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COMPARATIVE STUDIES ON NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE FROM DIFFERENT SOURCES

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SUMMARY

1. Nicotinamide nucleotide transhydrogenases in submitochondrial particles from beef heart mitochondria, chromatophores from *Rhodospirillum rubrum* and membrane preparations from *Escherichia coli* and *Pseudomonas aeruginosa* have been compared with respect to the following properties: stereospecificity for the 4-hydrogen of NADH, reactivity with 3′-NADP, inhibition by palmityl-CoA, sensitivity tot ryp-sin, and effects of Ca^{2+} and 2′-AMP on the reaction rates.

2. Transhydrogenases from submitochondrial particles, *R. rubrum* chromato-phores and *E. coli* membrane preparations have A-side stereospecificity for NADH, do not react with 3′-NADP, are inhibited by palmityl–CoA and are extremely sen-sitive to trypsin treatment. No effects of Ca^{2+} or 2′-AMP on the reaction rates were observed. In *R. rubrum* chromatophores trypsin-sensitive sites are present both in the soluble transhydrogenase factor and in the membrane preparation devoid of transhydrogenase factor.

3. In contrast, *P. aeruginosa* transhydrogenase is allosterically regulated by Ca^{2+} and 2′-AMP, is reactive with 3′-NADP, has B-side stereospecificity for NADH and is insensitive to palmityl–CoA or trypsin treatment.

4. It is concluded that the properties characterizing the transhydrogenase in *E. coli*, *R. rubrum* chromatophores and submitochondrial particles are closely con-nected with the interaction of the enzyme with an energy-conserving membrane system.

INTRODUCTION

Nicotinamide nucleotide transhydrogenase catalyzes the reversible transfer of hydrogen between the oxidized and reduced forms of NAD and NADP. In mammalian systems the enzyme is strongly bound to the inner mitochondrial membrane and is functionally linked to the enzyme system catalyzing oxidative phosphorylation [1] (see [2] for a review). Energy, generated either by the respiratory chain or from ATP, enhances both the rate and the extent of the NADH-dependent reduction of $NADP^+$ (the "forward" reaction) and inhibits the reverse reaction [3, 4]. Attempts to solu-bilize the system from mitochondria have so far been only partially successful [5, 6].

More recently, transhydrogenase activities have been demonstrated in mem-

brane preparations of both photosynthetic [7–9] and respiring bacteria [10–12]. These transhydrogenases resembled the mammalian system in their ability to interact with the energy-conserving system. Studies of these bacterial transhydrogenases have been concentrated mainly on their relationship with energy-conserving reactions [13–16]. From chromatophores of *Rhodospirillum rubrum* a protein factor has been solubilized [17–19] which, though itself devoid of transhydrogenase activity, was essential for the occurrence of both the energy-linked and the non-energy-linked transhydrogenase reactions in these particles.

In *Pseudomonas* and *Azotobacter* species, there occur transhydrogenases that can easily be solubilized. These enzymes have extensively been purified and studied in detail by Kaplan and coworkers [20–24] and by Van den Broek and coworkers [25–29] (see also [30]). No functional relationship with the energy-conserving system was found for these enzymes, and they differ from the mammalian enzyme in several properties, e.g. in their stereospecificity to NADH [21, 31, 32–34], their reactivity to 3′-NADP [35, 36] and in the effects of 2′-AMP, Ca^{2+}, NADPH and $NADP^+$ on the reaction rates [20, 23, 29, 30].

In this paper, transhydrogenases from different sources were studied with respect to several properties that distinguish the mammalian transhydrogenase from the enzyme in *P. aeruginosa* preparations.

MATERIALS AND METHODS

Submitochondrial particles from beef-heart mitochondria were prepared according to Löw and Vallin [37] as described by Teixeira da Cruz et al. [38].

Escherichia coli K 12 and *P. aeruginosa* were grown on a minimal medium with succinate as the sole energy source, as described by Sweetman and Griffiths [39]. Membrane preparations from *E. coli* and *P. aerigunosa* were made by sonication according to the procedure of Sweetman and Griffiths [39].

