Involvement of Histidine-91 of the β Subunit in Proton Translocation by the Pyridine Nucleotide Transhydrogenase of *Escherichia coli*†

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ABSTRACT: The pyridine nucleotide transhydrogenase (EC 1.6.1.1) carries out transmembrane proton translocation coupled to transfer of a hydride equivalent between NAD^+ and $NADP^+$. Mutations were made in histidine-91 of the β subunit of the pyridine nucleotide transhydrogenase of *Escherichia coli*. This amino acid is the only conserved charged residue in the transmembrane domains of this enzyme and thus potentially is involved in proton translocation by the transhydrogenase. The mutant βH91N retained 80% of the hydride transfer activity while proton translocation was reduced to 7%. This behavior is consistent with a role for βHis91 in the proton translocation pathway. Other mutations at this residue affected the conformation of the enzyme. Thus, the enzyme in mutants βH91C, βH91T, and βH91S was unable to undergo the conformational change that occurred on binding of the substrates $NADP^+$ or NADPH. By contrast, the enzyme in the βH91K mutant was present in the NADP(H)-induced conformation even in the absence of these substrates. Further evidence for the linkage between βHis91 and the conformation of the β subunit was obtained by labeling the transmembrane domain of the β subunit with [^{14}C]*N,N'*-dicyclohexylcarbodiimide (DCCD). Labeling occurred most readily with the enzyme of βH91K. It is concluded that βHis91 is a component of the proton translocation pathway of the transhydrogenase and that its state of protonation is probably linked to conformational changes induced by binding/debinding of substrates during the catalytic cycle of the enzyme.

Pyridine nucleotide transhydrogenase (EC 1.6.1.1) is a proton pump which catalyzes the reversible reaction

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

where reduction of $NADP^+$ by NADH is linked to an inward translocation of protons from the periplasm (in bacteria) or cytosol (mammalian cells) into the cytosol or mitochondrial matrix, respectively. In the presence of an electrochemical proton gradient generated by another proton pump, the apparent equilibrium of the reaction is strongly shifted toward formation of NADPH and NAD^+, and the rate of reduction of $NADP^+$ by NADH is increased 10-fold (Rydström, 1977; Fisher & Earle, 1982; Rydström et al., 1984, 1987; Jackson, 1991; Hatefi & Yamaguchi, 1992).

The transhydrogenase of *Escherichia coli* has been extensively investigated (Ahmad et al., 1992a,b, 1993; Clarke & Bragg, 1985; Clarke et al., 1986; Glavas et al., 1993; Holmberg et al., 1994; Homyk & Bragg, 1979; Hou et al., 1990; Olausson et al., 1993; Tong et al., 1991). It is composed of two subunits, α (510 residues) and β (462 residues), organized as an $\alpha_2\beta_2$ dimer (Hou et al., 1990). The relative simplicity of this structure makes the enzyme an excellent system in which to study the mechanism of proton pumping. Studies of the topology of the enzyme in the membrane of *E. coli* have led to a model for the transmembrane region which is illustrated in Figure 1 (Tong et al., 1991; Holmberg et al., 1994). The C-terminal 100 residues of the α subunit are organized as four transmembrane α-helices. The transmembrane domain of the β subunit is composed of the N-terminal 260 residues with six transmembrane α-helices. A significant feature of these transmembrane domains is the paucity of charged groups which could carry protons across the membrane. Aspartic and glutamic acid residues have been implicated in this role in such well-characterized proton pumps as the bacterial reaction center, bacteriorhodopsin, cytochrome *bo*, and the F_1F_o ATPase (Paddock et al., 1994; Henderson et al., 1990; Khorana, 1993; Thomas et al., 1993; Fillingame, 1990).

We have shown previously by site-directed mutagenesis that of all the conserved charged groups in the transmembrane domain of the *E. coli* transhydrogenase, only βHis91 is important for the activity of the enzyme (Holmberg et al., 1994). This residue is located in a transmembrane α-helix. Only two other charged groups, βGlu85 and βGlu224, are found in transmembrane helices, but these are not conserved in the transhydrogenases of other organisms. In this paper, we further explore the role of βHis91 in the proton pumping transhydrogenase of *E. coli* using site-directed mutagenesis and covalent labeling with *N,N'*-dicyclohexylcarbodiimide. We show that βHis91 is a component of the proton translocation pathway and that its state of protonation is

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[1] Abbreviations: CAT-1, 4-(trimethylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; CAT-16, 4-(*N,N*-dimethyl-*N*-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; DCCD, *N,N'*-dicyclohexylcarbodiimide; 5-DSA, 5-doxylstearic acid; 7-DSA, 7-doxylstearic acid; 12-DSA, 12-doxylstearic acid; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NCD-4, *N*-cyclohexyl-*N'*-(4-dimethylaminonaphthyl)carbodiimide; SDS, sodium dodecyl sulfate; TCS, 3,3',4',5-tetrachlorosalicylanilide.

