheroides* is only active in reconstituting the light-driven reaction. (b) In contrast to the *R. spheroides* factor, dithiols do not substitute for the THI factor in *R. rubrum*.

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REFERENCES
1. Keister, D. L., and Yiike, N. J., *Biochemistry*, 6, 3847 (1967).
2. Fisher, R. R., and Guillory, R. J., *J. Biol. Chem.*, 244, 1075 (1969).
3. Fisher, R. R., and Guillory, R. J., *J. Biol. Chem.*, 246, 4679 (1971).
4. Lee, C. P., and Ernstier, L., *Biochim. Biophys. Acta*, 81, 187 (1964).
5. Kaufman, B., and Kaplan, N. O., *J. Biol. Chem.*, 236, 2133 (1961).
6. Lee, C. P., Simard-Duquesne, N., Ernstier, L., and Hoberman, H. D., *Biochim. Biophys. Acta*, 105, 397 (1965).
7. Kawasaki, T., Satoy, K., and Kaplan, N. O., *Biochim. Biophys. Acta*, 17, 648 (1964).
8. Kaplan, N. O., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology*, Vol. X. Academic Press, New York, 1967, p. 317.
9. Griffiths, D. E., and Robertson, A. M., *Biochim. Biophys. Acta*, 115, 453 (1966).
10. Fisher, R. R., and Guillory, R. J., *Fed. Eur. Biochim. Soc. Lett.*, 3, 27 (1969).
11. Keister, D. L., and Hemmes, R. B., *J. Biol. Chem.*, 241, 2829 (1966).
12. Cohen, P. T., and Kaplan, N. O., *J. Biol. Chem.*, 245, 2825 (1970).
13. Weber, M. M., and Kaplan, N. O., *J. Biol. Chem.*, 225, 909 (1957).
14. Orlando, J. A., *Arch. Biochem. Biophys.*, 124, 433 (1967).
15. Thomas, J. O., Fisher, R. R., and Guillory, R. J., *Biochim. Biophys. Acta*, 238, 204 (1970).
16. Racker, E., *Fed. Proc.*, 26, 1335 (1967).
17. Copley, M., and Fromm, H. J., *Biochemistry*, 6, 3503 (1967).
18. Lynn, W. S., Jr., Brown, R. H., and Mullins, J., *J. Biol. Chem.*, 239, 995 (1964).
19. Mansour, T. C., Wikid, N., and Sprouse, H. M., *J. Biol. Chem.*, 411, 1512 (1966).
20. Lynne, A., and Racker, E., *J. Biol. Chem.*, 244, 1339 (1969).
21. Pesch, L. A., and Peterson, J., *Biochim. Biophys. Acta*, 98, 390 (1965).
22. Mitchell, P., *Biol. Rev. (Cambridge)*, 41, 445 (1966).
23. Orlando, J. A., *Arch. Biochem. Biophys.*, 141, 111 (1970).
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