Chromatophores from *Rhodospirillum rubrum* (Strain S 1) were prepared as described by Baltscheffsky [40]. The "light" chromatophore fraction ($100\,000 \times g$ precipitate) was used for measuring transhydrogenase activity. The final suspension contained 0.47 mg bacteriochlorophyll and 21 mg protein per ml. The transhydrogenase factor was prepared by repeated washing of the chromatophores, according to Konings and Guillory [19] and further purified as described by these authors, omitting the final DEAE-Sephadex chromatography step; the purified factor had a specific activity of 12 nmoles/min per mg. Specific activity is expressed as the stimulation of the reverse transhydrogenase activity (i.e. reduction of NAD^+ by NADPH) obtained on recombination with excess of the depleted chromatophore preparation.

Transhydrogenase activity was determined as described previously [30, 38].

The stereospecificity of the transhydrogenase with respect to NADH was determined by measuring the loss of radioactivity of [4B-^{3}H]NADH into water (cf. [32]). Water was isolated from the reaction mixture as described by Lee et al. [32]. ^3H_2O radioactivity was measured in a Beckman liquid scintillation counter.

Protein was determined by the biuret method. Chromatophores were pretreated with 0.2 M trichloroacetic acid in 80% acetone at 70 °C for 3 min and subsequently washed with 96% ethanol at 70 °C for 3 min in order to remove carotenoids, prior to protein determination.

Bacteriochlorophyll was determined at 880 nm, using an extinction coefficient of $1.4 \cdot 10^5\ M^{-1} \cdot cm^{-1}$.

An equimolar mixture of 3'- and 2'-NADP was prepared according to Shuster and Kaplan [35].

[4-^{3}H]NAD$^+$ was obtained from the Radiochemical Centre, Amersham. Other chemicals and enzymes were obtained from Sigma Chem. Co. or Boehringer, Mannheim.

RESULTS

Stereospecificity with respect to NADH

In submitochondrial particles the transhydrogenase reactions, both energy-linked and non-energy-linked, involve the 4B hydrogen atom of NADPH and the 4A hydrogen atom of NADH [32–34]. Similar findings have been reported for the transhydrogenase reaction in chromatophores from *R. rubrum* [18]. In contrast, the purified transhydrogenases from *A. vinelandii* and *P. aeruginosa* were found to be 4B specific for both NADH and NADPH [21, 31].

The stereospecificity of the transhydrogenase in *E. coli* was studied in the experiment shown in Fig. 1, by the method of Lee et al. [32]. Membrane particles were incubated with [4-^{3}H]NAD$^+$ and unlabeled NADPH (Curve A). Under these conditions ^3H_2O is produced at a high rate, presumably by detritiation of the [4B-^{3}H]NADH, formed in the reverse transhydrogenase reaction, via the respiratory chain-linked NADH dehydrogenase, analogous to the reaction in submitochondrial particles (cf. ref. 32). In the control experiments (Curves B and C), it is demonstrated that a rapid detritiation of [4B-^{3}H]NADH occurs, when [4-^{3}H]NAD$^+$ is reduced by alcohol dehydrogenase (which is specific for the 4A hydrogen of NADH), but not with glutamate dehydrogenase (which is 4B specific). This confirms that NADH dehydrogenase in *E. coli* catalyzes an exchange of the 4B hydrogen of NADH with water, similar to the mitochondrial enzyme. Thus it is concluded that the transhydro-

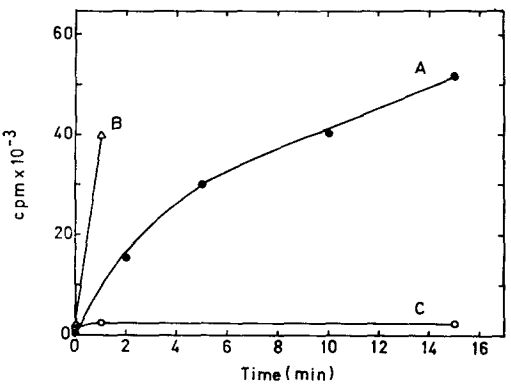

Fig. 1. Stereospecificity of NADPH-dependent NAD$^+$ reduction by *E. coli* membrane preparations. The incubation medium (2.0 ml) contained 50 mM Tris-HCl (pH 8.0), 250 mM sucrose, 2 mM Na_2S, 150 μM NAD$^+$ containing 1.25 μC [4-^{3}H]NAD$^+$ and 0.5 mg *E. coli* particles. Further additions: A, 100 μM NADPH; B, 130 mM ethanol, 6 mM hydrazine, 0.1 mg yeast alcohol dehydrogenase; C, 5 mM glutamate, 6 mM hydrazine, 0.1 mg glutamate dehydrogenase (solution in glycerol).

