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Energy-linked transhydrogenase. Characterization of a nucleotide-binding sequence in nicotinamide nucleotide transhydrogenase from beef heart

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Purified nicotinamide nucleotide transhydrogenase from beef heart was investigated with respect to labeling and subsequent sequence analysis of a nicotinamide nucleotide-binding site. A photo-activated azide derivative, 8-azidoadenosine 5′-monophosphate, was used as an active-site-directed photoaffinity label, which was shown to be specific for the NAD(H)-binding site in the dark. Light-activated incorporation of the label in transhydrogenase was accompanied by an inactivation, which approached 100% at the incorporation of about 1 mol label/mol transhydrogenase monomer. As expected from the assumed site-specificity of the label, NADH prevented both labeling and inactivation to some extent. However, NADPH also prevented labeling and inactivation marginally. The oxidized substrates NAD^+ and $NADP^+$ were inhibitory by themselves under these conditions, and the substrate analogs 5′-AMP and 2′-AMP were also poor protectors. The NAD(H)-site specificity of the azido compound was thus largely lost upon illumination and covalent modification. Radioactive labeling of transhydrogenase with 8-azido-[2-^{3}H]-adenosine 5′-monophosphate followed by protease digestion, isolation of labeled peptides and amino-acid sequence analysis showed that Tyr 1006 in the sequence 1001–1027 close to the C-terminus was labeled. This sequence shows homologies with nucleotide-binding sequences in, e.g., F_1-ATPase. On the basis of sequence homologies with other NAD(P)-dependent enzymes it is proposed that transhydrogenase contains 4 nucleotide-binding sites, of which 2 constitute the adenine nucleotide-binding domains of the catalytic sites for NAD(H) and NADP(H) close to the N- and C-terminals, respectively. Each of these domains has an additional vicinal nucleotide-binding sequence which may constitute a non-catalytic nucleotide-binding site or the nicotinamide nucleotide-binding domain of the catalytic site. The present results indicate that 8-azidoadenosine 5′-monophosphate is kinetically specific for the catalytic NAD(H)-binding site, but reacts covalently with Tyr 1006 of the putative non-catalytic site or nicotinamide nucleotide-binding domain formed by the 1001–1027 amino acid sequence of the catalytic NADP(H)-binding site. Interactions between the catalytic NAD(H) and NADP(H) binding sites, and the assumed non-catalytic sites, may be facilitated by a ligand-triggered formation of a narrow pocket, which normally allows an efficient hydride ion transfer between the natural substrates.

Introduction

The mitochondrial nicotinamide nucleotide transhydrogenase is a proton pump associated with the inner membrane, which catalyzes the reversible transfer of hydrogen between NAD(H) and NADP(H) according to the reaction (for reviews see Refs. 1–3):

$$H^+_{out} + NADH + NADP^+ \rightleftharpoons H^+_{in} + NAD^+ + NADPH \quad (1)$$

Transhydrogenase is composed of a single polypeptide with a molecular weight of about 115 000 and contains separate binding sites for NAD(H) and NADP(H). The enzyme purified from beef heart has been characterized extensively with respect to steady-state kinetics and regulation, and has been reconstituted in phospholipid vesicles with or without other proton pumps such as the mitochondrial ATPase [4] or bacteriorhodopsin [5]. In such vesicles the stoichiometry of protons pumped per hydride ion transferred in the reaction

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Abbreviations: FSBA, *p*-fluorosulfonylbenzoyl-5′-adenosine; FSBI, *p*-fluorosulfonylbenzoyl-5′-inosine; SBA, sulfonylbenzoyl-5′-adenosine; 8-N_3-AMP, 8-azido-adenosine 5′-monophosphate; 8-N_3-[2-^{3}H]AMP, 8-azido-[2-^{3}H]adenosine 5′-monophosphate; 5′-AMP, adenosine 5′-monophosphate; 2′-AMP, adenosine 2′-monophosphate; $AcPyAD^+$, 3-acetylpyridine adenine nucleotide; DCCD, *N,N′*-dicyclohexylcarbodiimide; TPCK-trypsin, trypsin treated with L-1-tosylamide-2-phenylethylchloromethyl ketone to remove chymotrypsin activity.

approaches 1 at neutral pH [3,4]. The generation of an electrochemical proton gradient by, e.g., ATPase, results in a 20–30-fold increase in the rate of the reduction of $NADP^+$ by NADH, and a concomitant increase in the nicotinamide nucleotide ratio [4,5]. These effects of ATP on the transhydrogenase reaction catalyzed by the reconstituted vesicles are similar to those seen in submitochondrial particles [3]. An analysis of the mode of coupling between transhydrogenase and other proton pumps, e.g., ATPase, suggests that these proteins interact through a delocalized electrochemical proton gradient [6].

Mitochondrial transhydrogenase from beef heart has recently been cloned and its cDNA sequence determined, indicating a molecular weight of 109212 [7]. Hydropathy profiles suggest the presence of at least 14 central hydrophobic sequences, some of which probably constitute membrane-traversing segments. The putative NAD(H) and NADP(H)-binding regions are located in relatively hydrophilic regions in the extreme ends of the peptide [7]. By using the reactive substrate analog FSBA, two sites have been identified after covalent binding followed by sequence analysis [7]. Site-specificity of the analog was introduced by blocking the second site by the natural substrate, and it was suggested that the amino-acid sequence 237–247 close to the N-terminus and the sequence 1001–1027 form part of the NAD(H) and NADP(H)-binding site, respectively [7].

The aim of the present investigation was to further characterize the NAD(H)-binding site by using a reactive photoaffinity analog of NAD(H), i.e., 8-N_3-AMP, which, on the basis of its structural resemblance with 5'-AMP [8], was assumed to bind specifically to the NAD(H) site. After covalent binding of the analog, the modified transhydrogenase was treated with trypsin, followed by peptide separation and sequence analysis of the analog-containing peptide(s). Covalent binding of the analog to the transhydrogenase was associated with an inactivation of the enzyme. The results show that 8-N_3-AMP binds to the amino-acid sequence previously assumed to correspond to the NADP(H) binding site. The consequences of this labeling for the identification of the NAD(H) and NADP(H) binding sites are discussed.

