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Biochimica et Biophysica Acta 1231 (1995) 297–303

BBA Biochimica et Biophysica Acta

The mechanism of hydride transfer between NADH and 3-acetylpyridine adenine dinucleotide by the pyridine nucleotide transhydrogenase of *Escherichia coli*

Natalie A. Glavas, Philip D. Bragg *

Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC, Canada, V6T 1Z3

Received 23 November 1994; accepted 13 May 1995

Abstract

The pyridine nucleotide transhydrogenase of *Escherichia coli* catalyzes the reversible transfer of hydride ion equivalents between NAD^+ and $NADP^+$ coupled to translocation of protons across the cytoplasmic membrane. Recently, transhydrogenation of 3-acetylpyridine adenine dinucleotide ($AcPyAD^+$), an analog of NAD^+, by NADH has been described using a solubilized preparation of *E. coli* transhydrogenase [Hutton, M., Day, J.M., Bizouarn, T., and Jackson, J.B. (1994) Eur. J. Biochem. 219, 1041–1051]. This reaction depended on the presence of NADP(H). We show that (a) this reaction did not require NADP(H) at pH 6 in contrast to pH 8; (b) the reaction occurred at pH 8 in the absence of NADP(H) in the mutant βH91K and in a mutant in which six amino acids of the carboxy-terminus of the α subunit had been deleted; (c) the mutant transhydrogenases contained bound $NADP^+$ and were in a conformation in which the β subunit was digestible by trypsin; (d) the conformation of the β subunit of the wild-type enzyme was made susceptible to trypsin digestion by NADP(H) or by placing the enzyme at pH 6 in the absence of NADP(H). It is concluded that reduction of $AcPyAD^+$ by NADH does not involve NADPH as an intermediate and that the role of NADP(H) in this reaction at pH 8 is to cause the transhydrogenase to adopt a conformation favouring transhydrogenation between NADH and $AcPyAD^+$.

Keywords: Ion transfer; Hydride transfer mechanism; NADH; 3-Acetylpyridine adenine dinucleotide; Pyridine nucleotide transhydrogenase; Transhydrogenation

1. Introduction

Pyridine nucleotide transhydrogenase, found in the cytoplasmic membrane of *Escherichia coli* and other bacteria, and in the inner mitochondrial membrane, catalyzes transmembrane proton translocation coupled to transfer of a hydride ion equivalent between NAD^+ and $NADP^+$ [1–5]. In *E. coli*, the enzyme is composed of two subunits, α (510 residues) and β (462 residues), organized as an $\alpha_2\beta_2$ structure [6,7]. It is likely that the NAD(H)- and NADP(H)-binding sites are on the α and β subunits, respectively [8,9]. Equivalent sites are present in the mitochondrial enzyme [10–12].

Transhydrogenase activity is most readily measured by following the reduction of the NAD^+ analog, $AcPyAD^+$ by NADPH ('NADPH → $AcPyAD^+$ transhydrogenation'). However, Wu et al. [13] described transhydrogenation between NADH and $AcPyAD^+$ ('NADH → $AcPyAD^+$ transhydrogenation'). This reaction depended on the presence of NADP(H) and was considered to be a partial reaction of NADPH → NAD^+ transhydrogenation with the participation of a reduced enzyme intermediate. Enander and Rydström [14] dismissed the possibility that a reduced enzyme was involved. Recently, Hutton et al. [15] have demonstrated NADH → $AcPyAD^+$ transhydrogenation with a solubilized preparation of the *E. coli* transhydrogenase. As with the mitochondrial enzyme, the reaction was dependent on the presence of NADP(H). They proposed that $NADP^+$ at the NADP(H)-binding site was reduced by NADH bound at the NAD(H)-binding site. The NADPH so formed was then reoxidized by $AcPyAD^+$ subsequently occupying the NAD(H) binding site.

In the present paper, we examine the involvement of NADP(H) in NADH → $AcPyAD^+$ transhydrogenation. We show that NADP(H) is not required if the reduction is

Abbreviations: $AcPyAD^+$, 3-acetylpyridine adenine dinucleotide; Mes, 2-[*N*-morpholino]ethanesulfonic acid; SDS, sodium dodecyl sulfate.

* Corresponding author. Fax: +1 604 8225227.

SSDI 0005-2728(95)00089-5

carried out at pH 6 and present evidence that the role of NADP(H) in this reaction at higher pH is to induce an appropriate conformational change in the enzyme likely leading to the binding of a proton.

