Site-Directed Mutagenesis of Tyrosine Residues at Nicotinamide Nucleotide Binding Sites of *Escherichia coli* Transhydrogenase†

Torbjörn Olausson,‡ Thomas Hultman,§ Erland Holmberg,‖ and Jan Rydström*,‡

Department of Biochemistry and Biophysics, University of Gothenburg and Chalmers University of Technology, S-412 96 Gothenburg, Sweden, Department of Biochemistry and Biotechnology, Royal Institute of Technology, S-100 44 Stockholm, Sweden, and Biochemistry Department, Peptide Hormones, Kabi Pharmacia, S-112 87 Stockholm, Sweden

Suhail Ahmad,⊥ Natalie A. Glavas,⊥ and Philip D. Bragg⊥

Department of Biochemistry, The University of British Columbia, 2146 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3, Canada

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ABSTRACT: Nicotinamide nucleotide transhydrogenase (E.C. 1.6.1.1) from *Escherichia coli* was investigated with respect to the role of specific conserved tyrosine residues of putative substrate-binding regions. The enzyme from *E. coli* is made up of two subunits, α (510 residues) and β (462 residues). The corresponding enzyme from bovine mitochondria is a single polypeptide (1043 residues) whose N-terminal region corresponds to the α subunit and whose C-terminal region corresponds to the β subunit. Tyrosines 245 and 1006 of the mitochondrial enzyme have been shown to react selectively with 5′-(*p*-fluorosulfonylbenzoyl)adenosine with inactivation of the enzyme. In *E. coli* these residues correspond to tyrosine 226 of the α subunit and tyrosine 431 of the β subunit. In addition, tyrosine 315 of the β subunit is of interest since mutation of an adjacent residue (glycine 314) leads to inactivation [Ahmad, S., Glavas, N. A., & Bragg, P. D. (1992) *Eur. J. Biochem. 207*, 733–739]. In order to assess the role of the aforementioned conserved tyrosine residues in the mechanism and structure of transhydrogenases, these were replaced by site-specific mutagenesis, using the cloned and overexpressed *E. coli* transhydrogenase genes [Clarke, D. M., & Bragg, P. D. (1985) *J. Bacteriol. 162*, 367–373]. Phenylalanine mutants of all three tyrosine residues showed approximately 50% activity or more with regard to catalytic activity assayed as reduction of 3-acetylpyridine–NAD^+ by NADPH. These mutants were also active in proton pumping assayed as quenching of 9-methoxy-6-chloro-2-aminoacridine or quinacrine fluorescence. With respect to catalytic activity these and other mutants were ranked as αY226F > L = H > N, βY431F ≫ L = H = N = I, and βY315F > N = H > L > D = I = V, indicating that the amino acids at position 226 of the α-subunit and positions 431 and 315 of the β-subunit should be aromatic and sufficiently large and hydrophobic or hydrophilic, but not charged or small (aliphatic) and hydrophobic. These results suggest that tyrosine 226 of the α subunit, and tyrosines 431 and 315 of the β subunit are not essential for catalytic activity or proton pumping.

The redox-driven proton pump nicotinamide nucleotide transhydrogenase (E.C. 1.6.1.1.) catalyzes the reversible transfer of a hydride ion between NAD and NADP and the concomitant translocation of *n* protons across the membrane according to the reaction

$$n\mathrm{H}^+_{\mathrm{out}} + \mathrm{NADP}^+ + \mathrm{NADH} \rightleftharpoons \mathrm{NADPH} + \mathrm{NAD}^+ + n\mathrm{H}^+_{\mathrm{in}}$$

where "out" and "in" denote the cytosol and the matrix, respectively, in mitochondria and the periplasmic space and the cytosol, respectively, in bacteria [for reviews, see Rydström (1977), Fisher and Earle (1982), Rydström et al. (1987), and Jackson (1991)]. Transhydrogenase is an integral membrane protein which has been purified from several sources, e.g., bovine heart mitochondria (Höjeberg & Rydström, 1977; Anderson & Fisher, 1978; Wu et al., 1982; Persson et al., 1984; Phelps & Hatefi, 1984) and *Escherichia coli* (Clarke & Bragg, 1985), and characterized extensively in the reconstituted form with and without other proton pumps in phospholipid vesicles (Wu et al., 1982; Persson et al., 1984; Clarke & Bragg, 1985a; Earle & Fisher, 1979, 1980; Pennington et al., 1981; Eytan et al., 1987a,b, 1990). By employment of reconstituted vesicles, the number *n* in the above reaction has been concluded to be 1 [cf. Rydström et al. (1987); see also Olausson et al. (1992) and Hoek and Rydström (1988)]. However, in chromatophores from *Rhodospirillum rubrum*, *n* has been shown to be 0.5 (Bizouarn & Jackson, 1993). The active bovine heart enzyme is a homodimer with a molecular mass of the monomer of about 109 kDa (Wu & Fisher, 1983; Persson et al., 1987; Ormö et al., 1992), whereas the *E. coli* enzyme has 2 subunits and a composition of $\alpha_2\beta_2$ in the active enzyme (Hou et al., 1990). The genes for the *E. coli* transhydrogenase (Clarke & Bragg, 1985a; Clarke et al., 1986) and the cDNA for bovine heart mitochondrial enzyme (Yamaguchi et al., 1988) have been cloned and sequenced. However, even though the complete bovine transhydrogenase gene has been synthesized from partial clones (Holmberg et al., 1992), only the genes for the α and β subunits of *E. coli* transhydrogenase have been expressed individually (Clarke & Bragg, 1986) or simultaneously (Clarke & Bragg, 1985b), using various *E. coli* strains as host cells. This expression represents approximately a 70-

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* To whom correspondence should be addressed.
‡ University of Gothenburg and Chalmers University of Technology.
§ Royal Institute of Technology.
‖ Kabi Pharmacia.
⊥ The University of British Columbia.

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fold overexpression compared to the wild-type activity. Several interesting studies regarding the structure and function of this transhydrogenase using membranes or purified transhydrogenase from overexpressing *E. coli* have been carried out (Clarke & Bragg, 1985b; Clarke & Bragg, 1986; Tong et al., 1991; Chang et al., 1992; Ahmad et al., 1992). In addition, because of the high sequence homology and nearly identical functional properties of the bovine and *E. coli* transhydrogenases, the latter enzyme may be assumed to serve as a general model system for proton-pumping transhydrogenases.