genase in *E. coli* particles specifically reduced the 4A side of NAD.

Similar experiments with a small-particle preparation from *P. aeruginosa* (not shown) demonstrated that the transhydrogenase in this system is 4B specific for NADH, in agreement with earlier reports [21].

Reactivity to 3'-NADP

Shuster and Kaplan [35] studied the reactivity of several NADP-dependent enzymes with the 3'-isomer of NADP. They observed that enzymes, which are reactive with NAD as well as NADP, can also react with 3'-NADP. In contrast, NADP-specific enzymes were found to be inactive with 3'-NADP. The transhydrogenase in *P. fluorescens* belonged to the former type [35]. Recently, Rydström [36] showed that the transhydrogenase in submitochondrial particles is unable to catalyze the transfer of hydrogen from NADH to 3'-$NADP^+$ or from 2'-NADPH to 3'-$NADP^+$.

In the experiment of Fig. 2 *E. coli* and *P. aeruginosa* preparations were compared in their ability to catalyze the reduction of 3'-$NADP^+$ by NADH or 2'-NADPH. In an equimolar mixture of 2'- and 3'-$NADP^+$, the *E. coli* particles were found to reduce only the 2'-isomer (Fig. 2A). Only on addition of alkaline phosphatase, converting 2'- and 3'-NADP(H) into NAD(H), a complete reduction of the nicotinamide nucleotides is obtained (catalyzed by alcohol dehydrogenase). Essentially the same results were obtained with chromatophores from *R. rubrum* (not shown). On the other

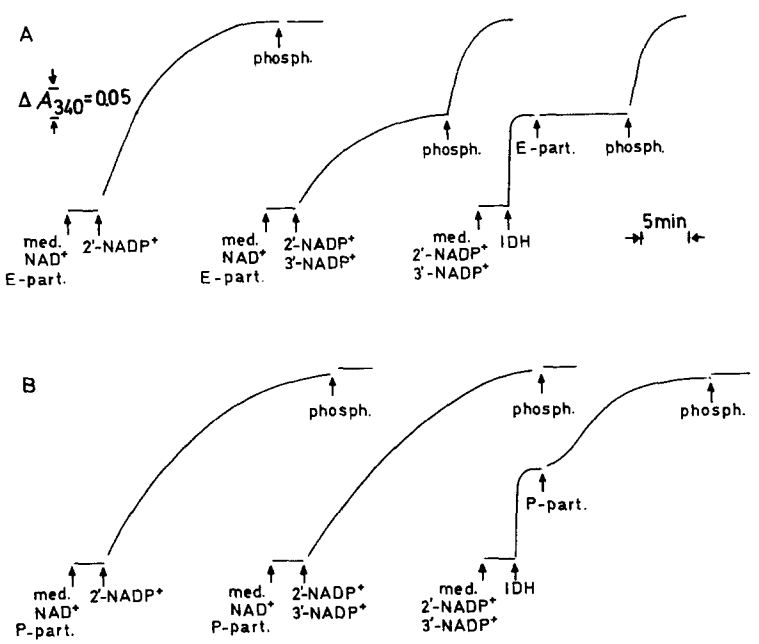

Fig. 2. Reactivity of *E. coli* and *P. aeruginosa* transhydrogenase with 3'-$NADP^+$. The medium (3 ml) contained 130 mM ethanol, 6 mM hydrazine and 0.1 mg alcohol dehydrogenase. Further additions (where indicated) were 100 μM NAD^+, 50 μM 2'-$NADP^+$ or 25 μM 2'-$NADP^+$ plus 25 μM 3'-$NADP^+$, 1 mM $MgCl_2$, 0.1 mg isocitrate dehydrogenase, 10 mM isocitrate, 20 units alkaline phosphatase (phosph.). Expt A, 0.8 mg *E. coli* particles (E-part.), Expt B, 1.0 mg *P. aeruginosa* particles (P-part.) plus 1 mM 2'-AMP.