2. Materials and methods

2.1. Preparation of wild-type and mutant transhydrogenase

E. coli JM109 cells containing wild-type (pSA2) or mutant plasmids were grown, and washed membranes purified, as described previously [16]. The enzyme was solubilized from unwashed membranes suspended in 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA and 1 M KCl by 30 mM sodium cholate and 30 mM sodium deoxycholate, and the enzyme partially purified by affinity chromatography [3].

2.2. Assay of trypsin activity

4 μL of 100 mM N^{α}-benzoyl-L-arginine ethyl ester was added to 1 mL of each of the following buffers: 50 mM Mes/KOH (pH 6), 1 mM DTT, 1 mM EDTA; 50 mM sodium phosphate (pH 7), 1 mM DTT, 1 mM EDTA; 50 mM Tris-HCl (pH 8), 1 mM DTT, 1 mM EDTA. Trypsin (10 μL of 1 mg/mL) was added and the increase in absorbance was measured at 255 nm. One unit equals a change in absorbance of 0.001.

2.3. Trypsin digestion

Washed membranes (1 mg/mL) in 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA were digested with 1:100 (w/w) trypsin:transhydrogenase for 30 min. The reaction was stopped by addition of soybean trypsin inhibitor at twice the weight of the trypsin. See the legend to Fig. 3 for the conditions for digestion at other pH values. The buffers used were those described in the section 'Assay of trypsin activity' above.

2.4. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [17]. Gels were stained with Coomassie blue [18].

2.5. Measurement of NADPH → AcPyAD$^+$ and NADH → AcPyAD$^+$ transhydrogenation activities

Transhydrogenation of AcPyAD$^+$ by NADPH was measured as described previously [3]. An appropriate amount of washed membrane (20–100 μg protein) was added to 1 mL of 50 mM sodium phosphate buffer (pH 7), 0.5 mM EDTA, 2 mM DTT, 0.01% Brij 35 containing AcPyNAD$^+$ at 1 mM and NADPH at 0.5 mM. Reduction of AcPyNAD$^+$ was monitored at 375 nm using a Perkin-Elmer Lambda 3A UV/VIS spectrophotometer. For assay of transhydrogenase activities at pH 6 and pH 8 the buffers were 50 mM Mes-KOH (pH 6.0) and 50 mM Tris-HCl (pH 8.0). These buffers contained also 0.5 mM EDTA, 2 mM DTT and 0.01% Brij 35. For transhydrogenation of AcPyAD$^+$ by NADH, 1 mM NADH or 0.5 mM NADH plus 0.5 mM NADP$^+$ were added instead of NADPH. Protein concentration was determined by the method of Lowry et al. [19]

2.6. Extraction of NAD$^+$ and NADP$^+$

Pyridine nucleotides were extracted as described by Klingenberg [20]. Washed membranes (up to 10 mg) were taken up in 1 mL 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 1 mM EDTA. 200 μL of 3 M $HClO_4$ was added. The mixture was vortexed and centrifuged for 5 min at 1200 rpm in a benchtop centrifuge. The supernatant was removed and 200 μL of 1 M K_2HPO_4 was added to the solution at 0°C. Then, 3 M KOH was added until the pH reached 7.2–7.4. The mixture was centrifuged for 2 min at 1200 rpm to remove the precipitate of $KClO_4$. The supernatant was removed and the content of NAD$^+$ and NADP$^+$ was assayed. This extraction method destroys NADH and NADPH.

2.7. Extraction of NADH and NADPH

The reduced pyridine nucleotides were extracted as described by Klingenberg [20]. Washed membranes (4 mg) were taken up in 1 mL 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 1 mM EDTA. 0.5 mL of 1 M KOH in ethanol was added and the solution was heated at 90°C for 5 min. The mixture was cooled on ice and 1 mL of 0.5 M triethanolamine-HCl, 0.4 M KH_2PO_4, 0.1 M K_2HPO_4 was added until the pH of the mixture was 7.8. Since NADH and NADPH are unstable in the neutral solution, the nucleotides were enzymatically oxidized as suggested by Klingenberg [20]. Glutathione reductase (6.25 μg), 100 μL of 100 mM oxidized glutathione, lactic dehydrogenase (20 μg) and 100 μL of 100 mM pyruvate were added to the mixture and kept at room temperature for 15 min. NAD$^+$ and NADP$^+$ were then extracted from the mixture as described above. The assayed amounts of NAD$^+$ and NADP$^+$ were a direct measure of the amounts of NADH and NADPH originally extracted, since any NAD$^+$ or NADP$^+$ originally present would have been destroyed during extraction with alkali.