Two important aspects of the characterization of transhydrogenases are the identification of the substrate-binding sites and the determination of their properties. The approximate sequence locations of these binding sites have been identified by the use of 5′-(*p*-fluorosulfonylbenzoyl)adenosine (FSBA)[1] which, by virtue of its adenosine moiety, binds as a substrate analogue to both the NAD(H) and the NADP(H) site, although with a preference for the former site (Yamaguchi et al., 1988; Phelps & Hatefi, 1985; Wakabayashi & Hatefi, 1987). However, in the presence of NAD(H) the binding is directed to the NADP(H) site (Yamaguchi et al., 1988; Phelps & Hatefi, 1985; Wakabayashi & Hatefi, 1987). The reactive amino acid residues identified as covalently modified by FSBA under these conditions were Tyr245 and Tyr1006 of the assumed NAD(H)- and NADP(H)-binding regions, respectively (Yamaguchi et al., 1988; Wakabayashi & Hatefi, 1987). These residues are conserved and correspond to αTyr226 and βTyr431 of the *E. coli* transhydrogenase. They constitute parts of possible nucleotide-binding folds (Brändén & Tooze, 1991; Hu et al., 1992). That the N-terminal nucleotide-binding region binds one NADH and the C-terminal nucleotide-binding region binds one NADPH per dimer in the bovine transhydrogenase was recently demonstrated by Hatefi and co-workers (Yamaguchi & Hatefi, 1993), strongly suggesting that the previous tentative site localization is correct (Hu et al., 1992). An additional conserved tyrosine residue of *E. coli* transhydrogenase, βTyr315, is located in a third putative nucleotide-binding fold, next to βGly314. The latter yields an inactive enzyme when replaced by a glutamic acid residue (Ahmad et al., 1992).

Chemical modification of tyrosine residues by FSBA and the resulting inactivation of the enzyme are either the result of a modification of an essential residue or a consequence of the introduction of a sterically hindering group. In order to establish a possible role of the conserved tyrosine residues of nucleotide-binding regions in the mechanism and structure of the *E. coli* transhydrogenase, these residues were modified by site-specific mutagenesis. The catalytic and proton-pumping activities as well as the sensitivities to FSBA of the resulting mutant transhydrogenases were determined.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *pnt* gene introduced in the pUC13 plasmid, giving a construct denoted pDC21 (Clarke & Bragg, 1985b), was transformed into *E. coli* K12 strain TG1 (Carter et al., 1985). Alternatively, the *pnt* gene was introduced in the pGEM-7Zf(+) plasmid, giving the construct pSA2, and subsequently transformed into *E. coli* K12 strain JM109 (Ahmad et al., 1992a,b). Normally, a 40–80-fold overexpression was obtained, depending on the plasmid and cell type used. Mutant *E. coli* transhydrogenase was made through PCR–mutagenesis on plasmid pDC21.

Site-Specific Mutagenesis. Mutants of *E. coli* transhydrogenase were constructed using two-step PCR amplification (Nordling et al., 1991). The template, pDC21, was prepared by following the Qiagen plasmid preparation protocol. The first PCR amplification was performed in 30-μL aliquots containing 250 μM dNTP, 1 μM each of mutant (degenerate) and flanking primers, 10 ng of template, buffer, and 4–5 units of Taq polymerase. The amplification was performed in 20 cycles, with denaturation at 94 °C for 1 min and extension at 72 °C for 1 min. The annealing temperature varied from 60 to 72 °C, in agreement with the melting temperature of each mutant primer. The amplified fragment was then purified through gel electrophoresis and DNA extraction according to Nordling et al. (1991). The second PCR amplification was performed as above, using the first PCR product and a second flanking primer for extension. The smallest possible fragment containing the desired mutation was then cut out of the final PCR product and was substituted for the corresponding fragment of the wild-type plasmid. The TAT codon encoding tyrosine at positions α226 and β431 was replaced with C/TA/TC encoding leucine, histidine, phenylalanine, and tyrosine. The αTyr226 mutants were cut by *Nae*I and *Xho*I, and the βTyr431 mutants were cut by *Bss*HII and *Bam*HI. In both cases the amplified fragment was approximately 465 bp in size.

After transformation, colonies carrying plasmids with inserts of the correct size were identified by PCR. Since degenerate mutant primers were used, several clones had to be sequenced in order to find all mutants. The entire subunit comprising the mutation was subsequently sequenced to detect any undesired substitutions.

Alternatively, plasmid pSA2 carrying wild-type *pnt* genes was used to isolate single-stranded phagemid DNA. Site-directed mutagenesis to convert selected tyrosine residues was performed by the method of Taylor et al. (1985) using degenerate primers. The reagents and protocols outlined in the Amersham mutagenesis kit were followed except that competent *E. coli* JM109 cells were used for transformation. Plasmid DNA was prepared from individual colonies, and the mutants were identified by double-stranded DNA sequencing. The entire coding region of the *pnt* gene from each mutant was completely sequenced, using overlapping synthetic primers, to eliminate the possibility of unwanted changes in the DNA sequence.

Sequencing of E. coli Transhydrogenase Mutant DNA. All mutants were resequenced with respect to the subunit subjected to mutagenesis, and more than half of the mutants were completely resequenced. DNA sequencing of mutants was routinely carried out by either of the two methods, A and B, described below.

(A) Using the dideoxy method of Sanger et al. (1977), as adapted by Amersham (Amersham International plc, Buckinghamshire, U.K.), sequencing was carried out by the so-called Multiwell sequencing system employing electrophoresis as described by Olsson et al. (1984). Alternatively, sequencing was performed by the dideoxy chain-termination method using Sequenase (Sanger et al., 1977).