Materials and Methods

Preparation and assay of transhydrogenase

Nicotinamide nucleotide transhydrogenase was prepared essentially as described in Ref. 9, with the modification that the medium used for wash and elution of the enzyme bound to the calcium phosphate gel was 2 mM sodium phosphate containing 0.5% sodium cholate, and 200 mM sodium phosphate and 0.5% sodium cholate, respectively. Purified, homogeneous transhydrogenase used in this study consisted of calcium phosphate pools with a specific activity of at least 7 μmol/min per mg protein and a purity of at least 90%. Protein was determined by a modified Lowry procedure as described [10] using beef serum albumin as standard.

Transhydrogenase activity was assayed as reduction of $AcPyAD^+$ by $NADPH^+$ at 375–420 nm, using a dual wavelength Aminco DW-2 spectrophotometer [11]. Temperature was 30°C. The assay medium contained 80 mM potassium phosphate (pH 6.3), 180 μM $AcPyAD^+$, 180 μM NADPH and 0.4 mg/ml lysophosphatidylcholine (egg yolk) in a volume of 1 ml. The reactions were started by the addition of enzyme.

SDS-PAGE was performed using a 7.5% polyacrylamide and bispolyacrylamide gel system [12]. Staining was accomplished using a modified silver staining method [13]. Densitometric scanning of the stained polyacrylamide gels at 633 nm was achieved by a LKB 2202 ultrascan laser densitometer. The gel was then cut into 2 mm slices, transferred into scintillation vials containing 0.5 ml 30% hydrogen peroxide, and incubated for 12 h at 60°C, after which 4 ml Packard 299 emulsifier scintillation liquid was added and the radioactivity of the digested slices determined in a 6 Beckman LS 1800 liquid scintillation counter.

Preparation and chemical characterization of 8-N_3-AMP

8-N_3-AMP and 8-N_3-[2-^{3}H]AMP were isolated and co-purified as hydrolyzed by-products in the synthesis of 8-N_3-ATP and 8-N_3-[2-^{3}H]ATP, respectively. The latter compounds were synthesized according to the procedure described by Schäfer et al. [14]. The preparations of the two AMP analogues did not contain any acid-labile phosphate in the form of ADP or ATP analogs [15]. TLC on cellulose MN-300 in n-butanol/acetic/acid water (5:2:3) showed the expected R_F value of 0.48. No impurities could be detected on TLC, and ultraviolet spectra in 25 mM Tris-HCl (pH 7.0) showed that the purity was more than 95%. The concentration of 8-N_3-AMP was estimated from the absorbance at 281 nm. The specific activity of the 8-N_3-[2-^{3}H]ATP was 100 dpm/pmol.

Modification of transhydrogenase with 8-N_3-AMP

Covalent labeling of purified transhydrogenase with 8-N_3-AMP or 8-N_3-[2-^{3}H]AMP was performed at 0°C in the dark or at a wavelength of 365 nm. The light source was a 100 W ultraviolet lamp. About 30 to 50 μg of transhydrogenase in a 10 mm Petri dish at a final concentration of 0.4 mg/ml, was irradiated for various times or at various concentrations of the photoaffinity label, at a distance of 3–15 cm to the lamp. The longer radiation distance was used in the measurements of inactivation of small samples of transhydrogenase by 8-N_3-AMP; the shorter distance was used for labeling

transhydrogenase with 8-N_3-[2-^{3}H]AMP. To prevent heat denaturation, the sample dish was placed on ice, and in cases of prolonged illumination, the light was turned off after each 5 min exposure and the sample was cooled. In order to remove unreacted probe after photolabeling of the transhydrogenase protein, Sephadex G-50 coarse columns (5 ml) [16] equilibrated with 20 mM potassium phosphate (pH 7.5) and 0.05% sodium cholate were used prior to electrophoretic analysis and peptide fractionation of the trypsin-digested transhydrogenase.

Tryptic digestion of the photoaffinity-labeled transhydrogenase

Photoaffinity labeling of 7.5 mg of transhydrogenase at a final concentration of 0.4 mg/ml, with 400 μM 8-N_3-[2-^{3}H]AMP for 25 min on ice, gave a close to fully inhibited enzyme. After removal of unreacted probe, as described above, the enzyme was treated with TPCK-trypsin (trypsin to sustrate ratio of approximately 1 : 30, w/w) and incubated at room temperature for 8 h. The peptides of the digested modified transhydrogenase were analyzed by PAGE and radioactivity counting as described above.

Fractionation of the tryptic digest of photoaffinity-labeled transhydrogenase

(Additional details of the purification of photoaffinity-labeled transhydrogenase are found in Fig. 5)

Step A: The tryptic digest of the 8-N_3-[2-^{3}H]AMP-labeled transhydrogenase was lyophilized, redissolved in 4 ml 30% acetonitrile containing 0.1 M ammonium acetate (pH 7.4), after which 4 × 1 ml aliquots were applied to an FPLC Superose 12 column equilibrated and run with 30% acetonitrile containing 0.1 M ammonium acetate (pH 7.4) at a flow rate of 0.3 ml/min. FPLC was carried out with an instrument purchased from Pharmacia (Uppsala, Sweden) equipped with a Liquid Chromatography Controller LCC-500 and P-500 pumps. Immediately before use the medium was filtered through a Millipore GUWP filter and degassed. The chromatography was carried out at room temperature. Fractions of 0.6 ml were collected and analyzed for radioactivity and relative absorbance at a wavelength of 280 nm.

Step B: Labeled peptides of fractions 24–31 obtained in the previous fractionation step were lyophilized, redissolved in 1.1 ml of 20 mM sodium tricine (pH 7.4) and 0.05% Lubrol PX, and applied on a 1 ml FPLC Mono Q column equilibrated with 20 mM sodium tricine (pH 7.4) and 0.05% Lubrol PX. The chromatography was carried out at room temperature. Immediately before use the medium was filtered through a Millipore filter (0.22 μm) and degassed. The applied flow rate was 0.8 ml/min. After washing with 6.4 ml of buffer the peptides recovered in the fractions 5–26 and 26–35 were eluted with a linear gradient of 0–0.5 M potassium chloride in equilibration buffer with a relative gradient slope of 17.8 mM and 27.8 mM potassium chloride per ml buffer, respectively. Fractions of 0.8 ml were collected and analyzed for radioactivity and relative absorbance at a wavelength of 280 nm.