2.8. Assay of NAD$^+$ and NADP$^+$

NAD$^+$ and NADP$^+$ were quantitated as described by Klingenberg [20]. NAD$^+$: 800 μL of extract was com-

bined with 800 μL 0.1 M sodium pyrophosphate-semicarbazide HCl buffer (pH 8.8). 10 μL of ethanol was added. The reaction medium was mixed and left to incubate for 15 min, when the absorbance at 340 nm was read (A_1). 10 μL of 1.4 mg/mL yeast alcohol dehydrogenase was added. The reaction was mixed and the absorbance was read after 6 min (A_2). The concentration of NAD^+ was calculated from $\Delta A = A_2 - A_1$ using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

$NADP^+$: 800 μL extract was combined with 4 μL 1 M $MgSO_4$ and 80 μL 50 mM glucose 6-phosphate. The reaction was incubated for 15 min at room temperature when the absorbance at 340 nm was read (A_1). 10 μL of 0.25 mg/mL glucose-6-phosphate dehydrogenase was added and after 15 min, the absorbance was read (A_2). The concentration of $NADP^+$ was calculated from $\Delta A = A_2 - A_1$ using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

These methods will measure down to 10^{-9} mol of pyridine nucleotide.

2.9. Materials

Electrophoresis reagents were supplied by Bio-Rad. All other biochemicals including NADH, $AcPyAD^+$, $NADP^+$ and NADPH were obtained from the Sigma Chemical Company.

3. Results

3.1. Transhydrogenation between NADH and 3-acetylpyridine adenine dinucleotide in mutant transhydrogenases

Hutton et al. [15] have shown recently that hydride equivalents can be transferred from NADH to 3-acetylpyridine adenine dinucleotide ($AcPyAD^+$) at pH 6.0 provided that small amounts of $NADP^+$ or NADPH are present. They postulated that binding of NADP(H) to the enzyme was stabilized at this pH and permitted enzyme-bound NADPH to be an intermediate in hydride transfer