(B) A single bacterial colony containing the plasmid to be sequenced was scraped from the plate into 10 μL of PCR buffer (20 mM Tris-HCl, pH 8.3 at 20 °C, 50 mM KCl, and 0.1% Tween 20) and lysed at 99 °C for 5 min. One microliter of this lysis solution was used as DNA source for the subsequent reactions. Polymerase chain reaction (PCR) amplification carried out in 50 μL of PCR buffer included 200 μM of each

[1] AcPyAD$^+$, reduced 3-acetylpyridine-NAD$^+$; FSBA, 5′-(*p*-fluorosulfonylbenzoyl)adenosine; FSBI, 5′-(*p*-fluorosulfonylbenzoyl)inosine; ACMA, 9-methoxy-6-chloro-2-aminoacridine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; 8-azido-AMP, 8-azidoadenosine 5′-phosphate; 8-azido-ATP, 8-azidoadenosine 5′-triphosphate; 2-azido-ATP, 2-azidoadenosine 5′-triphosphate; TCS, 3,3′,4′,5-tetrachlorosalicylanilide.

dNTP, 1 unit of ampliTaq DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA), and 5 pmol of oligonucleotides complementary to regions downstream and upstream of the multilinker region of pUC-derived vectors, respectively. The temperature cycle consisted of denaturation at 95 °C for 30 s and annealing of primers and chain extension at 70 °C for 2 min. The reaction was carried through 30 cycles on a GenAmp PCR 9600 system (Perkin-Elmer).

Amplified biotin-labeled clones were subjected to automated solid-phase sequencing, using a robot work station (Biomek-1000, Beckman Instrument, Fullerton, CA) as described (Hultman et al., 1989, 1991). In brief, the PCR mixture was immobilized on streptavidin-coupled magnetic beads (Dynabeads, Dynal, Oslo, Norway). After being washed, the immobilized double-stranded DNA was converted to single-stranded templates by treatment with NaOH, resulting in a single-stranded template immobilized at the 5'-end to the beads while the complementary strand remained in the NaOH supernatant. Each clone was thus sequenced from both directions: the immobilized strand, by using a fluorescence-labeled M13 forward primer, and the neutralized eluted strand, by using a fluorescence-labeled reverse primer. The sequencing reactions were analyzed on an automated laser fluorescent (ALF) sequencer (Kabi-Pharmacia AB, Stockholm, Sweden).

Preparation of Membrane Vesicles. *E. coli* cells carrying wild-type or mutant plasmids pDC21 or pSA2 were grown in LB medium supplemented with 0.1 g/L ampicillin, to an OD_{550} of 1.0 before harvest essentially as described (Tong et al., 1991; Ahmad et al., 1992b). Cells were sedimented at 4400*g* for 20 min and washed as described (Tong et al., 1991), after which the cell pellets were resuspended in TMD buffer, composed of 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, and 2 mM DTT, and kept on ice. Cells were then disrupted by 5-s pulse sonication (Sonics and Materials, Inc., Danbury, CT; setting 6) for an accumulated time of 1 min at 0–4 °C or by passage through a French press at 1400 kg/cm^2. The debris was sedimented at 10000*g* for 20 min, followed by ultracentrifugation of the supernatant at 100000*g* for 1 h. Pellets were resuspended in TMD buffer and stored on ice at a concentration of about 10 mg/mL. The vesicles were used fresh or kept frozen below –20 °C.

Catalytic Activity. The catalytic activity of transhydrogenase was measured spectrophotometrically at 375 nm as reduction of $AcPyAD^+$ by NADPH at pH 7.0 according to Rydström (1979) as modified by Clarke and Bragg (1985b) in the absence or in the presence of 1 μM CCCP or 2.5 μM TCS. Rotenone (1 μg/mL) and KCN (1 mM) added to prevent oxidation of the AcPyADH formed through the respiratory chain increased the rate of AcPyADH formation less than 5% and were therefore normally excluded from the assay medium. The reaction was started by the addition of membrane vesicles or NADPH, and the initial rate was calculated. Activities of membrane vesicles derived from TG1 and JM109 cells were 0.014 and 0.3 μmol/min/mg of protein, corresponding to basal activities of 1% and 2%, respectively, of the membrane vesicles derived from pDC21/TG1 and pSA2/JM109 cells.

Proton Pump Activity. Initial relative rates of proton pumping catalyzed by freshly prepared membrane vesicles obtained from transformed TG1 or JM109 cells were assayed fluorimetrically as quenching of ACMA fluorescence (using excitation at 418 nm and emission at 480 nm) or as quenching of quinacrine fluorescence (using excitation at 430 nm and emission at 505 nm) (Persson et al., 1984; Clarke & Bragg, 1985a). All activity-dependent quenching was sensitive to uncouplers, e.g., CCCP. Rates were expressed in terms of a decrease in arbitrary fluorescence units/min/mg of membrane protein relative to the rate of pumping obtained by membrane vesicles obtained from pDC21/TG1 or pSA2/JM109 cells. The proton-pumping activity of membrane vesicles from wild-type TG1 cells was less than 1% of that of membranes from pDC21/TG1 cells.

Modification of Tyrosine Residues by FSBA. Treatment of membrane vesicles of pDC21/TG1 cells with FSBA dissolved in DMSO was carried out at room temperature in the absence and the presence of 2 mM $AcPyAD^+$ or NADPH as described by Phelps and Hatefi (1985) using 1 mM FSBA at pH 7.5. Final concentration of DMSO was 1%. The treatment was initiated by the addition of FSBA, and aliquots of the reaction mixture were removed at the times indicated, maximally after about 3 h. As a control incubation, vesicles were incubated in the presence of 1% DMSO. Alternatively, purified transhydrogenase (Tong et al., 1991) or membrane vesicles of pSA2/JM109 cells were treated with 1 mM FSBA in the presence or the absence of 0.5 mM NADH or NADPH. The protein concentration was 1 mg/mL. The final concentration of MeOH was 4%.