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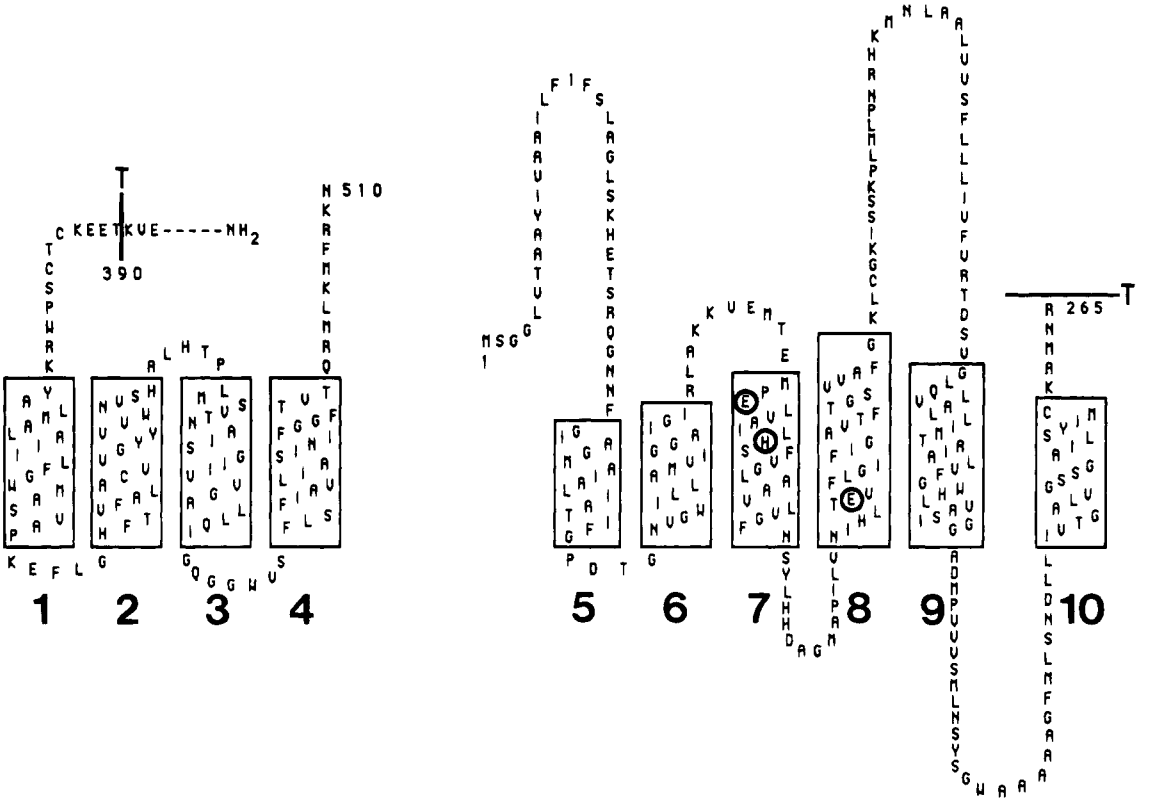

FIGURE 1: Model of the distribution of amino acid residues in the transmembrane α-helices of the α and β subunits of the *E. coli* transhydrogenase (Holmberg et al., 1994). βGlu85, βHis91, and βGlu124 are circled. T, trypsin cleavage site.

probably linked to conformational changes induced by binding/debinding of substrates during the catalytic cycle of the enzyme.

MATERIALS AND METHODS

Materials. Materials were obtained from the following suppliers: (Sigma) trypsin (bovine pancreatic), soybean trypsin inhibitor; (Bio-Rad) electrophoresis reagents; (Pharmacia) Sephadex G50 fine, electrophoresis low molecular weight standards; (Nutritional Biochemicals Corporation) quinacrine; (Fisher Scientific) TCS;[1] (Amersham) [^{14}C]-DCCD (50 μCi, 1 mL, 54 mCi/mmol); (Molecular Probes) NCD-4, 5-DSA, 7-DSA, 12-DSA, CAT-1, CAT-16.

Preparation of Membrane Vesicles Containing Transhydrogenase. JM109 cells containing the multicopy plasmid pSA2 (Ahmad et al., 1992b), or a mutant plasmid which also encodes the transhydrogenase genes, were grown in a medium of 1% bactotryptone, 0.5% yeast extract, 1% NaCl, 50 μg/mL proline, and 0.1 mg/mL ampicillin. The cultures were shaken at 37 °C for 16 h at 250 rpm in a New Brunswick Scientific Controlled Environment Incubator Shaker. The cell cultures were harvested by centrifugation at 4400*g* for 20 min. The cell pellets were washed by resuspension in 0.9% NaCl followed by centrifugation at 12000*g* for 15 min. Cell pellets were resuspended in buffer A (50 mM Tris-HCl, pH 7.8, 1 mM DTT, 1 mM EDTA) at 1 g wet weight/5 mL. All steps were performed at 0−4 °C. The cells were lysed by passage through an AMINCO French Pressure Cell at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at 12000*g* for 10 min. The supernatant was centrifuged at 252000*g* for 2 h, and the membrane pellet containing everted membrane vesicles was suspended in buffer A at 1 g wet weight/5 mL.