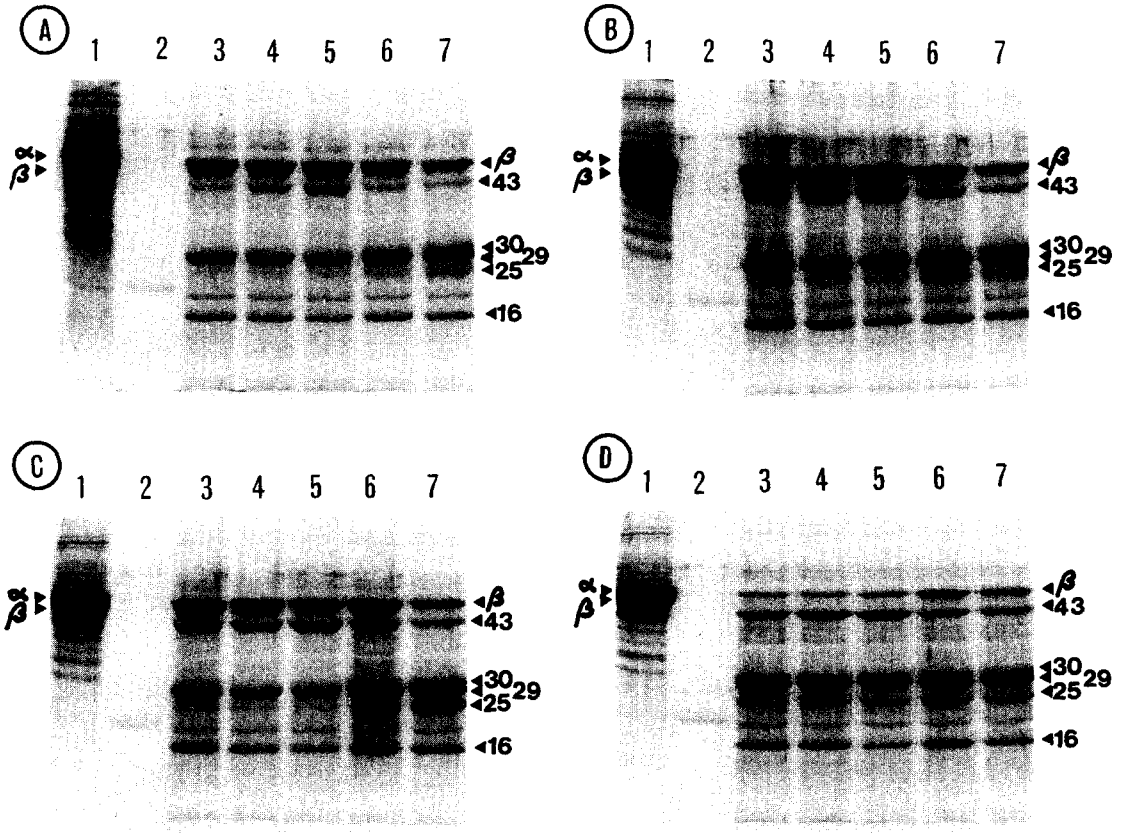

Fig. 1. Trypsin digestion of the transhydrogenases of mutants in the carboxy-terminal region of the α subunit in the presence of pyridine nucleotides. Washed membranes (1 mg/mL) in buffer (50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA) were treated with trypsin at a trypsin:transhydrogenase weight ratio of 1:100 for 30 minutes at room temperature in absence (lane 3) or presence of 0.5 mM NAD^+ (lane 4), NADH (lane 5), $NADP^+$ (lane 6) or NADPH (lane 7). The reactions were terminated by addition of soybean trypsin inhibitor at a weight twice that of the trypsin. The samples were then examined by SDS-polyacrylamide gel electrophoresis. Lane 1, untreated membranes. Lane 2, membranes omitted. The positions of migration of the α and β subunits and of the trypsin cleavage fragments (in kDa) are indicated. A, wild-type; B, QRMLKMFREN; C, QRMLKMF; D, QRML.

with NADH and AcPyAD$^+$ alternately occupying the NAD$^+$-binding site. This reaction was measured in wild-type and mutant transhydrogenases.

Three point mutants of the β subunit (βH91K, βC260S, βG314A) [21,22] and three mutants of the α subunit were chosen for study. These mutants assemble to normal levels in the cell membrane. The carboxy-terminal ten residues of the α subunit are important for subunit interactions in the *E. coli* transhydrogenase [22]. In the wild-type enzyme, the ten terminal amino acids are QRMLKMFRKN and thus contain four positively-charged residues. The following mutants were examined: QRMLKMFREN (αK509E), in which one positively charged residue had been replaced by a negatively-charged residue, and the deletion mutants QRMLKMF and QRML. The transhydrogenase activities of these mutants are shown in Table 1. Normal NADPH → AcPyAD$^+$ transhydrogenase activity is reduced by about 50% in the βC260S, QRMLKMFREN (αK509E) and QRMLKMF mutants, and by 94–96% in the βH91K, βG314A and QRML mutants. In the first set of mutants (βC260S, αK509E and QRMLKMF) NADH → AcPyAD$^+$ transhydrogenation in the presence of NADP$^+$ is near wild-type levels. The requirement for NADP$^+$ in this reaction is clearly indicated. The second set of mutants (βH91K, βG314A, QRML) show two types of behaviour. The βG314A mutant showed negligible NADH → AcPyAD$^+$ transhydrogenation even in the presence of NADP$^+$. By contrast, βH91K and QRML mutants showed very high rates of NADH → AcPyAD$^+$ transhydrogenation *in the absence of NADP$^+$*.

Table 1
Transhydrogenase activities of membranes from wild-type and mutant strains

Mutant	Transhydrogenase specific activity		
	NADPH → AcPyAD$^+$	NADH → AcPyAD$^+$	NADH(+NADP$^+$) → AcPyAD$^+$
Wild-type	5.2	0.31	5.5
βH91K	0.22	12.0	14.3
βC260S	2.3	0.33	7.2
βG314A	0.35	0	0.18
αQRMLKMFREN	2.6	0.52	11.1
αQRMLKMF	2.6	0.95	13.9
αQRML	0.32	10.9	20.4

The activities were assayed as described in Materials and Methods. The averages of values from two separate membrane preparations are given.