Step C: Eluted peptides in fractions 2–13 of the previous step were pooled, lyophilized, redissolved in 0.3 ml 30% formic acid and further diluted to 0.9 ml in 0.1% trifluoroacetic acid in double-distilled water after which the sample was applied on a HPLC reverse-phase column, equilibrated with 0.1% trifluoroacetic acid in double distilled water. HPLC was carried out using a Waters instrument equipped with a Waters Model 660 Solvent Programmer. The applied flow rate was 1 ml/min. The transhydrogenase peptides were immediately separated by a linear gradient of 0–70% acetonitrile in equilibration medium equivalent to a relative gradient slope of 0.58% per ml medium. A volume of 1 ml per fraction was collected during the first 66 fractions, after which the fraction size was reduced to 0.6 ml. The collected fractions were analyzed for radioactivity and relative absorbance at a wavelength of 280 nm.

Step D: Labeled peptides of fraction 99–100 from the previous HPLC chromatography step were pooled, lyophilized, redissolved in 0.2 ml 30% formic acid and further diluted to 0.4 ml with 0.1% trifluoroacetic acid in double-distilled water. The sample was then rechromatographed in the same HPLC system as in the previous step, with the exception that a linear two-step gradient of acetonitrile was applied, i.e., 0–15% and 15–40%, corresponding to a relative gradient slope of 2.5% per ml medium and 0.21% per ml medium, respectively. A volume of 5 ml per fraction was collected during the first 11 fractions, after which the fraction size was reduced to 1 ml and the fractions analyzed for radioactivity and relative absorbance at a wavelength of 280 nm.

Step E: Fraction 17, eluted by 29% acetonitrile in the previous HPLC chromatography step containing a highly labeled transhydrogenase peptide, was lyophilized, redissolved in 0.2 ml 30% formic acid and further diluted to 0.4 ml with 0.1% trifluoroacetic acid in double-distilled water. The fraction was then rechromatographed under the same conditions as in step D, except that the applied two-step acetonitrile gradient now was 5–20% and 20–35%, corresponding to a relative gradient of 1.0% per ml medium and 0.25% per ml medium, respectively. Fractions of 5 ml were collected during the first nine fractions after which the fraction size was reduced to 1 ml. The collected fractions were analyzed for radioactivity and relative absorbance at a wavelength of 280 nm.

Fraction 13, eluted with 29% acetonitrile and which contained a highly labeled peptide, was lyophilized and the amino-acid content of the peptide and its amino-acid sequence were determined.

Amino acid sequence analysis

The amino-acid sequence of the isolated transhydrogenase peptide was determined in an Applied Biosystems 470 A amino acid gas-phase sequencer with detection of phenylthiohydantoin amino acids by high-performance liquid chromatography as described in Ref. 17. The radioactivity of each amino-acid fraction was measured. For determination of total composition the peptide in 6 M HCl/0.5% phenol was hydrolyzed in evacuated tubes at 110°C for 24 h, and amino acids were determined with a Beckman 121 M analyzer.

Sequence retrieval and alignment

The primary structure of the bovine transhydrogenase was obtained from the EMBL databank 'Swissprot' as NNTM$BOVIN. The 43-amino-acid N-terminal signal sequence was excluded from the mature sequence. Primary structures of the *E. coli* sequences for the α and β subunits were obtained from Prof. P.D. Bragg (Department of Biochemistry, University of British Columbia, Vancouver, Canada), since the sequences available from the Swissprot databank (PNTA$ECOL and PNTB$ECOLI) are incorrect. Sequences for aligning the nucleotide-binding regions were obtained from the Swissprot databank: GSHR$ECOLI (glutathione reductase from *E. coli*), GSHR$HUMAN (glutathione reductase from human erythrocytes), G3P$PIG (glyceraldehyde 3-phosphate dehydrogenase from pig), DLDH$HUMAN (human dihydrolipoamide dehydrogenase from pig), TRXB$ECOLI (thioredoxin reductase from *E. coli*), TYTR$TRYCO (trypanothione reductase from *Trypanosoma congolense*), MERA$SHIGFL (mercuric reductase from *Shigella flexneri*) and DDHM$PIG (L-lactate dehydrogenase, M-chain from pig).

The alignment of the sequences was carried out manually.

Chemicals

8-N_3-AMP and 8-N_3-[2-^{3}H]AMP were prepared and analyzed by Mr. A.F. Hartog, University of Amsterdam, The Netherlands. Sephadex G-50 coarse, Superose 12 and Mono Q FPLC columns were purchased from Pharmacia, Uppsala Sweden. HPLC 218TPS reverse-phase column was purchased from Vydac, USA. Trypsin (beef pancreas) treated with TPCK and other biochemicals were of analytical grade and obtained from Sigma or Boehringer.

Results

Fig. 1 shows the effect of the photoaffinity inhibitor 8-N_3-AMP on purified beef heart transhydrogenase in the dark, catalyzing the reverse reaction in the presence of varying concentrations of the substrates AcPyAD$^+$ (Fig. 1A) and NADPH (Fig. 1B). The inhibition patterns indicate that the analog was a competi-