Washing of Membrane Vesicles. The membrane vesicles were further purified by detergent extraction. Although approximately 70% of the transhydrogenase is solubilized by this procedure, the membrane vesicles are depleted of other proteins which interfere during the evaluation of the results of trypsin digestion. The membrane vesicles show unimpaired energy-coupled reactions (e.g., energy-dependent transhydrogenation, NADH oxidation- and ATP hydrolysis-driven proton translocation). Membrane vesicles (1.5 mL) were layered on a 6 mL sucrose cushion [45% sucrose (w/w) in buffer A] and centrifuged in a Beckman Type 65 fixed angle rotor at 40000 rpm (139000*g*) for 1 h. The outer membrane fraction pelleted to the bottom of the tube and was discarded while the everted membrane vesicles banded at the interface and were removed by a syringe. The vesicles were diluted 2-fold with buffer A. Triton X-100 was added to 1% (w/v) and the membranes were stirred at 0 °C for 5 min and then centrifuged at 218000*g* for 1 h. The pellet was resuspended in buffer A, and sodium cholate was added to 50 mM. The mixture was stirred at 0 °C for 5 min and then centrifuged at 218000*g* for 1 h. The washed membrane pellet was suspended in buffer A.

Assay of Transhydrogenase Catalytic (Hydride Equivalent Transfer) Activity. The assay was performed at ambient temperature (20 °C) as described by Clarke and Bragg (1985). The assay medium was 50 mM sodium phosphate pH 7 buffer, 2 mM DTT, 0.5 mM EDTA, and 0.01% (w/v) Brij 35. 3-Acetylpyridine-NAD$^+$ (AcNAD$^+$) was added to 1 mM, and NADPH was added to 0.5 mM. Reduction of AcNAD$^+$ was measured as an increase of absorbance at 375 nm which was followed with a Perkin-Elmer Lambda 3A UV/Vis spectrophotometer. The absorbance maximum of AcNADH is 363 nm; therefore, by monitoring the reaction

at 375 nm, there is little contribution from NADH or NADPH. The extinction coefficient of 5.1 mM^{-1} cm^{-1} was used to calculate specific activity (units/mg) where 1 unit equals the conversion of 1 μmol of $AcNAD^+$ to AcNADH per minute.

Energy-Linked Assay of Transhydrogenase Catalytic Activity. This assay was performed at ambient temperature (20 °C) as outlined by Fisher and Sanadi (1971). Approximately 100 μg of unwashed everted membrane vesicles were taken up in 1 mL total volume of 50 mM Tris-HCl, pH 7.8, 10 mM $MgSO_4$, 1 mM DTT, and 0.25 M sucrose containing 1% (v/v) ethanol, 200 μg yeast alcohol dehydrogenase, and 0.068 mM NAD^+. Absorbance changes were followed at 340 nm. After 1 min, 0.78 mM $NADP^+$ was added to give the NADH oxidation-driven rate of transhydrogenation. When the dissolved O_2 was exhausted, the slope decreased and leveled off giving the energy-independent rate of transhydrogenation. Then 0.65 mM ATP, pH 7, was added, increasing the slope to give the rate of ATP-driven transhydrogenation. The extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate specific activity (units/mg) where 1 unit equals the conversion of 1 μmol of $NADP^+$ to NADPH per minute.

Proton Translocation Assays. Proton translocation was measured at ambient temperature (20 °C) as the quenching of the fluorescence of quinacrine (excitation 430 nm and emission 505 nm) with a Turner model 430 spectrofluorometer. The assay medium consisted of 10 mM HEPES/KOH, pH 7.5, 0.3 M KCl, 5 mM $MgCl_2$, and 50−100 μg of membranes in a 2 mL volume. The fluorescence baseline was established by adding 2.5 μM quinacrine. The fluorescence was quenched when 0.5 mM each of NADPH and $AcNAD^+$ were added. Fluorescence was reestablished by addition to 2.5 μM of the uncoupler TCS. Proton translocation was calculated as the percentage of quenching (change in fluorescence after TCS addition divided by the total fluorescence). Energy-linked proton translocation was also followed using the same assay medium to which 1 mM ATP was added to set up a proton gradient measured by the quenching of the fluorescence of quinacrine. Fluorescence was reestablished when 0.5 mM each of $NADP^+$ and NADH were added to consume protons in the energy-linked reaction.