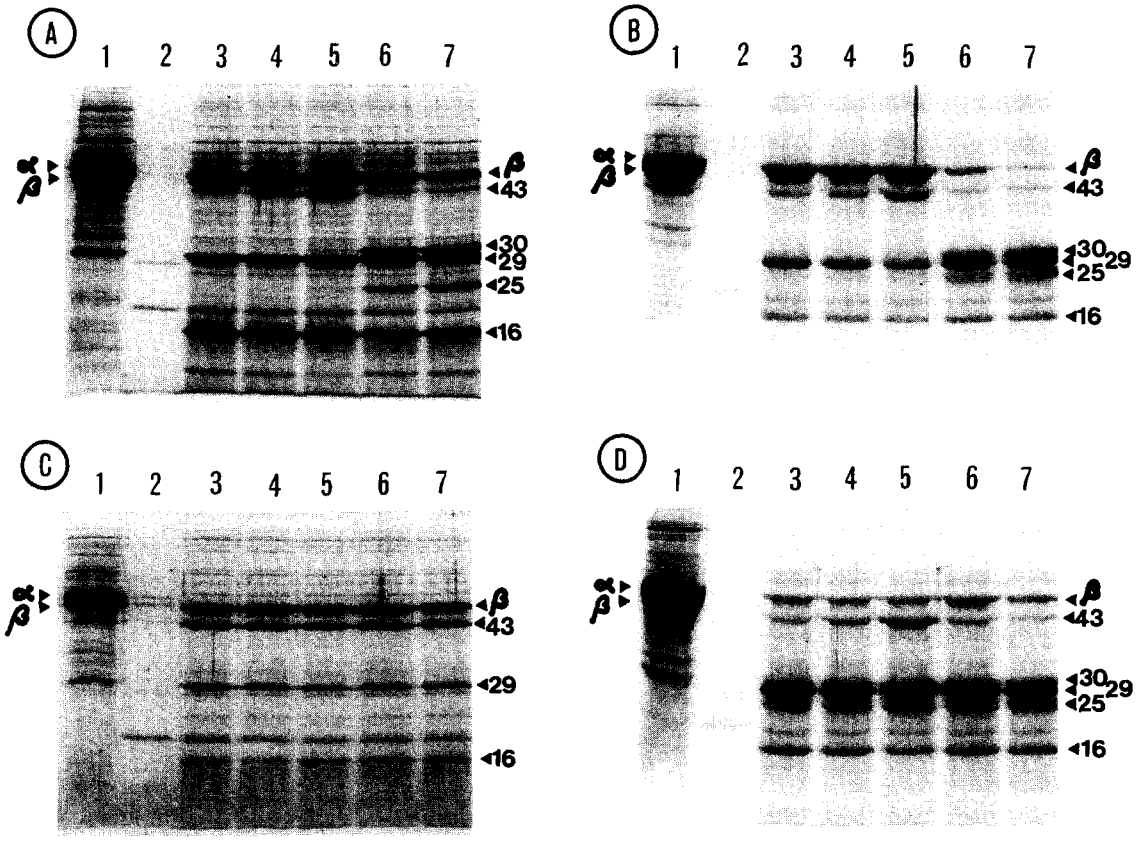

Fig. 2. Trypsin digestion of wild-type transhydrogenase (A) and of the mutants βC260S(B), βG314A(C) and βH91K(D) in the presence of pyridine nucleotides. The experiment was carried out as described in the legend to Fig. 1. Lane 1, untreated membranes; lane 2, membranes omitted; lane 3, trypsin digestion in the absence of nucleotides. Lanes 4–7, digestion in the presence of 0.5 mM NAD$^+$, NADH, NADP$^+$ and NADPH, respectively. The positions of migration of the α and β subunits and of the trypsin cleavage fragments (in kDa) are indicated.

3.2. Trypsin digestion of mutant transhydrogenases

The trypsin digestion patterns of the mutant transhydrogenases are shown in Figs. 1 and 2. Trypsin does not cleave the β subunit of wild-type transhydrogenase to a significant extent unless $NADP^+$ or NADPH is present when 30 kDa C-terminal and 25 kDa N-terminal fragments are formed [16]. This pattern of digestion was shown also by βC260S, QRMLKMFREN and QRMLKMF mutants. The β subunit of the βG314A mutant was resistant to cleavage even in the presence of NADP(H). By contrast, the βH91K and QRML mutants showed digestion of the β subunit in the absence of added nucleotides.

The digestibility of the β subunit correlates with the NADH → $AcPyAD^+$ transhydrogenase activity of the mutant enzymes. Thus, wild-type and βC260S, QRMLKMFREN and QRMLKMF mutants show NADP(H)-induced trypsin cleavage of the β subunit and have significant NADH → $AcPyAD^+$ transhydrogenase activity in the presence of $NADP^+$. The βH91K and QRML mutants show cleavage of β in the absence of NADP(H) and have very high NADH → $AcPyAD^+$ transhydrogenase activity in the absence of $NADP^+$. The βG314A mutant shows little transhydrogenase activity and the β subunit is resistant to digestion by trypsin even in the presence of NADP(H).