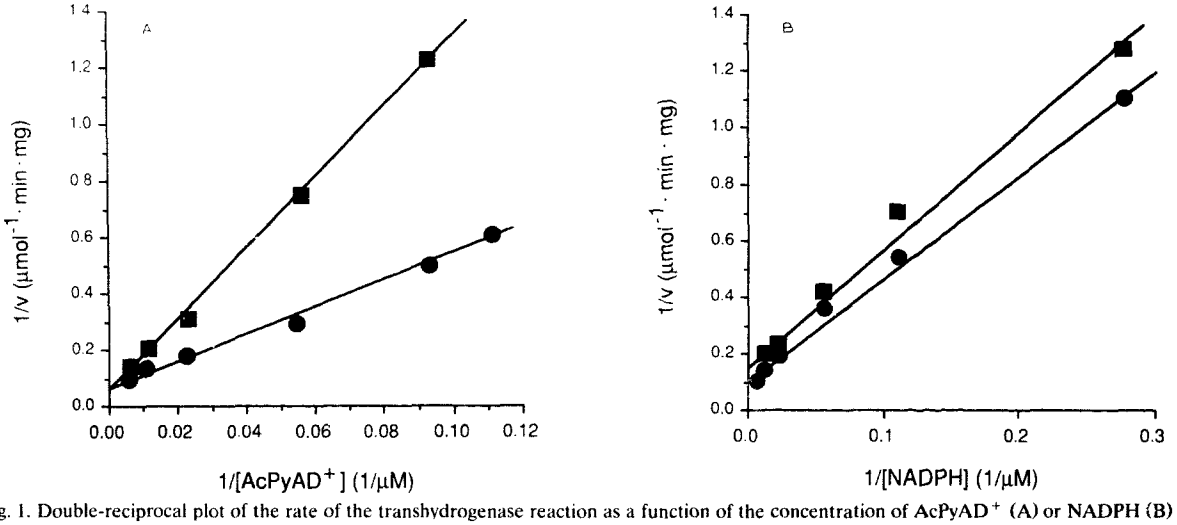

Fig. 1. Double-reciprocal plot of the rate of the transhydrogenase reaction as a function of the concentration of AcPyAD$^+$ (A) or NADPH (B) in the absence and in the presence of 8-N_3-AMP. The catalytic activity of 0.5 μg transhydrogenase in a final volume of 1 ml was titrated with 3.6–180 μM AcPyAD$^+$ at a constant concentration of NADPH of 180 μM, and with 3.6–180 μM NADPH at a constant concentration of AcPyAD$^+$ of 180 μM, in the absence (●) and in the presence (■) of 1.26 mM non-covalently bound 8-N_3-AMP. Other conditions were as described in Materials and Methods. The results are shown as reciprocal values of the catalytic activity vs. reciprocal values of the concentration of the variable substrate.

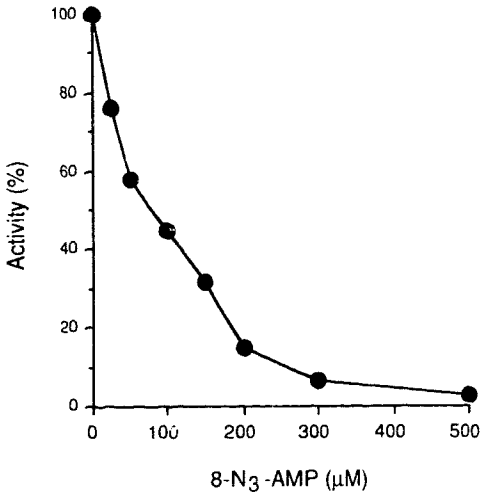

Fig. 2. Photoaffinity labeling of transhydrogenase with 8-N_3-AMP as a function of the concentration of the analog. Labeling was achieved by adding varying concentrations of the photoaffinity analog, incubating for 15 min, after which an aliquot of the sample was withdrawn and the resulting inhibition of the transhydrogenase activity was determined spectrophotometrically. In this experiment the distance between the sample and the light source was 15 cm. Other conditions were as described in Materials and Methods. The result is shown as normalized values of the catalytic activity as a function of the concentration of 8-N_3-AMP.

tive inhibitor with respect to $AcPyAD^+$. This specificity of the analog is in agreement with earlier results obtained with 5'-AMP [8]. In the presence of saturating concentrations of $AcPyAD^+$ and non-saturating concentrations of NADPH the inhibition was essentially abolished, suggesting that the analog was essentially specific for the NAD(H)-binding site. The small extent of inhibition in the latter case was mainly due to the fact that the 180 μM $AcPyAD^+$ used in the assay did not completely eliminate the interaction of 8-N_3-AMP with the NAD(H) site. However, very high concentrations of 8-N_3-AMP, above 10 mM, showed varying degrees of interactions also with the NADP(H) site (not shown, see Discussion). The K_i-value for 8-N_3-AMP with respect to $AcPyAD^+$ was about 400 μM which may be compared to that found with 5'-AMP and NADH using submitochondrial particles as the source of transhydrogenase, 300 μM and 30 μM, respectively [8].

Treatment of transhydrogenase with 8-N_3-AMP in the presence of light but in the absence of substrates (see Materials and Methods) resulted in a concentration-dependent inhibition of the enzyme (Fig. 2). At a fixed time of incubation, 15 min, 50% inactivation of transhydrogenase was obtained with 75 μM 8-N_3-AMP (Fig. 2). At a fixed concentration of the inhibitor the inhibition was time-dependent (not shown). Treatment of transhydrogenase in the dark inhibited the enzyme only slightly after more than 90 min (not shown).

Attempts to protect transhydrogenase against light-induced inactivation by 8-N_3-AMP were carried out with NAD(H) and NADP(H). Both NADH (Fig. 3A) and NADPH (Fig. 3B) apparently protected the enzyme efficiently. However, as indicated by the S-shaped and thus non-Michaelis-Menten patterns of protection by NADH and NADPH above 50 μM, higher concentrations of nicotinamide nucleotides may interfere with the light-requirement of the activation of 8-N_3-AMP and therefore give an overestimation of the extent of protection. Based on the protection at a concentration of 50 μM and below, the protective effects of NADH and NADPH at higher concentrations were derived by extrapolation. It may then be seen that NADH pro-

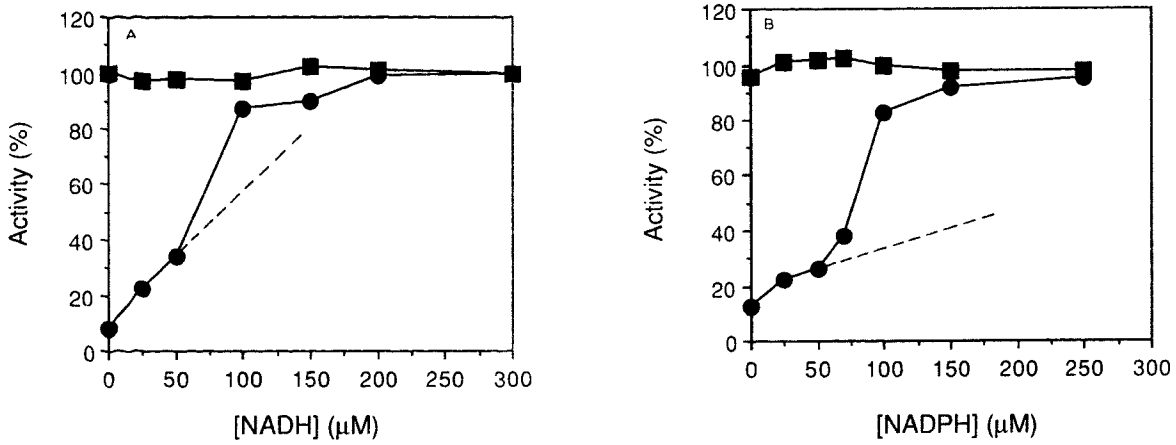

Fig. 3. Protection by NADH (A) or NADPH (B) against light-dependent inactivation of transhydrogenase by 8-N_3-AMP. Transhydrogenase was incubated with 200 μM 8-N_3-MP for 15 min in the absence or presence of varying concentrations of NADH or NADPH in the light (●) or in the dark (■). Conditions were as described in the legend of Fig. 2. The result is shown as normalized values of the catalytic activity as a function of varying concentrations of NAD(P)H. Dotted lines denote extrapolated values of protection.