Protein Determination. Protein content was measured by the method of Lowry et al. (1951) using 0−100 μg of bovine serum albumin to construct a standard curve. Absorbance was measured at 500 nm.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. This was performed by the method of Laemmli (1970) using a Bio-Rad mini-protean II gel apparatus. Gel concentration was 12%. The gels were stained with Coomassie blue (Fairbanks et al., 1971). Pharmacia low molecular weight standards were used containing phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Trypsin Digestion of Transhydrogenase. Membrane vesicles (1 mg/mL) were digested at ambient temperature (20 °C) with trypsin at a 1:100 (w/w trypsin/transhydrogenase ratio for 30 min in the absence or presence of 0.5 mM of NAD^+, NADH, $NADP^+$, or NADPH. The reaction was stopped by the addition of soybean trypsin inhibitor (trypsin inhibitor/trypsin = 2:1 weight ratio).

Modification of Transhydrogenase by [^{14}C]DCCD. Washed membranes (1 mg/mL) were treated at ambient temperature (20 °C) with 0.1 mM [^{14}C]DCCD (specific activity 54 μCi/mmol) for 1.25 h. The reaction was terminated by centrifugation through 1 mL columns of G50 Sephadex. The transhydrogenase was either left undigested or was digested with trypsin at a trypsin/transhydrogenase weight ratio of 1:100 for 30 min in the presence of 0.5 mM NADPH. Trypsin digestion was terminated by addition of trypsin inhibitor (weight ratio of inhibitor/trypsin = 2:1). Samples were examined by SDS−polyacrylamide gel electrophoresis, followed by staining and autoradiography using Kodak XAR5 film. The film was exposed to the gel for 5 days.

Labeling of Transhydrogenase with NCD-4. Washed membranes (1 mg/mL) were incubated at ambient temperature (20 °C) with 0.5 mM NCD-4 [final concentration of ethanol = 2% (w/v)]. At timed intervals, aliquots were removed and hydride transfer activities were measured.

Washed membranes (1 mg/mL) in buffer A were also labeled with 0.5 mM NCD-4 in the absence or presence of 0.5 mM NAD^+, NADH, $NADP^+$, or NADPH for 5 h at room temperature. After labeling, transhydrogenase was left undigested or was digested with trypsin (1:100 trypsin/transhydrogenase weight ratio) in the presence of 0.5 mM NADPH for 30 min. Digestion was stopped with trypsin inhibitor (inhibitor/trypsin = 2:1 weight ratio). The samples were examined by SDS polyacrylamide gel electrophoresis. The gels were fixed in 40% methanol/10% acetic acid for 1 h and were photographed on a 254 nm UV light source. The gels were then stained with Coomassie blue and destained as usual.

Interaction of NCD-4 Labeled Transhydrogenase with Spin Labels. Washed membranes (4 mg/mL) in buffer A were labeled with 4 mM NCD-4 overnight at 4 °C. The reaction was stopped by removing excess NCD-4 by centrifugation through columns of Sephadex G-50. Fluorescence excitation and emission spectra for the labeled membranes were determined. Maximum fluorescence was observed at an excitation wavelength of 320 nm and an emission wavelength of 460 nm. Fluorescence was measured with a SLM Aminco SPF-500C spectrofluorometer with 200 μg of membranes in 2 mL of a buffer of 10 mM HEPES-KOH, pH 7.5, 0.3 M KCl, and 5 mM $MgCl_2$. The spin labels were dissolved in ethanol to give the following stock solutions: 20 mM 5-DSA, 20 mM 7-DSA, 20 mM 12-DSA, 20 mM CAT-16, and 50 mM CAT-1 (in 50% ethanol). These spin probes were added to the reaction mixtures to give final concentrations of 10, 30, 50, 70, and 100 μM, respectively. The final fluorescence values were measured after a 5 min incubation at room temperature.

RESULTS

Properties of Mutants of βHis91. Figure 1 shows the predicted amino acid sequence and arrangement of the transmembrane regions of the α and β subunits of the *E. coli* transhydrogenase (Holmberg et al., 1994). These sequences are notable for the paucity of protonatable groups in the transmembrane helices. Site-directed mutagenesis identified βHis91, located near the centre of helix 7, as an important residue for catalytic activity. Conversion to serine, threonine, or cysteine resulted in loss of catalytic activity and associated proton pumping. The result of further

Table 1: Catalytic and Proton Pumping Activities of the Membranes of the βH91 Mutants[a]

mutant	% catalytic activity of membranes	% catalytic activity of washed membranes	% proton pumping activity
control	100	100	100
βH91S	5	19	11
βH91T	3	11	8
βH91C	3	12	7
βH91K	1	4	20
βH91N	21	80	7
βH91D	9	15	9

[a] Catalytic activities are specific activities of hydride transfer expressed as a percentage of the activity of control membranes or washed membranes where 100% activity of membranes = 5.0 μmol/(min·mg) and 100% activity of washed membranes = 2.7 μmol/(min·mg of protein). Proton pumping activities are expressed as a percentage of the quenching in control membranes where 100% proton pumping activity = 89.6% quenching/100 μg of protein. The assays were carried out as described in Materials and Methods.