3.3. Pyridine nucleotide content of transhydrogenases

The possibility that the NADH → $AcPyAD^+$ transhydrogenase activity shown by the mutant enzymes in the absence of added $NADP^+$ could be due to the presence of bound pyridine nucleotide was examined. Washed membranes instead of purified enzyme were analyzed for NAD^+ and $NADP^+$ since large amounts of protein were required. Transhydrogenase composes greater than 90% of the total protein in washed membranes. As shown in Table 2, NAD^+ was not present in washed membranes. Insignificant amounts of NADH, NADPH and $NADP^+$ were found in wild-type and mutant membranes with the exception of those from βH91K and αQRML mutants. Approximately 0.2 mol $NADP^+$/mol transhydrogenase was found. This suggests that the trypsin-sensitivity of the β subunits in these mutants was likely due to the presence of this bound nucleotide. It also raises the possibility that the bound $NADP^+$ plays a similar role in NADH → $AcPyAD^+$ transhydrogenation as does free $NADP^+$ with the wild-type enzyme and those of the other mutants.

Table 2
Pyridine nucleotide content of membrane-bound transhydrogenases

Mutant [a]	mol nucleotide/mol transhydrogenase			
	NAD^+	NADH	$NADP^+$	NADPH
Wild-type	0	0	0.0098 ± 0.0017 [b]	0
βH91K	0	0.027	0.22 ± 0.02 [b]	0
βC260S	0	0	0.036	0
βG314A	0	0	0.015	0
αQRMLKMFREN	0	0.031	0.019	0
αQRMLKMF	0	0	0.025	0
αQRML	0	0.027	0.19 ± 0.03 [b]	0.015
Plasmid absent	0	0	0.007	0

Washed membranes were analyzed as described in Materials and Methods.
[a] The mutation was present in the plasmid-encoded gene of the transhydrogenase.
[b] Mean ± standard deviation. Two-four analyses were performed.

Table 3
Effect of pH on transhydrogenase activities

Transhydrogenase	pH	Specific activity		
		NADPH → $AcPyAD^+$	NADH → $AcPyAD^+$	NADH (+$NADP^+$) → $AcPyAD^+$
Membrane-bound	6	1.1	27.1	45.5
	7	6.3	0.2	4.6
	8	4.2	0.2	4.4
Partially purified	6	0.7	4.4	17.9
	7	2.2	0	1.7
	8	1.1	0	1.9

Activities were assayed as described in Materials and Methods and expressed as μmol substrate reduced/min per mg protein.

3.4. Effect of pH on transhydrogenase activities

Normal NADPH → $AcPyAD^+$ transhydrogenation and transhydrogenation of $AcPyAD^+$ by NADH was measured at pH 6, 7 and 8 with washed membranes and partially purified transhydrogenase (Table 3). Activities of NADPH → $AcPyAD^+$ transhydrogenation were higher at pH 7 and 8 than at pH 6. The reverse was true for NADH → $AcPyAD^+$ transhydrogenation, where the activity at pH 6 was very much greater than at pH 7 and 8. In contrast to the results of Hutton et al. [15], we found very high NADH → $AcPyAD^+$ activity at pH 6 even in the absence of added $NADP^+$. Since these enzyme preparations were free of $NADP^+$, it is clear that $NADP^+$ is not required as an intermediate hydride carrier in NADH → $AcPyAD^+$ transhydrogenation although it increases the rate of this reaction, probably by inducing an appropriate conformation.

3.5. Trypsin cleavage of transhydrogenase at different pH

Effect of pH on the conformation of the enzyme was examined by trypsin digestion of washed membranes in the presence and absence of $NADP^+$ and NADPH (Fig. 3). The extent of trypsin digestion at pH 6, 7 and 8 was equalized either by varying the duration of incubation with the proteinase (Fig. 3A) or by varying the amount of trypsin added to give the same number of units of activity in each sample (Fig. 3B). (This required prior assay of