Affinity Chromatography of Trypsin-Digested Mutant Transhydrogenases. Soluble wild-type or mutant transhydrogenase was prepared as described (Tong et al., 1991). Transhydrogenase (5 mg of protein) was digested for 15 min with trypsin (treated with diphenylcarbamyl chloride to inhibit chymotryptic activity) at a 1:100 (trypsin:transhydrogenase) ratio. The reaction was terminated by addition of soybean trypsin inhibitor. The trypsin-digested transhydrogenase was applied to a NAD- or NADP-agarose column (1 × 6 cm) equilibrated with a buffer containing 10 mM sodium phosphate (pH 7), 1 mM dithiothreitol, 1 mM EDTA, 20 mM NaCl, and Brij-35 (0.5 mg/mL). The column was eluted successively with buffer (15 mL), with either 5 mM NADPH or 10 mM NADH in buffer (5 mL), and with buffer (10 mL) again at a flow rate of 1 mL/min. The fractions were examined by SDS–PAGE.

Polyacrylamide Gel Electrophoresis. SDS–PAGE of membrane vesicles was performed using a 7.5% polyacrylamide and bis(polyacrylamide) gel system (Laemmli, 1970). Gels were stained by a modified silver staining method (Merril et al., 1981). Both electrophoresis and staining were carried out using a Pharmacia Phast-Gel instrument (Kabi-Pharmacia, Uppsala, Sweden). Densitometric scanning of the α and β subunits of *E. coli* transhydrogenase was achieved using an LKB Ultroscan XL laser densitometer (LKB-Pharmacia, Bromma, Sweden).

Chemicals. Unless otherwise specified, biochemicals were of analytical grade and purchased from Sigma Chemical Co. or Boehringer Mannheim. NADP-agarose type 3 was obtained from Kabi-Pharmacia.

RESULTS

As shown in Figure 1, transhydrogenase of membrane vesicles obtained from TG1 cells transformed with pDC21 was inhibited by FSBA. Inhibition was rapid initially but declined with time. Similar to the bovine transhydrogenase (Phelps & Hatefi, 1985; Wakabayashi & Hatefi, 1987), addition of $AcPyAD^+$ protected *E. coli* transhydrogenase by about 25–70%. However, in contrast to the bovine enzyme, which is more rapidly inactivated by FSBA in the presence of NADPH (Wakabayashi & Hatefi, 1987), the addition of NADPH to the *E. coli* transhydrogenase had a slight protecting effect.

Figure 2 shows the sequences of the *E. coli* transhydrogenase containing the three tyrosine residues αTyr226, βTyr431, and

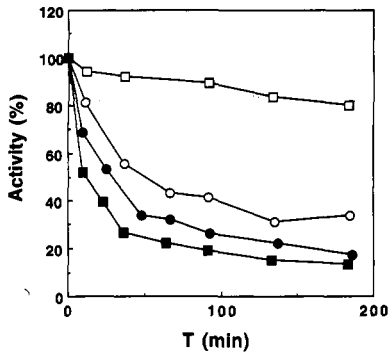

FIGURE 1: Inhibition of pDC21/TG1 transhydrogenase by FSBA in the absence and in the presence of substrates. Membrane vesicles isolated from pDC21/TG1 cells were incubated with 1 mM FSBA at room temperature for the times indicated, as described in Materials and Methods. Additions were (□) 1% DMSO; (■) 1 mM FSBA and 1% DMSO; (○) 1 mM FSBA, 1% DMSO, and 2 mM AcPyAD$^+$; and (●) 1 mM FSBA, 1% DMSO, and 2 mM NADPH. One hundred percent (100%) corresponds to a specific activity of 1.3 μmol/min/mg.

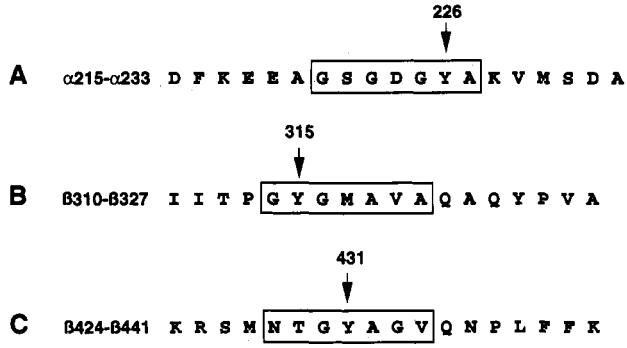

FIGURE 2: Sequences of *E. coli* transhydrogenase containing assumed FSBA-reactive tyrosine residues subjected to site-specific mutagenesis. The tyrosine residues αTyr226, βTyr315, and βTyr431 chosen for site-specific mutagenesis are indicated in the sequences α215–α233 (A), β310–β327 (B), and β424–β441 (C). Boxed sequences are GXGXXG/A or GXGXXG/A-like sequences of βαβ folds of possible nucleotide-binding domains [cf. Brändén and Tooze (1991) and Hu et al. (1992)].

βY315. The first two residues correspond to Tyr245 and Tyr1006 of the bovine transhydrogenase, which have been shown to react covalently with FSBA, resulting in an inhibition of activity (Yamaguchi et al., 1988; Phelps & Hatefi, 1985; Wakabayashi & Hatefi, 1987). βTyr315, another tyrosine residue located in or close to a nucleotide-binding region, is also potentially reactive with FSBA. βTyr315 has recently been shown to be located next to βGly314. The latter residue has been shown to be essential for catalytic activity and for NADPH-induced conformational changes in the enzyme (Ahmad et al., 1992b).

Site-specific mutagenesis of the *pnt* gene of pDC21 expressed in TG1 cells was carried out with the FSBA-reactive tyrosine residues αTyr226 and βTyr431, which were replaced by His, Leu, and Phe. In site-specific mutagenesis of pSA2 expressed in JM109 cells, αTyr226 was replaced by His, Phe, and Asn; βTyr431, by His, Ile, Phe, and Asn; and βTyr315, by His, Ile, Leu, Phe, Asn, Asp, and Val. *E. coli* transhydrogenase mutants were grown, membrane vesicles were isolated in a manner identical to that of the pDC21-expressed enzyme, and catalytic activity was measured.

Table I shows the specific catalytic activities of membrane vesicles derived from mutant-transformed TG1 and JM109 cells. Replacement of the tyrosine residue in the α226 position in the mutants αY226H, αY226L, and αY226F, i.e., in the NAD(H)-binding region, caused an inhibition of about 67–55%. With the same host cell type a different picture emerged for substitutions of the tyrosine in the β431 position, i.e., in the NADP(H)-binding region. In this case βY431F was still very active, but the βY431H and βY431L mutants were essentially inactive. Although the specific activities were generally about 10 times higher, essentially the same results were obtained using the pSA2/JM109 system.