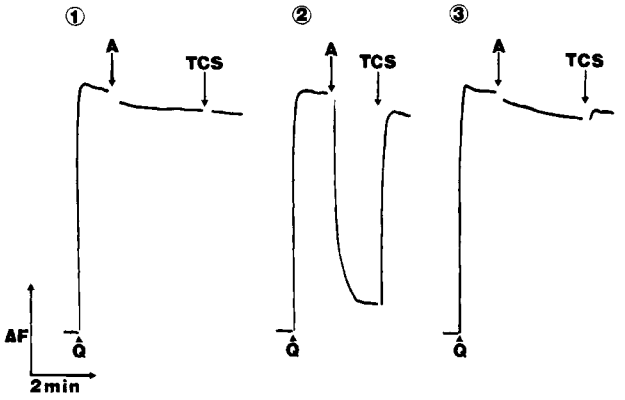

FIGURE 2: Quinacrine assay of proton pumping during hydride transfer from NADPH to AcNAD$^+$ by washed membrane vesicles of JM109 (1) or JM109 transformed with plasmid containing genes for normal (2) or βH91N mutant (3) transhydrogenases. The assay was performed as described in Materials and Methods using 100 μg of membrane protein. Q, addition of 2.5 μM quinacrine; TCS, addition of 2.5 μM TCS; A, addition of 0.5 mM AcNAD$^+$. ΔF, fluorescence intensity in arbitrary units.

substitutions for βHis91 are shown in Table 1. Representative assays of proton pumping are shown in Figure 2. The contribution to proton pumping by the chromosomally-encoded transhydrogenase was negligible under the conditions of our assay. The contribution to hydride transfer was 2% at most. Conversion of this βHis91 to lysine and aspartic acid caused drastic loss in both catalytic and proton pumping activities. However, catalytic activity was largely retained in the βH91N mutant in which about 80% of the control hydride transfer activity was retained by washed membrane vesicles. Significantly, proton translocation was reduced by 93% suggesting that βHis91 has a role in proton pumping by the transhydrogenase.

The effect of mutation of βHis91 on energy-dependent transhydrogenase activity was examined. In energy-linked transhydrogenation, the proton electrochemical gradient established by substrate oxidation through the respiratory chain or by hydrolysis of ATP by the ATP synthase is utilized to displace the equilibrium between the pyridine nucleotides to result in net formation of NADPH (Bragg et al., 1972). Mutation to asparagine or lysine completely abolished NADH oxidation-driven and ATP-driven energy-linked transhydrogenase activities.

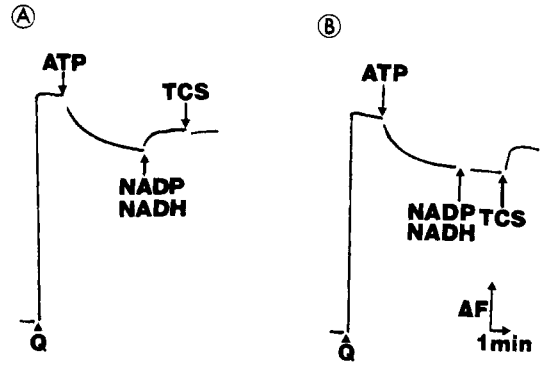

FIGURE 3: Utilization by transhydrogenase of proton gradient established by ATP hydrolysis in control and mutant membranes. The assay was performed as described in Materials and Methods using 200 μg of membranes containing wild-type (A) or βH91N mutant (B) transhydrogenases. Q, addition of 2.5 μM quinacrine. ΔF, fluorescence intensity in arbitrary units.

The proton gradient established by ATP hydrolysis in everted membrane vesicles can be measured qualitatively using quinacrine. Acidification of the vesicle interior by proton translocation results in reequilibration of the quinacrine across the vesicle membrane with quenching of fluorescence of the dye. As shown in Figure 3, addition of NADP$^+$ and NADH restore fluorescence as intravesicular protons are used to drive the reduction of NADP$^+$ by NADH. This reaction is absent in the βH91N mutant, although reequilibration of protons across the membrane can be brought about by the addition of the uncoupler TCS.