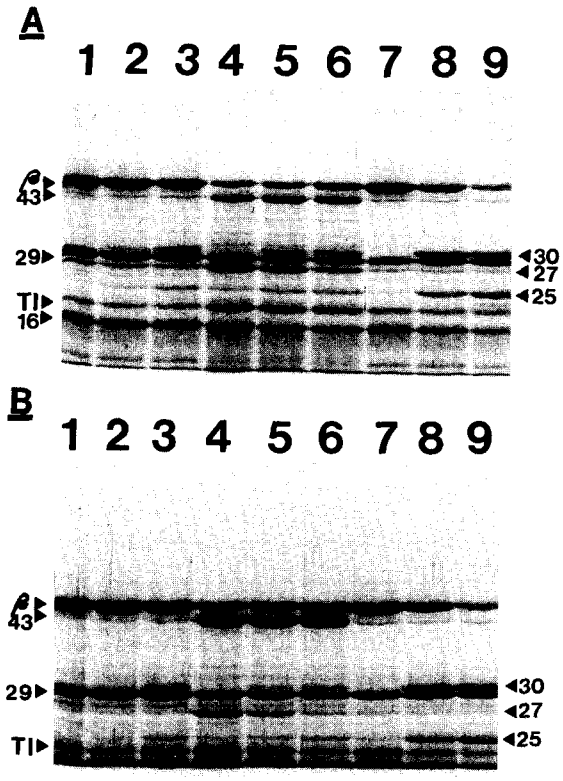

Fig. 3. Trypsin digestion of transhydrogenase in washed membranes at pH 6, 7 and 8. The membranes were digested as described in Materials and Methods except that in (A) the time of digestion and units of trypsin activity were: pH 6, 164 min (22 units); pH 7, 60 min (60 units); pH 8, 69 min (52 units). In (B), 60 units of trypsin activity were added. Incubation was for 60 min. The assayed activities of trypsin (units/mg protein) at pH 6, 7, and 8 were 11000, 30000, 26000, respectively. The buffers used were 50 mM sodium phosphate (pH 7.0), containing 1 mM DTT and 1 mM EDTA (lanes 1, 2, 3), 50 mM Mes-KOH (pH 6.0), containing 1 mM DTT and 1 mM EDTA (lanes 4, 5, 6), and 50 mM Tris-HCl (pH 8.0), containing 1 mM DTT and 1 mM EDTA (lanes 7, 8, 9). Trypsin digestion was carried out in the absence of added nucleotide (lanes 1, 4, 7) or in the presence of 0.5 mM $NADP^+$ (lanes 2, 5, 8) or 0.5 mM NADPH (lanes 3, 6, 9). The products of digestion were examined by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. (A) 12% polyacrylamide; (B) 10% polyacrylamide. The positions of migration of the β subunit and of the trypsin cleavage fragments (in kDa) are indicated. TI, soybean trypsin inhibitor.

trypsin activity at the different pH values.) As shown by both methods, the α subunit was readily cleaved by trypsin to give the usual 43, 29 and 16 kDa fragments [16]. Interestingly, further cleavage of the 43 kDa fragment to the 29 and 16 kDa fragments was retarded somewhat at pH 6. As previously shown [6,15], cleavage of the β subunit (to 30 and 25 kDa fragments) at pH 7.8 in washed membranes occurred only in the presence of $NADP^+$ or NADPH. The β subunit was essentially uncleaved at pH 7, even in the presence of these nucleotides. By contrast, cleavage of the β subunit to a 27 kDa fragment, derived from the N-terminal domain, proceeded actively at pH 6. This cleavage did not depend on the presence of $NADP^+$ or NADPH. In fact, these nucleotides slightly retarded the digestion. It can be concluded that, at pH 6, the enzyme showed a change in the conformation of the β subunit. Presumably, this conformation favoured $NADH \rightarrow AcPyAD^+$ transhydrogenation.

4. Discussion

Two major conclusions can be made from the results described in this paper. First, $NADP^+$ is not a necessary intermediate hydride carrier between NADH and $AcPyAD^+$ as had been proposed by Hutton et al. [15]. Second, $NADH \rightarrow AcPyAD^+$ transhydrogenation is favoured by the adoption of a certain conformation by the β subunit. (The α subunit may also change conformation but we have not been able to measure if this occurs.) That $NADP^+$ is not a necessary intermediate hydride carrier between NADH and $AcPyAD^+$ is demonstrated by the occurrence of this activity in a NADP(H)-free enzyme at pH 6 (Table 3). This reaction is not caused by the presence of other enzymes in the washed membrane preparations since this activity is absent in membranes from strain RH1, a mutant which lacks transhydrogenase activity [23,24] but which would contain these other enzymes. It is unlikely that any NADP(H) present as a contaminant in the NADH and $AcPyAD^+$ used in the assay reaction would contribute significantly to the reaction. Thus, the samples of NADH used in our experiments contained from 0.02–0.1% $NADP^+$ and up to 0.1% NADPH. No NADP(H) was detected in $AcPyAD^+$. The maximum level of contamination would give no more than 1 μM NADP(H). This amount would be expected to give about 15% of the reaction rate observed at pH 7 at saturating concentrations of NADP(H). In actuality we observed no more than 2.5% of the maximum rate in experiments in which NADP(H) was not added. Clearly the level of contaminating NADP(H) in the NADH was insufficient to significantly effect the rate of transhydrogenation. In contrast to the results presented here, Hutton et al. [20] were not able to detect $NADH \rightarrow AcPyAD^+$ activity at pH 6. This discrepancy is puzzling but may be attributable to either the nature of the enzyme preparation or to the conditions of the assay. Hutton et al. used a purified solubilized enzyme in contrast to the membrane-bound enzyme used in our studies. Furthermore, the concentrations of nucleotides used in their assay system were approximately one-fifth of those used in our study. We found that the lower concentrations gave submaximal rates. A further difference was the inclusion of lysophosphatidylcholine in their assay medium. It is likely that detergents have an effect on the $NADH \rightarrow AcPyAD^+$ transhydrogenation. We have been informed recently by Professor Jan Rydström (University of Göteborg, Sweden) (personal communication) that Brij 35 is required in the assay system to show $NADP^+$-independent reduction of $AcPyAD^+$ by NADH at pH 6.