hand, the transhydrogenase in *P. aeruginosa* preparations catalyzed the reduction of both 2'- and 3'-NADP$^+$ (Fig. 2B). No reduction of nicotinamide nucleotides occurred in the absence of NADH or 2'-NADPH (not shown).

Inhibition by palmityl-CoA

Rydström [36] demonstrated that palmityl-CoA is an inhibitor of both the forward and reverse transhydrogenase reaction in submitochondrial particles. The inhibition is competitive with respect to NADP(H) ($K_i = 0.15\ \mu M$). In Fig. 3 the effect of palmityl–CoA on the reverse transhydrogenase reaction at low substrate concentrations is compared in membrane preparations from different organisms. The transhydrogenase activity in *P. aeruginosa* preparations was not influenced by palmityl–CoA in the concentration range investigated. The reverse transhydrogenase reaction in *E. coli* particles is inhibited by palmityl–CoA, although much higher concentrations of the inhibitor are required than in submitochondrial particles (half-maximal inhibition at 7 μM palmityl–CoA under these conditions).

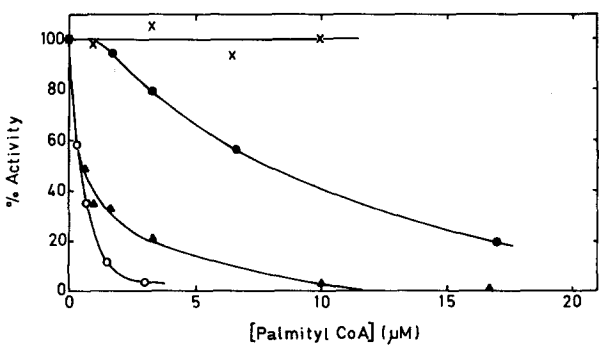

Fig. 3. Effect of palmityl–CoA on transhydrogenase from different sources. The activity of the reverse transhydrogenase reaction was measured as described under Materials and Methods, in the presence of different concentrations of palmityl–CoA. Further additions: 10 μM NAD$^+$, 20 μM NADPH and 0.3 mg *P. aeruginosa* particles (×–×), 0.3 mg *E. coli* particles (●–●), 0.1 mg *R. rubrum* chromatophores (▲–▲) or 0.2 mg submitochondrial particles (○–○).

The reverse transhydrogenase reaction in chromatophores is inhibited by palmityl–CoA equally strongly as is the mammalian system. No major differences in the K_m for NADPH were observed for the transhydrogenases from the different organisms; values varied between 20 to 50 μM (cf. [23, 38]).

Inactivation by trypsin

Juntti et al. [41] reported that the transhydrogenase in submitochondrial particles is characterized by a relatively high sensitivity to trypsin treatment as compared to various catalysts involved in oxidative phosphorylation. This finding was interpreted to indicate a relatively superficial localization of the enzyme in the mitochondrial membrane. Konings and Guillory [19] found that the transhydrogenase factor isolated from chromatophores lost all transhydrogenase-reconstituting capacity on incubation at 30 °C with 5 μg trypsin per mg protein.