Table I: Effects of Site-Specific Mutagenesis of Tyrosine Residues in NAD(P)-Binding Regions on the Catalytic Activity of Membrane Vesicles of pDC21/TG1 and pSA2/JM109 Cells[a]

mutant	activity (%) pDC21/TG1	pSA2/JM109
control	100	100
α-subunit, NAD(H)-binding region		
αY226H	33	51
αY226L	38	
αY226F	45	
αY226N		42
β-subunit, NADP(H)-binding region		
βY315H		28
βY315I		4
βY315F		62
βY315D		6
βY315N		25
βY315V		4
βY315L		13
βY431H	2	5
βY431L	6	
βY431F	60	80
βY431N		4
βY431I		4
others		
βG314E (RH1)	0	0
βY315F/βY431F		62
αY226F/βY315F/βY431F		50

[a] Activities are specific activities expressed as percent of the activity of control membranes, where 100% corresponds to 1.3 and 14.1 μmol/min/mg for pDC21/TG1 and pSA2/JM109 membranes, respectively. Measurements were made in duplicate. Conditions were as described in Materials and Methods.

Like αY226F and βY431F, replacement of the tyrosine residue in the β315 position by a phenylalanine, i.e., βY315F, resulted in an active enzyme. As expected, the triple mutant αY226F/βY315F/βY431F therefore showed about 50% activity compared to pSA2 (Table I). Other substitutions in the β315 position gave mixed results. βY315H and βY315N were relatively active, whereas βY315I, βY315D, and βY315V were essentially inactive. These results are interesting in view of the strongly inhibitory effect of a substitution of the neighboring residue, βGly314, by, e.g., a glutamic acid residue, on the transhydrogenase activity [Table I; see also Hu et al. (1992)]. Presumably, the inhibition is caused by a change of the βαβ fold of the adenosine nucleotide-binding fold of the NADP(H) site (see Discussion). Although site-specific substitutions at αTyr226, βTyr315, and βTyr431 had varying effects on the catalytic activity of the transhydrogenase, the incorporation of the two subunits into the membrane in these mutants was nearly identical, as evidenced by gel electrophoretic analysis of membrane proteins (not shown).

The apparent K_m values of the *E. coli* transhydrogenase for AcPyAD$^+$ and NADPH were determined for pDC21/TG1 (Table II). This plasmid/host cell was chosen rather than pSA2/JM109 because of the low wild-type background activity of the TG1 cells. The K_m values of all αTyr226 mutants for AcPyAD$^+$ were at least twice that of the control pDC21, whereas the corresponding values for NADPH were unchanged. Surprisingly, also the K_m of the βY431F mutant for AcPyAD$^+$ was increased with an unchanged value for NADPH. However, the triple mutant αY226F/βY315F/

Table II: Michaelis–Menten Constants of *E. coli* Transhydrogenase Mutants for AcPyAD$^+$ and NADPH, Respectively[a]

mutant	K_m(AcPyAD$^+$) (μM)	K_m(NADPH) (μM)
control (pDC21/TG1)	83	52
α-subunit, NAD(H)-binding region		
αY226H	250	46
αY226L	156	73
αY226F	252	80
β-subunit, NADP(H)-binding region		
βY431F	153	74
other		
αY226F/βY315F/βY431F (pSA2/JM109)	250	125

[a] The concentration of one substrate was varied in the presence of a fixed concentration of the second substrate, essentially as described in Materials and Methods, and the K_m values were calculated. The concentration of the fixed substrate was 1 mM AcPyAD$^+$ or 0.5 mM NADPH. Mutants which are not listed in the table either exhibited a too-low activity or gave nonlinear plots and thus unreliable constants. All mutants, except the triple-Phe mutant αY226F/βY315F/βY431F, were based on the pDC21/TG1 system.

Table III: Effects of Site-Specific Mutagenesis of Tyrosine Residues in NAD(P)-Binding Regions on the Proton-Pumping Activity of Membranes of pDC21/TG1 and pSA2/JM109 Cells[a]

mutant	activity (%) pDC21/TG1	pSA2/JM109
control	100	100
α-subunit, NAD(H)-binding region		
αY226H	28	42
αY226L	13	
αY226F	31	
αY226N		51
β-subunit, NADP(H)-binding region		
βY315F		60
βY315H		63
βY315N		20
βY431H	11	
βY431L	8	
βY431F	19	113
others		
βG314E (RH1)	0	

[a] Activities are specific proton-pumping activities expressed as percent of the activity of membranes of pDC21/TG1 or pSA2/JM109 cells. Measurements were made in duplicate. Conditions were as described in Materials and Methods.

βY431F showed pronounced increases in the K_m of both substrates. As expected, the V_{max} values were close to those shown in Table I (not shown).

As shown in Table III, using the pDC21/TG1 or the pSA2/JM109 system, most mutant *E. coli* transhydrogenases pump protons, as indicated by quenching of the fluorescence of the transmembrane pH probe ACMA or quinacrine, respectively. In general, the proton-pumping activities were related to the specific catalytic activities, and identical mutants expressed in different host cells showed similar activities. However, the leucine mutant αY226L and the phenylalanine mutant βY431F showed considerably lower proton-pumping activities compared to the corresponding catalytic activities (cf. Table I). Also, for unknown reasons, the βY431F mutant expressed in JM109 cells gave much higher proton-pumping activity than that expressed in TG1 cells. Quenching of ACMA can normally not be used to determine proton pumping quantitatively, but is essentially a qualitative method. Nevertheless, these results show that the transhydrogenase mutants produced with the pDC21/TG1 system do incorporate properly in the membrane, as has been shown previously with pDC21 (Clarke & Bragg, 1985a), and that they pump protons.