The second major conclusion from our work is that NADH → AcPyAD$^+$ transhydrogenation is favoured by the adoption of a certain conformation by the β subunit. This conclusion is supported by the correlation between the ability of trypsin to digest the β subunit in the presence or absence of NADP(H) and the activity of NADH → AcPyAD$^+$ transhydrogenation in various mutants. Binding of NADP(H) to the transhydrogenase induces a conformational change in the enzyme to permit trypsin cleavage of the β subunit at βArg265 to give 25 kDa and 30 kDa fragments. Since the β subunit of the βH91K and QRML mutants will undergo cleavage at the same position in the absence of added NADP(H), it is likely that the conformation of the enzyme in these mutants resembles that induced by NADP(H) in the wild-type enzyme. Furthermore, whereas the wild-type enzyme requires the presence of NADP(H) to show NADH → AcPyAD$^+$ transhydrogenase activity, the βH91K and QRML mutants do not need this nucleotide to show significant activity. Presumably, the presence of bound NADP$^+$ in the mutant enzymes has caused them to adopt the conformation favouring NADH → AcPyAD$^+$ transhydrogenation and tryptic cleavage of the β subunit. Supporting this conclusion are the results of the measurements made at different pH values with wild-type transhydrogenase. Thus, the occurrence of high NADH → AcPyAD$^+$ activity at pH 6 in the absence of NADP$^+$ or NADPH correlates with the ability of trypsin to cleave the β subunit at this pH in the absence of these nucleotides, although the site of cleavage is not the same. Trypsin cleavage at pH 6 yielded a 27 kDa N-terminal fragment of the β subunit compared with the 25 kDa N-terminal fragment of the β subunit obtained at pH 7 in the presence of NADP(H). Since NADPH is not an intermediate on the pathway of hydride transfer from NADH to AcPyAD$^+$, in contrast to the mechanism proposed by Jackson and colleagues [15], it is likely that the role of NADP(H) in this reaction is to induce the enzyme to adopt a favourable conformation for NADH → AcPyAD$^+$ transhydrogenation. Thus, in addition to binding sites for NADH and AcPyAD$^+$, there must be an additional site for NADP(H). Whether this site is an additional site to the known NAD(H) and NADP(H) binding sites on the α and β subunits [9] is not known at present. It is possible, since the native enzyme has the dimeric $\alpha_2\beta_2$ structure [7] that transhydrogenation can be induced between the NAD(H) sites of each of the $\alpha\beta$ monomers. This requires further investigation.

Finally, the ability of low pH and of NADP(H) (at pH 8) to induce a conformational change in the enzyme, such that the β subunit is now susceptible to the action of trypsin and NADH → AcPyAD$^+$ transhydrogenation can occur, raises the possibility that binding of NADP(H) might induce binding of a proton as part of the process of coupled proton translocation. Binding of a proton would be favoured also by placing the enzyme at pH 6. In this respect, it is interesting that we have identified βHis91 as a putative proton carrier in transhydrogenase action (Glavas, Hou and Bragg, unpublished data; [21]). It is likely that this group would be protonated at pH 6. Hutton et al. [15] have speculated also on the relationship between the binding/debinding of NADP(H) and the binding and release of a proton by the transhydrogenase.

Acknowledgements

This work was supported by a grant from the Medical Research Council of Canada and by the award of a MRC Studentship to N.A.G.

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