Fig. 4 shows the effect of incubation of particle preparations from different

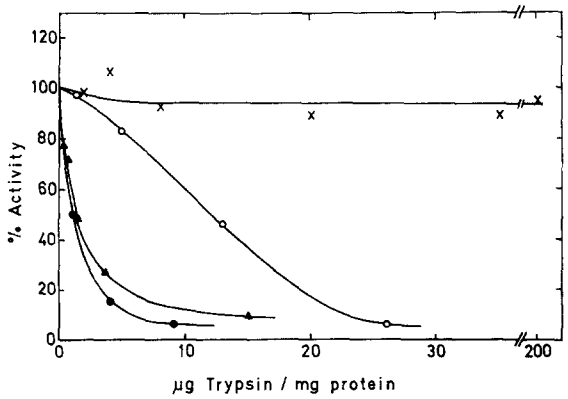

Fig. 4. Effect of trypsin on transhydrogenase from different sources. 0.3 mg of particles from *E. coli* or *P. aeruginosa* preparations, 0.3 mg submitochondrial particles or 0.9 mg reconstituted chromatophores were incubated for 10 min at 30 °C in the standard medium (3 ml) used for the transhydrogenase assay, without nicotinamide nucleotides, in the presence of different concentrations of trypsin. After 10 min, 200 μg of trypsin inhibitor was added, followed by 100 μM NADPH and 100 μM NAD^+. In the control incubations, trypsin inhibitor was added before addition of trypsin. Reconstituted chromatophores were prepared by recombination of depleted chromatophores and the purified transhydrogenase factor in a proportion that was just sufficient to give a maximal activity of the transhydrogenase reaction. The specific activity of the reconstituted chromatophores was 6.9 nmoles/min per mg. ×–×, *P. aeruginosa* particles; ●–●, *E. coli* particles; ▲–▲, *R. rubrum* chromatophores; ○–○, submitochondrial particles.

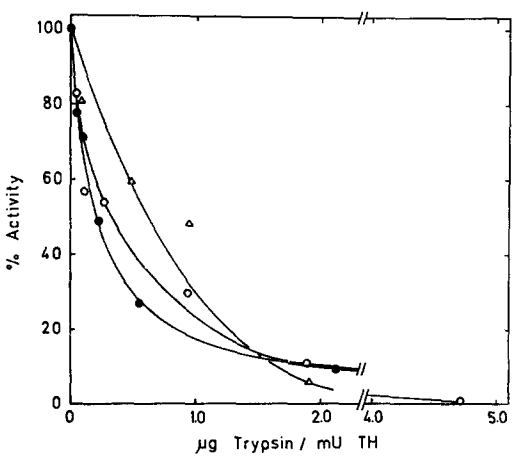

Fig. 5. Effect of trypsin on transhydrogenase reconstituting capacity of transhydrogenase factor and depleted chromatophores from *R. rubrum*. Incubation with trypsin was carried out as described in the legend to Fig. 4, with 0.1 mg depleted chromatophores (△–△), 0.8 mg transhydrogenase factor (○–○) or 0.9 mg reconstituted chromatophores (●–●; see legend to Fig. 4). Sensitivity to trypsin treatment was compared on the basis of the transhydrogenase activity obtained after recombination (1 unit = 1 μmole NADPH oxidized per min per mg protein under the assay conditions for the reverse transhydrogenase reaction; see [30, 38]).

sources with varying concentrations of trypsin on the transhydrogenase activity. The reaction in *P. aeruginosa* preparations was not significantly affected by treatment with trypsin up to 200 μg per mg protein. In contrast, the transhydrogenase in both *E. coli* particles and reconstituted chromatophores was extremely sensitive to trypsin, about 50% inactivation being obtained by incubation with 1 μg trypsin per mg protein. The transhydrogenase in submitochondrial particles was 50% inactivated by incubation with 11 μg trypsin per mg protein. This amount is somewhat larger than that reported by Juntti et al. [41], who observed 50% inactivation of this reaction in submitochondrial particles with 2–4 μg trypsin per mg protein.

The site of action of trypsin on chromatophores was investigated further in the experiment of Fig. 5. Purified transhydrogenase factor and depleted chromatophores were treated separately with different amounts of trypsin. Excess trypsin inhibitor was then added and the fractions were recombined with the active complementary component. In the control experiment, transhydrogenase factor and depleted chromatophores were recombined before the treatment with trypsin. The results were compared on the basis of the transhydrogenase activity obtained after recombination. In agreement with the observations of Konings and Guillory [19], the transhydrogenase factor was found to lose its capacity for reconstitution on treatment with trypsin. Similarly, treatment of the depleted chromatophores with trypsin resulted in a loss of the capacity to reconstitute transhydrogenase activity. Both components were about equally sensitive to trypsin treatment. A similar inactivation of the transhydrogenase was observed when the chromatophores were treated with trypsin after the recombination. These results indicate that the transhydrogenase in *R. rubrum* chromatophores contains at least two separate trypsin-sensitive sites.