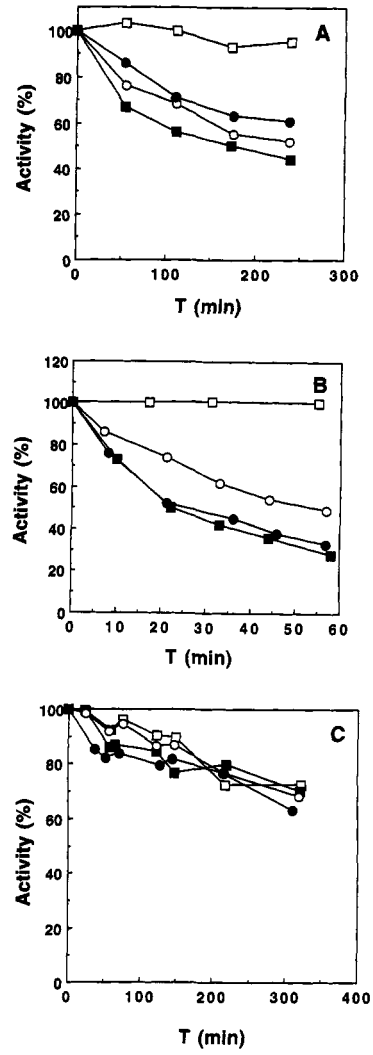

FIGURE 3: Inhibition of the transhydrogenase mutants αY226F (A), βY431F (B), and αY226F/βY315F/βY431F (C) by FSBA in the absence and in the presence of substrates. Membrane vesicles isolated from αY226F/TG1, βY431F/TG1, and αY226F/βY315F/βY431F/JM109 cells were incubated with 1 mM FSBA at room temperature for the times indicated, as described in Materials and Methods. Additions were (□) 1% DMSO; (■) 1 mM FSBA and 1% DMSO; (○) 1 mM FSBA, 1% DMSO, and 2 mM AcPyAD$^+$; and (●) 1 mM FSBA, 1% DMSO, and 2 mM NADPH. One hundred percent (100%) activities of αY226F, βY431F, and αY226F/βY315F/βY431F correspond to those shown in Table I.

Figure 3 shows the sensitivities of the phenylalanine mutants of the transhydrogenase to FSBA in the absence and in the presence of the substrates AcPyAD$^+$ and NADPH. αY226F was inactivated relatively slowly by FSBA with about 70% activity remaining after 60 min, compared to less than 30% remaining activity in the control pDC21 transhydrogenase (cf. Figure 1). In addition, protection by AcPyAD$^+$ was less pronounced in the case of the αY226F mutant compared to control, and NADPH gave initially more than 50% protection compared to a smaller effect in the control (Figure 3A; cf. Figure 1). Similar results were obtained with αY226L and αY226H (not shown). On the other hand, the rate of inactivation of the βY431F mutant by FSBA was very similar to that of the control (Figure 3B; cf. Figure 1). In addition, this mutant showed up to 50% protection by AcPyAD$^+$ but no protection by NADPH (Figure 3B). Using pSA2/JM109, the sensitivity of the βY315F mutant to FSBA was essentially as that of wild-type transhydrogenase (not shown), and the triple mutant αY226F/βY315F/βY431F was essentially

insensitive to FSBA regardless of the presence of substrates, although AcPyAD$^+$ still had a tendency to protect in the latter case (Figure 3C). In a parallel set of experiments using pSA2/JM109 and a modified procedure, the effects of FSBA on the transhydrogenase activities of vesicles of the mutants βY315F and βY315F/βY431F were compared to wild type. The mutants βY315F and βY315F/βY431F behaved in a similar manner to wild-type enzyme, with NADH offering some protection against inactivation, whereas NADPH was ineffective. However, NADH offered a more extensive protection than AcPyAD$^+$ (not shown).

The β subunit of purified wild-type transhydrogenase digested with trypsin binds strongly to columns of NAD- and NADP-agarose, from which it can be eluted by application of NADH or NADPH (Tong et al., 1991; Ahmad et al., 1992). By this procedure the substrate-binding ability of the α and β subunits of the mutant transhydrogenases may be estimated. Purified mutant transhydrogenases (βY315F; βY315N; βY315I/βY431I) treated in this manner showed a strong binding of the β subunit to the NAD- and NADP-agarose columns, which could be eluted by NADH or NADPH (not shown). Thus, these mutations in the β subunit do not abolish the ability of the transhydrogenase to bind its substrate(s).

DISCUSSION

The present investigation shows that tyrosine residues α226 and β431 of *E. coli* transhydrogenase are essential neither for catalytic activity nor for proton pumping. This is particularly interesting with regard to proton pumping since these tyrosine residues have been considered previously as candidates for proton-carrying residues in the mechanism of transhydrogenase-catalyzed proton pumping (J. Rydström, unpublished). Tyrosine residue β315 is also not essential for catalytic activity or proton pumping. With respect to catalytic activity, the various mutants of the *E. coli* transhydrogenase, regardless of expression system, were ranked as αY226F > L = H > N and βY431F ≫ L = H = N = I. Mutants of the tyrosine residue β315 showed a ranking order of βY315F > N = H > L > D = I = V. These results suggest that the α226, β431, and β315 residues should be aromatic and sufficiently large and hydrophobic or hydrophilic, but not charged or small (aliphatic) and hydrophobic. Further, the results allow the conclusion that inactivation by FSBA is caused by the covalent incorporation of a bulky ligand, producing a sterically hindered transhydrogenase which is unable to function catalytically.

The apparent K_m values of pDC21/TG1 transhydrogenase for AcPyAD$^+$ and NADPH were 83 and 52 μM, respectively, i.e., somewhat higher than those published previously for pDC21/JM83 transhydrogenase (Clarke & Bragg, 1985a). The affinities of the mutant transhydrogenases for AcPyAD$^+$ and NADPH were altered essentially as predicted, considering that the mutations occurred in the substrate-binding regions. Apparent K_m values for AcPyAD$^+$ were increased at least twice for αTyr226 mutants and the triple mutant αY226F/βY315F/βY431F. The latter, but not the former, also showed an increased K_m for NADPH. Unexpectedly, the βTyr431 mutant, which would be expected to have an increased apparent K_m for NADPH but not for AcPyAD$^+$, behaved similarly to the αTyr226 mutants. This may be the result of the required close interaction between the binding sites of the two substrates. Large changes, i.e., 2-fold and above, in the apparent K_m values of mutant transhydrogenases are considered significant. However, more exact changes in K_m have to be quantitated using purified and reconstituted mutant transhydrogenases.