vided at least twice the protection obtained with NADPH (Fig. 3A and B). In the case of the oxidized substrates NAD^+ and $NADP^+$, these protected partially against inactivation as well, but also caused a time- and concentration-dependent inactivation of the enzyme during incubation, even in the absence of 8-N_3-AMP (not shown). At concentrations up to 2.5 mM, i.e., more than a 10-fold excess over 8-N_3-AMP, the NAD(H) analog 5'-AMP and the NADP(H) analog 2'-AMP had no significant protective effect (not shown); both analogs are site-specific and competitive inhibitors [8]. The latter results thus clearly show that, under the conditions used, excess of an inhibitory adenine nucleotide does not inhibit modification of transhydrogenase by 8-N_3-AMP.

Fig. 4A shows transhydrogenase treated with 400 μM 8-N_3-[2-^{3}H]AMP and inhibited to about 85%, followed by analysis of the enzyme on PAGE, densitometric scanning of the silver-stained gel, slicing of the gel and radioactivity counting of the slices. The bulk of the radioactivity was recovered at the position of the transhydrogenase. The low-molecular-weight material in slice 8–10 was mainly of non-protein origin, i.e., residual detergent and phospholipid. After trypsin treatment of the labeled transhydrogenase, followed by the same treatment as in the experiment of Fig. 4A, most of the label was recovered in the 66000 molecular weight peptide, and several unresolved low-molecular-weight peptides (Fig. 4B). The 45000 molecular weight peptide was essentially unlabeled. Calculation of the number of mol analog bound per mol of transhydrogenase monomer gave a value of 0.79 mol 8-N_3-[2-^{3}H]AMP/mol transhydrogenase monomer.

In order to identify the amino-acids in the transhydrogenase peptide which react with 8-N_3-[2-^{3}H]AMP, a larger amount of transhydrogenase (7.5 mg) was incubated with 8-N_3-[2-^{3}H]AMP to 85% inhibition, followed by trypsin treatment (Materials and Methods).

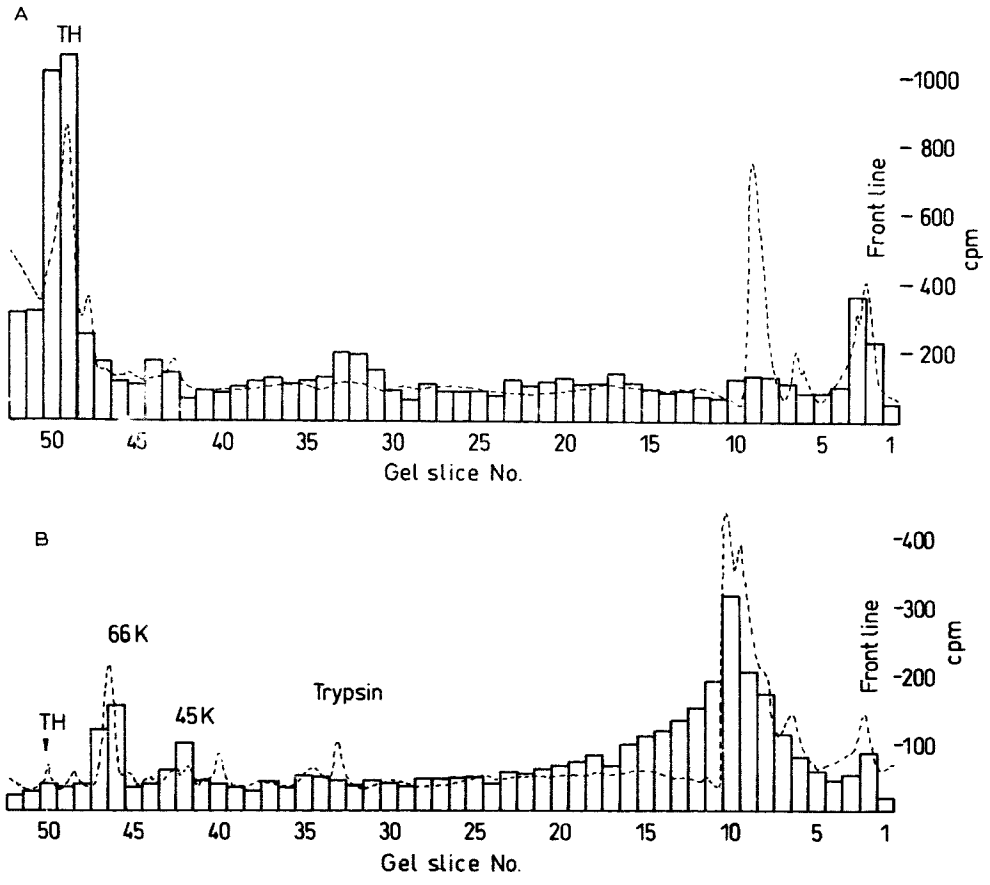

Fig. 4. PAGE analysis of transhydrogenase labeled with 8-N_3-[2-^{3}H]AMP without (A) or with trypsin digestion (B). Transhydrogenase was photoaffinity labeled in the presence of 400 μM 8-N_3-[2-^{3}H]AMP and treated with trypsin as described in Materials and Methods. Dashed lines denote absorbance and bars denote radioactivity, plotted against slice number.