Thus, the ability to use a preformed proton gradient, as well as to pump protons across the membrane, is greatly abolished or impaired on substitution of asparagine for βHis91, yet with retention of hydride transfer catalytic activity. This behavior is similar to the classical property of uncoupling, e.g., of ATP formation from substrate oxidation in oxidative phosphorylation. Thus, βH91N appears to be an uncoupled mutant. Furthermore, the ability of ATP hydrolysis to establish a proton gradient in vesicles of βH91N (Figure 3) indicates that the loss of proton translocation was not due to leakage of protons across the membrane caused by the mutant protein.

Trypsin Digestion Patterns of Mutant Transhydrogenases. Trypsin digestion of transhydrogenase has proved to be a useful technique to examine the conformational state of the enzyme. Digestion of nonmutant enzyme yields a defined series of peptides originating from the α subunit. The β subunit is resistant to digestion in the absence of pyridine nucleotides and in the presence of NAD$^+$ or NADH but is cleaved at βArg265 near the C-terminal end of the transmembrane domain (Figure 1) if digestion is carried out in the presence of NADP$^+$ or NADPH (Tong et al., 1991). Mutation at βGly314 prevents the cleavage of β (Ahmad et al., 1992b) whereas cleavage will occur in the absence of added nucleotide in certain mutant enzymes from which a few amino acids have been removed from the C-terminal end of the α subunit (Ahmad et al., 1993). Washed membranes of mutants at βHis91 were treated with trypsin in the absence or presence of pyridine nucleotide substrates. The cleavage pattern was examined by SDS−polyacrylamide gel electrophoresis. Figure 4 shows that mutation of βHis91 to serine, threonine, cysteine, lysine, or asparagine did not affect incorporation of the subunit of the enzyme into the membrane. Panel H shows the resistance to cleavage of the