Protection by substrates was tested with AcPyAD$^+$ and NADPH, i.e., substrates which are used in the assay of transhydrogenase activity. Binding of these substrates, in contrast to NADP$^+$ and NADH, may be assumed to be efficient in order to give the observed high activity in control membrane vesicles. Both AcPyAD$^+$ and NADPH were protective in a manner which varied for the different mutants. Thus, AcPyAD$^+$ protected most efficiently the βY431F mutant and the pDC21-expressed enzyme against FSBA inactivation and gave a marginal protection of the αY226F mutant. NADPH provided little or no protection of the pDC21 enzyme or the βY431F mutant, but did protect the αY226F mutant partially. These patterns of inactivation of the transhydrogenase mutants in the absence and the presence of substrates are consistent with a high reactivity of αTyr226 with FSBA and a much lower reactivity of βTyr431 with FSBA. In addition, protection by substrates indicates that the αTyr226 and βTyr431 residues are indeed located in the NAD(H)- and NADP(H)-binding regions, respectively. However, the fact that αY226F still showed some AcPyAD$^+$-protectable inhibition by FSBA indicates that FSBA may react slowly with other residues in the NAD(H)-binding region. On the other hand, the lack of protection by NADPH in the βY431F mutant suggests that FSBA inhibition does not affect other sites in the NADP(H)-binding region, indicating that, e.g., βTyr315 is relatively inaccessible to FSBA; the latter was shown directly by incubating FSBA with the βY315F mutant. These conclusions are also supported by the results with the double mutant βY315F/βY431F, where NADH, but not NADPH, afforded protection against inactivation by FSBA. As expected, the rate of inhibition of the triple mutant αY226F/βY315F/βY431F in the absence and the presence of substrates was very low.

Earlier experiments with radiolabeled FSBA on the bovine transhydrogenase in the absence and the presence of substrates, followed by identification of labeled residues (Yamaguchi et al., 1988; Wakabayashi & Hatefi, 1987), showed that Tyr245 of the NAD(H)-binding region was much more reactive with FSBA than Tyr1006 of the NADP(H)-binding region; apparently, no significant reactivity was detected with Tyr890. The present finding that the order of reactivity of the corresponding tyrosine residues of *E. coli* transhydrogenase with FSBA is αTyr226, βTyr431, and βTyr315 is in complete agreement with the results of Hatefi and co-workers. However, there are some noticeable similarities between the bovine and *E. coli* enzymes with respect to effects of substrates. NADPH had a strongly stimulating effect on FSBA inactivation in the bovine enzyme, whereas NADP$^+$ was protective (Phelps & Hatefi, 1985). In the present investigation NADPH also showed a stimulating effect on the wild-type transhydrogenase. Both AcPyAD$^+$ and AcPyADH were protective with the bovine transhydrogenase, but much more so than with the *E. coli* transhydrogenase, at least in the case of AcPyAD$^+$.

One of the drawbacks with FSBA as a nucleotide-site-specific agent is that it is an adenosine derivative missing both a 5′- and a 2′-phosphate moiety which confers specificity to either the NAD(H) or the NADP(H) site. Therefore, unless a competing dinucleotide is present, FSBA can not distinguish between the NAD(H) and NADP(H) sites. Rydström and co-workers (Hu et al., 1992) attempted to solve this problem by using a light-activated 8-azido-AMP, a derivative of the known competitive and NAD(H)-site-specific inhibitor and derivative of NAD(H), adenosine 5′-phosphate (Rydström, 1972). At this time it was suggested that transhydrogenase probably only contains two substrate-binding sites per bovine monomer or *E. coli* α+β subunit, one for NAD(H) and one for NADP(H) (Hu et al., 1992), which was consistent with the steady-state kinetics (Rydström, 1977; Rydström et al.,