The complete sequence of separation steps (steps A–E) is shown in Fig. 5. The major radioactively labeled peptide in the transhydrogenase-trypsin mixture was purified by FPLC-gel filtration (step A), FPLC-anion exchange (step B), and repeated HPLC reverse-phase (steps C–E). The final peptide used for amino-acid

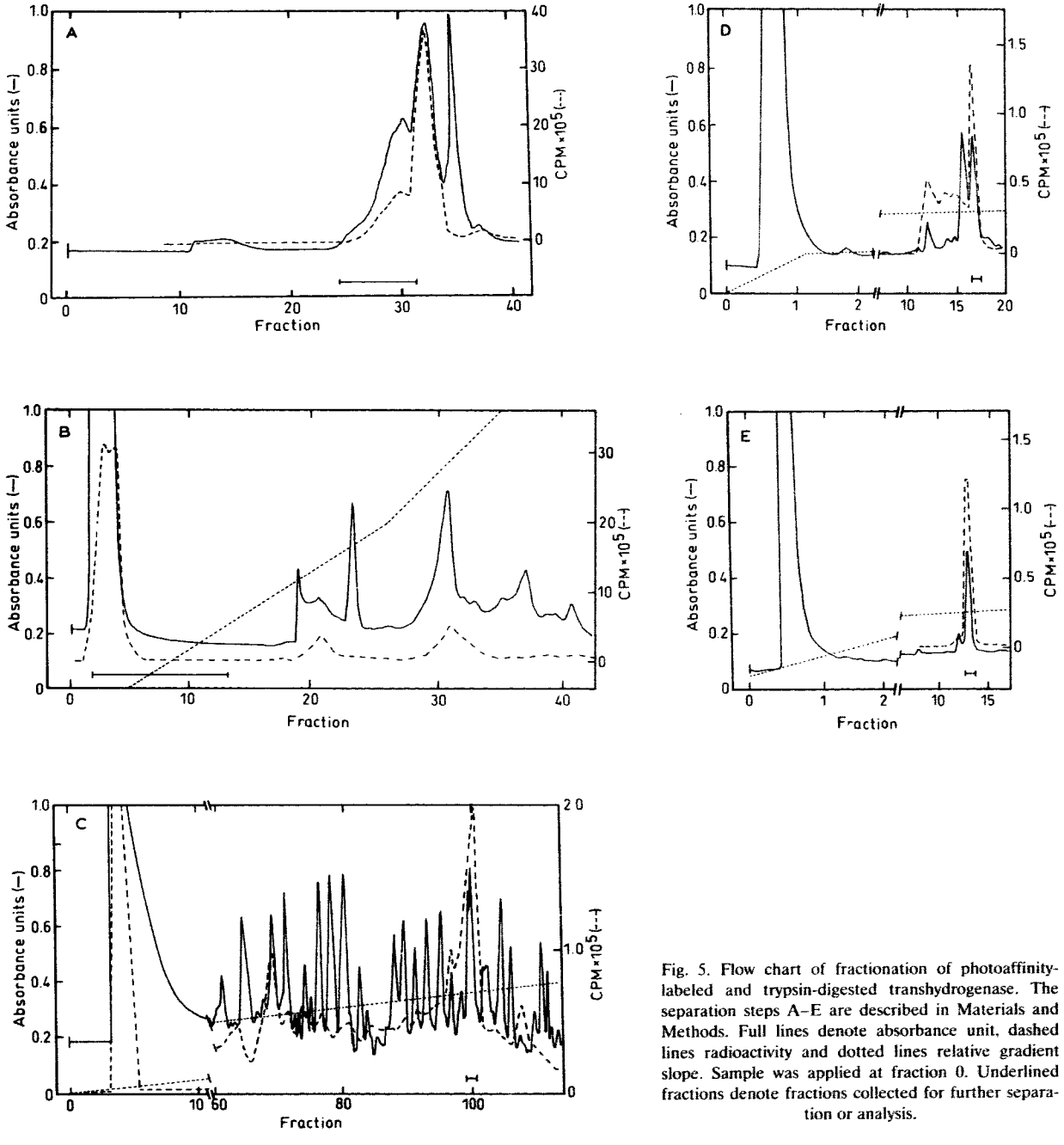

Fig. 5. Flow chart of fractionation of photoaffinity-labeled and trypsin-digested transhydrogenase. The separation steps A–E are described in Materials and Methods. Full lines denote absorbance unit, dashed lines radioactivity and dotted lines relative gradient slope. Sample was applied at fraction 0. Underlined fractions denote fractions collected for further separation or analysis.

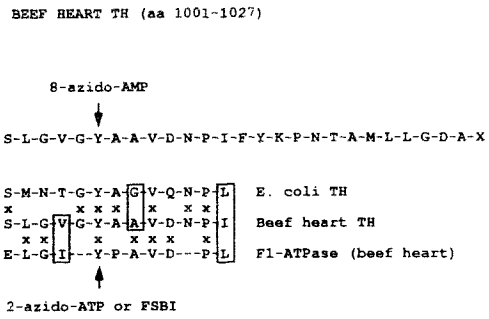

Fig. 6. Amino-acid sequence of isolated peptide containing an 8-N_3-[2-^{3}H]AMP-modified tyrosine residue. Alignment of the amino-acid sequences of the beef heart transhydrogenase, *E-coli* transhydrogenase and beef heart β-subunit of F_1-ATPase. Sequence analysis of the peptide recovered in fraction 13, step E (cf. Fig. 5), was carried out as described in Materials and Methods. The labeled Tyr is boxed. Boxes have been placed around identical and conservatively substituted residues. Crosses indicate identical matches.

sequence analysis was collected in fraction 13, step E. After sequence analysis of this peptide, more than 90% of the radioactivity of the photolytic product of 8-N_3-[2-^{3}H]AMP was found to be associated with a residue in a sequence of 27 amino-acids (Fig. 6), corresponding to Tyr 1006 in the amino-acid sequence 1001–1027 of beef heart transhydrogenase [7]. No other residues contained significant amounts of radioactivity and all other amino-acids chromatographed normally in the amino-acid analyzer.