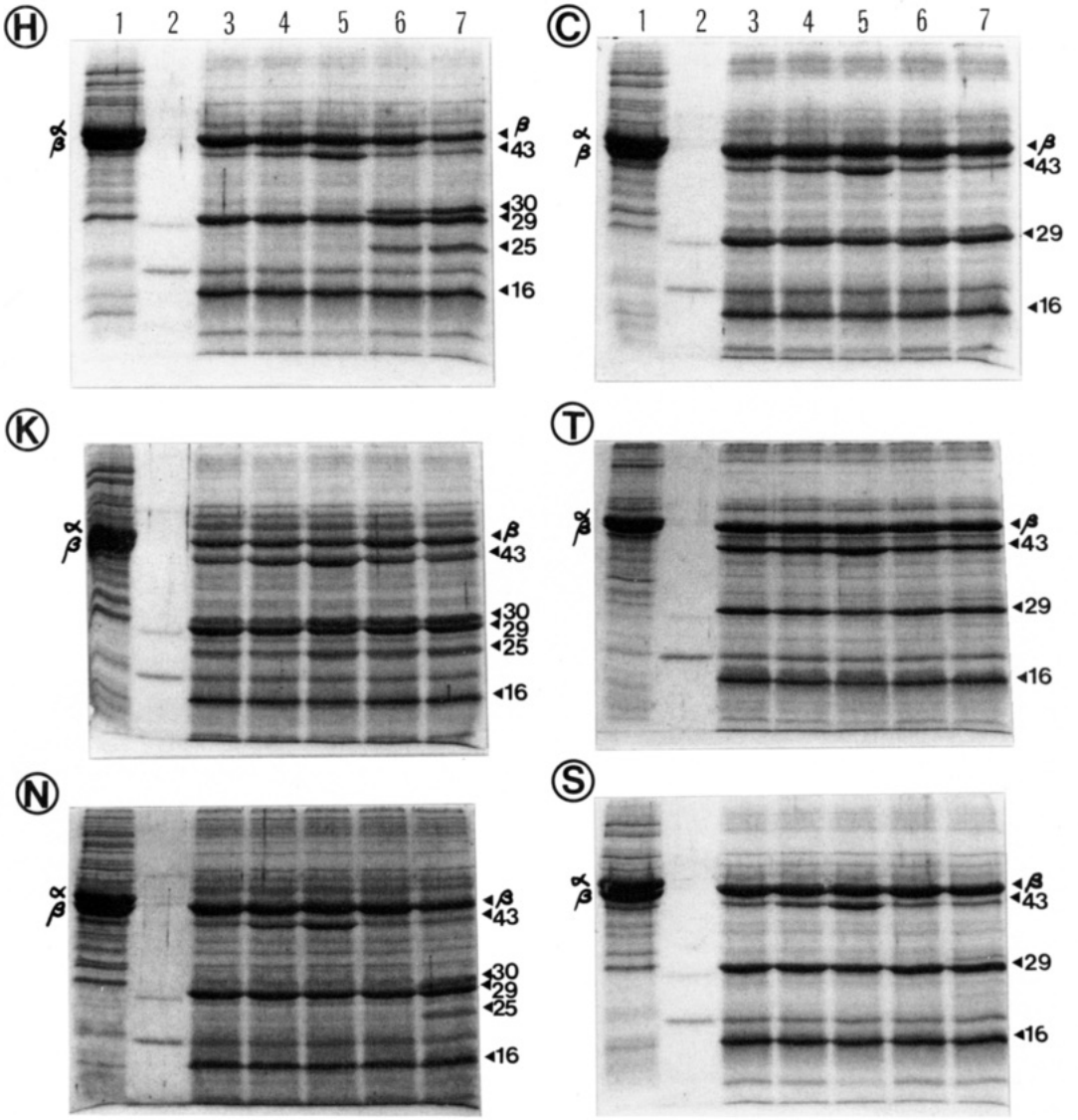

FIGURE 4: Effect of substrates on the trypsin digestion of the βHis91 mutants. Washed membrane preparations (1 mg/mL in buffer A) of wild-type transhydrogenase βH91 or the βH91C, βH91K, βH91T, βH91N, or βH91S mutants were digested with trypsin at a ratio of 1:100 (w/w) trypsin/transhydrogenase for 30 min in the 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 stopped by addition of twice the trypsin weight of soybean trypsin inhibitor. Samples were examined by SDS−polyacrylamide gel electrophoresis. Lane 1, undigested transhydrogenase; lane 2, trypsin and trypsin inhibitor control. The subunits and digestion fragments (in kDa) are indicated.

β subunit of the nonmutant enzyme in the absence of $NADP^+$ or NADPH and its cleavage to 30 and 25 kDa fragments occurring in the presence of these pyridine nucleotides. The 43 kDa polypeptide was the initial cleavage product of the α subunit. The 16 and 29 kDa polypeptides resulted from further cleavage of it. The transmembrane polypeptide of the α subunit was not readily detectable on SDS−polyacrylamide gels. The β subunit was not cleaved by trypsin in the presence of NADP or NADPH in the serine, threonine, and cysteine mutants (Figure 4, panels S, T, and C) or in the βH91D mutant (results not shown). By contrast, the β subunit of the βH91K mutant was susceptible to digestion even in the absence of pyridine nucleotide (panel K). The β subunit of the βH91N mutant showed significant cleavage only in the presence of NADPH. These results suggest that the nature of the group at βHis91 must profoundly affect the conformation of the β subunit to make the peptide bond at βArg265 more or less accessible to the action of trypsin.

Labeling of β Subunit by Carbodiimides. *N,N'*-Dicyclohexylcarbodiimide (DCCD) has been used as a useful probe to detect groups, usually glutamic or aspartic acid residues in nonpolar locations in the protein, involved in proton translocation pathways (Casey et al., 1980; Clejan & Beattie, 1983; Esposti et al., 1983; Senior & Wise, 1983). Treatment of the *E. coli* transhydrogenase with DCCD resulted in labeling of αAsp232, αGlu238, and αGlu240 (Glavas et al., 1993). The mitochondrial transhydrogenase was labeled at Glu257 (equivalent to *E. coli* αGlu238) (Yamaguchi et al., 1988; Wakabayashi & Hatefi, 1987). It is assumed that these sites are in proximity to the NAD^+ and NADH binding site of the transhydrogenase since NAD^+ or NADH protected the enzyme from labeling by DCCD, although it is conceivable that protection is afforded by a conformational change induced by substrate binding. A second site of labeling was detected in the transmembrane (25 kDa tryptic fragment) region of the β subunit of the *E. coli* transhydrogenase (Glavas et al., 1993). In spite of intense effort, we have not been able to characterize this site of labeling. The highly hydrophobic nature of the 25 kDa polypeptide and its cyanogen bromide or proteolytic (proteinase K, trypsin, V8