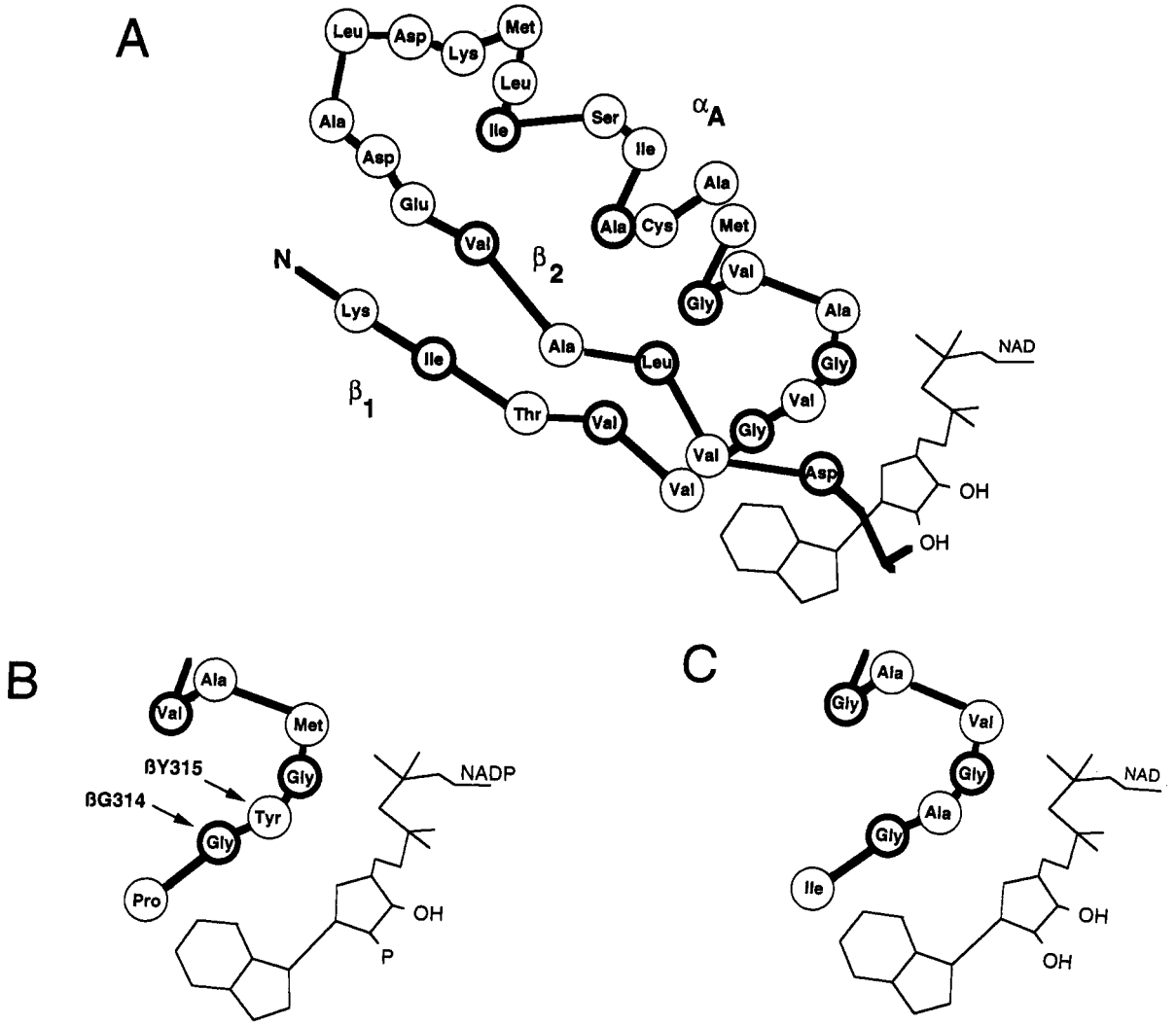

FIGURE 4: Models of adenine nucleotide-binding $\beta\alpha\beta$ folds of lactate dehydrogenase (A) and of the NADP(H)- (B) and the NAD(H)-binding site (C) of *E. coli* transhydrogenase. The model of the adenine nucleotide-binding domain (A) was based on the structure of dogfish muscle lactate dehydrogenase and was adapted from Brändén and Tooze (1991). In (B) and (C) are shown the GXGXXG/A sequences of the assumed NADP(H)- and NAD(H)-binding sites of *E. coli* transhydrogenase [cf. Hu et al. (1992)]. Conserved residues are shown by bold circles. Panels B and C show the sequences β313–319 and α171–177, respectively.

1987; Jackson, 1991). The basis for this suggestion was the occurrence of only two complete adenine nucleotide-like binding folds in both the bovine and *E. coli* enzymes, as deduced by a comparison with structurally known nucleotide-dependent enzymes (Hu et al., 1992). However, 8-azido-AMP proved to bind to Tyr1006 of the bovine enzyme, corresponding to βTyr431 in the *E. coli* transhydrogenase (Hu et al., 1992), which, like αTyr226, may be part of a possible nicotinamide nucleotide-binding region rather than an adenine nucleotide-binding region (Hu et al., 1992). This shows that an assumed site-specific labeling of transhydrogenase may be influenced by a variety of factors, leading to unexpected results. Similar conclusions have been reached with ATPases using FSBA, FSBI, 8-azido-ATP, and 2-azido-ATP (Esch & Allison, 1978; Hollemans et al., 1983; Colman, 1983; Jacobson & Colman, 1984; Bullough & Wilson, 1986; Cross et al., 1987). Labeling of βY331 of *E. coli* ATPase (corresponding to Tyr345 of bovine ATPase and βTyr431 of *E. coli* transhydrogenase) caused inactivation, but this tyrosine residue was recently shown not to be essential (Odaka et al., 1990; Wise, 1990; Weber et al., 1992).

The recent characterization of the sequence location and number of substrate-binding sites in the bovine transhydrogenase by Yamaguchi and Hatefi (1993) can be further substantiated by the calculation of a probability score for a certain amino acid sequence in the *E. coli* transhydrogenase as a binding sequence for the ADP moiety of NAD(H) (Wierenga et al., 1986). A score of 11 out of 11 possible was obtained for the sequence α167–195, i.e., sequence 1 according to Hu et al. (1992). A second sequence of the α subunit, α215–245, sequence 2 according to Hu et al. (1992), showed a score of only 6. Sequences close to the C-terminus of the β subunit, β309–351 and β423–461, i.e., sequences 3 and 4 of Hu et al. (1992), assumed to correspond to the NADP-(H)-binding region, were too different for a meaningful score to be calculated.

The available evidence thus suggests that, of the three tyrosine residues αTyr226, βTyr315, and βTyr431 studied in the present investigation, only βTyr315 constitutes part of a complete (adenine) nucleotide-binding $\beta\alpha\beta$ fold. It remains to establish whether αTyr226 and βTyr431 form part of another (nicotinamide) nucleotide fold. The βTyr315 residue is interesting because it is located next to the essential βGly314 residue forming part of the conserved GXGXXG/V sequence of the $\beta\alpha\beta$ fold, which is replaced by a glutamic acid residue in the inactive mutant RH1 (Ahmad et al., 1992b; Hanson & Rose, 1979). The fact that the latter mutant is inactive supports the assumption that βGly314 is indeed an essential component of a $\beta\alpha\beta$ fold. It is also clear that the tyrosine residue is not essential for substrate binding by the β subunit

since replacement of βTyr315 and/or βTyr431 does not prevent binding of this subunit to NAD- and NADP-agarose.

On the basis of the known structure of dogfish muscle lactate dehydrogenase shown in Figure 4A and the proposal that a larger hydrophobic residue than a glycine, i.e., an alanine, is required in GXGXXG/A for binding NADP(H) (Scrutton et al., 1990), the GXGXXG/V segments of the βαβ folds of the NADP(H) and NAD(H) sites of *E. coli* transhydrogenase may be modeled (Figure 4B and C), assuming that alanine can be replaced by a valine in the NADP(H) site.

In conclusion, replacement of the residues αTyr226, βTyr315, and βTyr431 of the *E. coli* transhydrogenase by other amino acids has been shown to produce mutant transhydrogenases which retain partially both catalytic and proton-pumping activity. These residues are therefore concluded not to be essential for activity.

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