Discussion

The present results indicate that both 5′-AMP and the photoaffinity label 8-N_3-AMP bound reversibly in the dark with approximately the same affinity for the NAD(H) site in purified beef heart transhydrogenase. As judged from the steady-state kinetics, in the mM concentration range of 8-N_3-AMP used, only a slight, if any, binding occurred to the NADP(H) site. This is in accordance with previous findings that 5′-AMP contains a ribose moiety with a 5′-phosphate required for a ligand to the NAD(H) site, but is devoid of the 2′- or 3′-phosphate group required for a ligand to the NADP(H) site [8]. Upon illumination, 8-N_3-AMP was activated and the reactive nitrene group combined covalently with e.g., amino, hydroxyl or thiol groups in amino-acid side-chains. Consequently, transhydrogenase exposed to 8-N_3-AMP in the light was inhibited, suggesting that the modified amino-acid(s) is essential for activity, or that introduction of a bulky ligand to this amino acid is inhibitory. Due to the assumed

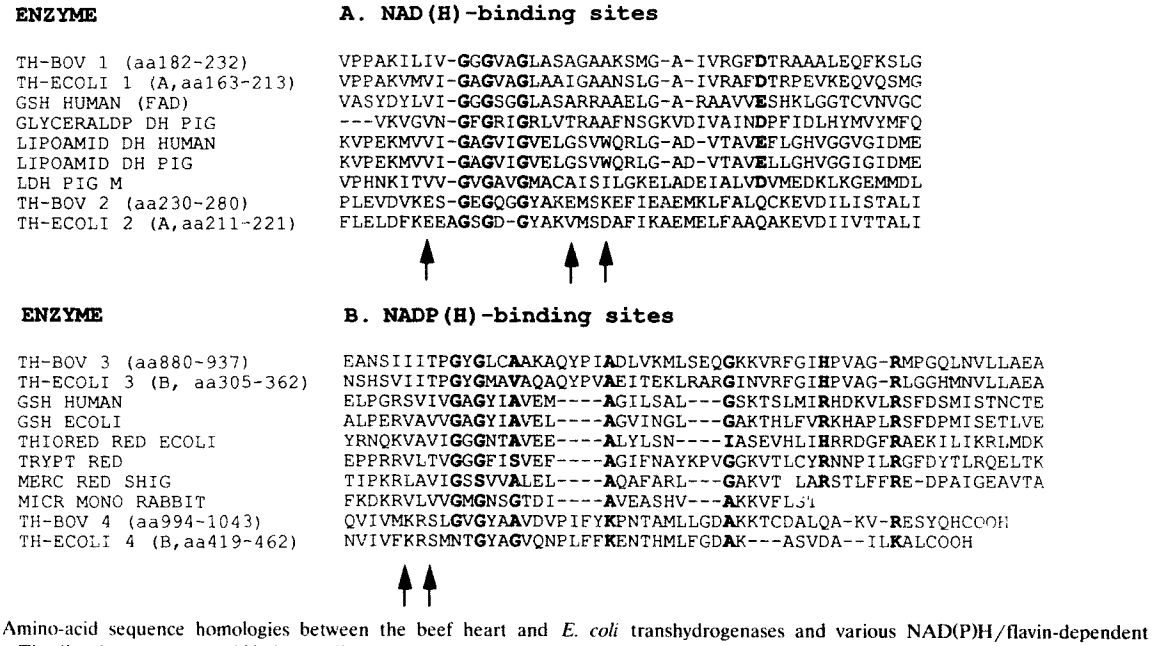

Fig. 7. Amino-acid sequence homologies between the beef heart and *E. coli* transhydrogenases and various NAD(P)H/flavin-dependent enzymes. The listed enzymes are: (A), NAD(H)/FAD-binding sequences: beef heart transhydrogenase, TH-BOV; *E. coli* transhydrogenase, TH-ECOLI; glutathione reductase, human (FAD-binding sequence), GRS HUMAN (FAD); glyceraldehyde-3-phosphate dehydrogenase, pig, GAPDH PIG; lipoamide dehydrogenase, human, LIPDH HUMAN; lipoamide dehydrogenase, pig, LIPDH PIG; lactate dehydrogenase, pig, M-form, LDH PIG M; (B), NADP(H)-binding sequences: beef heart transhydrogenase, TH-BOV; *E. coli* transhydrogenase, TH-ECOLI; glutathione reductase, human erythrocyte (NADPH-binding sequence), GRS HUMAN; glutathione reductase, *E. coli*, GRS ECOLI; thioredoxin reductase; THIORED RED ECOLI; trypanothione reductase, trypanosoma, TRYPAN RED; mercuric reductase, Shigella, MERC RED SHIG; microsomal monooxygenase, rabbit, MICR MONO RABBIT. Arrows indicate hydrophobic sequences suggested to be essential for nucleotide binding (cf. Ref. 35).

site-specificity of 8-N_3-AMP, NADH, as predicted, protected the enzyme against inactivation. NAD^+ tended to have the same protective effect, but inactivated the transhydrogenase by itself after prolonged incubation times. The mechanism of this latter inactivation remains unknown, but may be related to the redox state of the vicinal dithiols previously found to be important for activity [18]. However, more surprising is the fact that NADPH, which does not interact with the NAD(H) binding site significantly, as judged by steady-state kinetics [cf. 1–3], also protected against inactivation by 8-N_3-AMP. At concentrations below 50 μM, protection of the enzyme by NADPH was about half as efficient as that obtained by NADH. The extent of protection by NADH, but not NADPH, agreed approximately with the expected effect considering that the K_m for NADH is about 30 μM [8]. Higher concentrations of NAD(P)H provided close to full protection, but this effect can at present not be distinguished from an indirect effect caused by the light absorption of the reduced nucleotide. Like NAD^+, $NADP^+$ had a partially protecting but also inhibiting effect by itself, whereas the NAD(H) and NADP(H) analogs 5′-AMP and 2′-AMP, which are site-specific and competitive inhibitors [8], had little effect. Taken together these results suggest that 8-N_3-AMP interacts kinetically with the catalytic NAD(H) site in a predictable manner and with the expected affinity in the dark. However, upon illumination this specificity is largely lost and results in covalent modification of especially reactive residues outside this site, possibly also with residues in the NADP(H) site. This was particularly evident with 5′-AMP and 2′-AMP, which are closely related with 8-N_3-AMP, but do not interfere with the light-activation of the reagent, and do not protect even at a large excess. Thus, under the conditions used, protection by substrates or substrate analogs against covalent modification of transhydrogenase by 8-N_3-AMP does not provide conclusive information about the possible site-specificity of the labeling. The formation of a ligand-triggered narrow pocket normally required for transhydrogenation between the natural substrates may contribute to the relatively unspecific protection against covalent modification seen with various substrates and substrate analogs.