Effects of Ca^{2+} and 2′-AMP

Kaplan and coworkers [23, 24] demonstrated that the transhydrogenase from *P. aeruginosa* is activated by 2′-AMP. As shown by Rydström et al. [30], this activation is strongly influenced by Ca^{2+}.

The effects of Ca^{2+} and 2′-AMP on the forward non-energy-linked transhydrogenase were studied in particle preparations from different sources. It was found that the transhydrogenase activity in *E. coli* membrane particles, *R. rubrum* chromatophores or submitochondrial particles was not affected by Ca^{2+} or 2′-AMP under conditions that were previously reported [30] to strongly enhance the rate of the reaction in *P. aeruginosa* preparations (not shown).

DISCUSSION

It is evident from the results reported in this paper that the transhydrogenases in *R. rubrum* chromatophores, *E. coli* membrane preparations and submitochondrial particles are qualitatively remarkably similar. The transhydrogenase in *P. aeruginosa* preparations, on the other hand, behaves essentially different in all respects investigated here.

The difference in stereospecificity to NADH and NADPH of the transhydrogenase in submitochondrial particles [32–34] is taken as strong evidence for the existence of separate binding sites for the two nucleotides in the mammalian enzyme [38]. This interpretation is in line with the kinetic data of Rydström et al. [4, 36, 38] and

is also supported by the lack of reactivity of the enzyme to 3′-NADP [36]. On similar grounds it seems likely that the transhydrogenases in *E. coli* and *R. rubrum* as well have different binding sites for NAD and NADP.

In this respect the quantitative difference in sensitivity for palmityl–CoA between the *E. coli* transhydrogenase and the enzyme in mitochondria and chromatophores is of interest. Rydström [36] suggested that the high effectivity of palmityl–CoA inhibition in submitochondrial particles is related to interaction of the fatty acyl moiety of the inhibitor with a hydrophobic region in the vicinity of the NADP-binding site of the transhydrogenase. Similar considerations may apply to the transhydrogenase in chromatophores. On the other hand, the NADP-binding site of *E. coli* transhydrogenase would appear to be less hydrophobic in character.

The effects of trypsin on the chromatophore enzyme suggests that at least two distinct proteins containing trypsin-sensitive sites are present in this system.

The difference in properties between the *P. aeruginosa* transhydrogenase, on one hand, and the enzymes occurring in *E. coli*, submitochondrial particles and chromatophores, on the other, suggests the generalization that at least two different classes of transhydrogenases can be distinguished: (i) a strongly membrane-bound enzyme, closely linked to the energy-conserving system, characterized by a high sensitivity to trypsin and palmityl–CoA, and by A-side stereospecificity with respect to NADH; (ii) an easily solubilized transhydrogenase*, subject to allosteric regulation by Ca^{2+}, 2′-AMP and NADP(H), and characterized by B-side stereospecificity with respect to NADH.

It should be mentioned that the similarities between transhydrogenases of the former class are mainly qualitative and that several quantitative differences have been observed between these membrane-bound enzymes, as appears from the data in Figs 3 and 4. A solubilization of a component of the transhydrogenase was possible only with chromatophores [19] but not with other systems. A difference in sensitivity to membrane-active agents such as detergents and chaotropic agents has also been observed (Rydström, J. and Hoek, J. B., unpublished); this will be the subject of a forthcoming publication. Nevertheless, on the basis of the data presented here, it may be suggested that the similarities observed reflect molecular properties of the transhydrogenase that are related to its capability of interacting with the energy-conserving system.

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* It is difficult to assess at present whether the transhydrogenase activity in the *P. aeruginosa* small-particle preparation represents a membrane-bound form of the enzyme or a high-molecular weight enzyme complex, not associated with the membrane structure, but precipitated together with the small-particle fraction (cf. [24–26]). This problem is presently subject to investigation.

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