About 0.8 mol of the photolytic product of 8-N_3-[2-^{3}H]AMP bound covalently per mol transhydrogenase monomer in the inactivated enzyme, suggesting that only one site per monomer was involved in the modification. Isolation and analysis of the amino-acid sequence modified covalently by 8-N_3-[2-^{3}H]AMP showed the involvement of the tyrosine residue in position 1006 (see Ref. 7). The amino-acid sequence isolated that contained bound 8-N_3-[2-^{3}H]AMP included 27 amino acids identical to the previously published residue sequence 1001–1027, close to the C-terminus [7]. Based on the reactivity with FSBA in the presence of NAD(H), this sequence has previously been suggested to be part of the NADP(H) binding site [7]. In a similar manner, the NAD(H) binding site was assumed to include the residues 237–247 that bind FSBA more rapidly in the presence of NADP(H) [7], a suggestion which is supported by the fact that DCCD apparently binds to a glutamate residue in the residue sequence 248–259, in close proximity downstream of the former sequence, only in the absence of NAD(H) or an analog of this substrate [19]. Apparently, Tyr 1006 exhibits a very high reactivity to 8-N_3-AMP, which leads to covalent modification, even if the time of interaction with proximal binding site is kinetically insignificant. In this regard, Tyr 1006 of the beef heart transhydrogenase is similar to Tyr 345 of the beef heart F_1-ATPase and Tyr 331 of *E. coli* F_1-ATPase (see below).

One reason for the different labeling patterns obtained with FSBA and with 8-N_3-AMP is that the substrate analog moiety of FSBA is adenosine which is an unspecific and poor inhibitor of transhydrogenase [8]. FSBA itself has not to our knowledge been characterized kinetically as an inhibitor of transhydrogenase and it is not surprising, therefore, that this compound shows an apparent specificity for enzymes with only ADP/ATP-binding sites, e.g., ATPase. A theoretical site specificity of FSBA in transhydrogenase therefore relies on the simultaneous presence of NAD(H) in the case of the NADP(H) site, and NADP(H) in the case of the NAD(H) site, conditions which, in view of the present data, may introduce ambiguities with regard to the expected site-reactivity of FSBA. However, FSBA may also react with transhydrogenase in a site-unrelated manner, even in the presence of both NADP(H). In fact, in the presence of NADPH, FSBA reacts faster with transhydrogenase when it would be expected to react more slowly under these conditions [19,20]. Thus, NADPH may promote the reactivity of several essential and reactive groups, e.g., thiols [18,21]. Some of these differences may also depend on the fact that the reactive moiety is localized in different parts of the FSBA and 8-N_3-AMP molecules.

FSBA, its analog FSBI, and 8-N_3-ATP and 2-N_3-ATP have previously been used extensively as reagents for nucleophilic amino-acid side-chains in enzymes that bind adenine nucleotides, e.g., ATPase [22–27], including the Tyr-345 in the sequence of the β-subunit of the beef heart F_1-ATPase [24] corresponding to Tyr 1006 in transhydrogenase (cf. Fig. 6). However, although an aromatic residue may be important in this position, it has recently been shown that Tyrβ331 in *E. coli* ATPase (corresponding to Tyrβ345 in beef heart F_1-ATPase) is not essential for activity [28–30]. Thus, inhibition of ATPase due to modification of this tyrosine by agents like 2-N_3-ATP or FSBI was concluded to be secondary because of the introduction of a bulky

ligand. These tyrosines may be covalently modified by a variety of analogs regardless of the position of the reactive group in the analog molecule, and it has been suggested that covalent labeling does not require a specific binding of the analog to the assumed binding site (see Ref. 30). A similar reasoning may thus be applied to the interaction of 8-N_3-AMP with transhydrogenase.

Dinucleotide-binding sites have been shown to contain the consensus sequence GXGXXG/A for adenine nucleotide binding, where GXGXXG has been proposed to be specific for NAD(H)-binding sites [31–33], and GXGXXA specific for NADP(H)-binding sites [33]. As shown in Fig. 7 both the beef heart and *E. coli* transhydrogenases contain four GXGXXG/A consensus sequences, numbered 1–4 after the sequences 182–232, 230–280, 880–937, and 994–1043 in the beef heart transhydrogenase and A163–213, A211–221, B305–362, and B419–462 in the α and β subunits of the *E. coli* transhydrogenase. A comparison with homologous sequences in other NADP(H)-dependent enzymes as well as flavoenzymes (Fig. 7) strongly suggests that the transhydrogenase sequence 1 constitutes the adenine nucleotide-binding domain of the NAD(H)-binding site in both transhydrogenases and that sequence 3 constitutes a similar domain in the NADP(H)-binding site also in both transhydrogenases (flavin-binding sequences are interesting in this context because they have been shown to be homologous to dinucleotide-binding sequences, see Ref. 34). Sequence 2 does not have the required hydrophobic sequences preceding and following the GXGXXG sequence (see Ref. 35), and it has Glu-Ala (EA) or Lys-Ala (KA), i.e., charged residues instead of the required hydrophobic Gly-Ala (GA) in the end of the GXGXXGXXXXXXXXXXG-A sequence. Also, sequence 2 does not have the charged Glu/Asp (E/D) downstream residues stabilizing NAD(P)H)-binding [33]. That sequence 3 rather than sequence 4 is the adenine nucleotide-binding domain of the catalytic NADP(H) site is indicated by the fact that the final Ala (A) in GXGXXA/GXXXXXXXA is changed to a Lys (K) and that the Arg (R) and His (H) required for stabilizing NADP(H)-binding are missing in sequence 4.

The finding that 8-N_3-AMP labeled Tyr 1006 in the beef heart transhydrogenase, i.e., sequence 4 in Fig. 7, agrees with the assumption that it does not label a catalytic adenine nucleotide-binding domain. However, the fact that the β-subunit of the *E. coli* transhydrogenase [35] binds to NAD-agarose may indicate that the sequence 4 indeed is capable of interacting weakly with NAD(H).

In conclusion, 8-N_3-AMP has been shown to bind covalently to Tyr 1006 in beef heart transhydrogenase, which may be assumed to be part of a nucleotide-binding site close to the C-terminal in the enzyme. The functional role of this sequence and sequence 2 remains unknown but they may constitute nicotinamide nucleotide-binding domains of the active site. Alternatively, due to their sequence homology with ADP/ATP-binding sequences of F_1-ATPases, it is tempting to suggest that they (cf. Fig. 7), constitute regulatory nucleotide-binding sites derived from a pair of hypothetical dehydrogenases or flavoenzymes, perhaps the flavin-containing soluble bacterial transhydrogenase which, in the course of evolution, have formed the membrane-bound proton-pumping transhydrogenase.

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