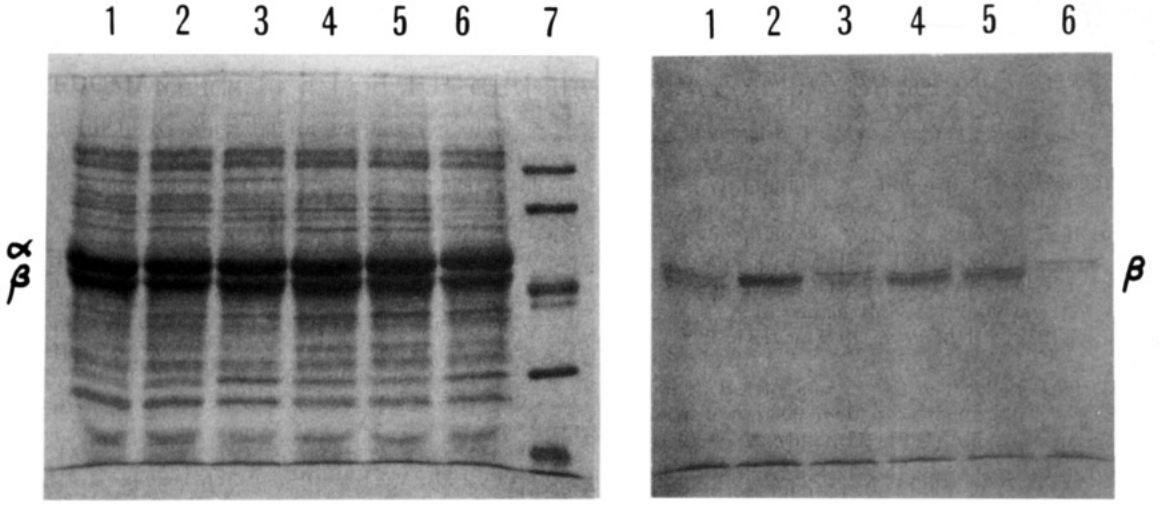

FIGURE 5: [^{14}C]DCCD labeling of washed membranes of βHis91 mutants. Washed membrane preparations (1 mg/mL in buffer A) were labeled with 0.1 mM [^{14}C]DCCD for 1.25 h. The reaction was stopped as described in Materials and Methods. Samples were examined by SDS−polyacrylamide gel electrophoresis on 8% gels. (Left panel) Coomassie blue stained gel; (right panel) radioautograph of stained gel. The position of migration of the β subunit is indicated. Lane 1, wild-type; lane 2, βH91K; lane 3, βH91N; lane 4, βH91S; lane 5, βH91T; lane 6, βH91C; lane 7, molecular mass markers (see Materials and Methods).

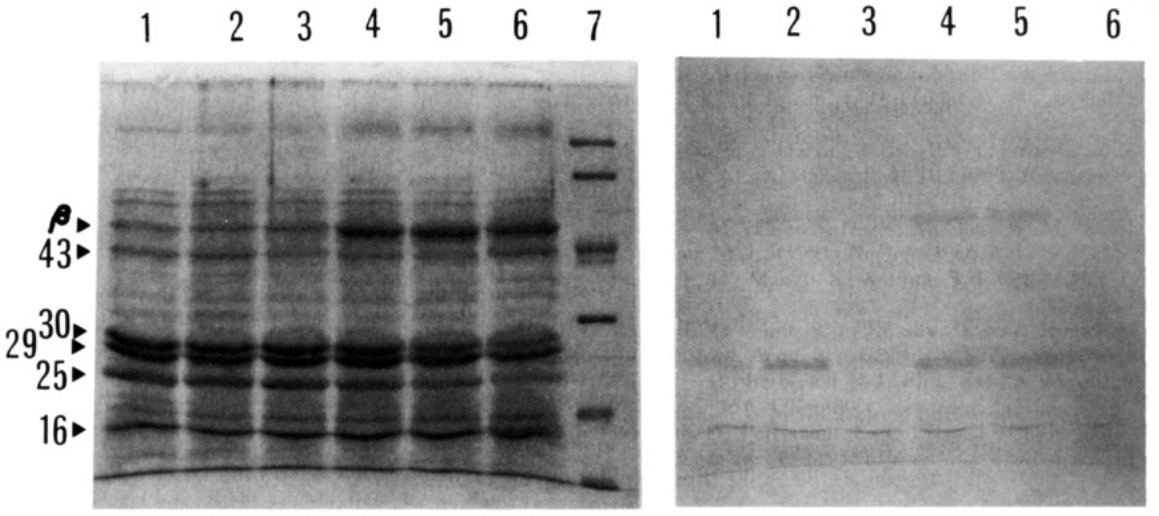

FIGURE 6: [^{14}C]DCCD labeling of tryptic fragments of βHis91 mutants. Washed membranes labeled with [^{14}C]DCCD were digested for 30 min with trypsin (trypsin/transhydrogenase, 1:100 weight ratio) in the presence of 0.5 mM NADPH. The reaction was terminated by addition of soybean trypsin inhibitor. Samples were examined by SDS−polyacrylamide gel electrophoresis on 12% gels. (Left panel) Coomassie blue stained gel; (right panel) radioautograph of stained gel. The position of migration of the β subunit and of the tryptic fragments of the α subunit (43, 29, 16 kDa) and β subunit (30, 25 kDa) are indicated. Lane 1, wild-type; lane 2, βH91K; lane 3, βH91N; lane 4, βH91S; lane 5, βH91T; lane 6, βH91C; lane 7, molecular mass markers (see Materials and Methods).

protease, pepsin, thermolysin, subtilisin) fragments interfered with conventional sequencing. The fragments adhered tightly to surfaces and were nonrecoverable.

Washed membranes of βHis91 mutants were treated with [^{14}C]DCCD and the constituent subunits were separated by SDS−polyacrylamide gel electrophoresis (Figure 5). The conditions for labeling were chosen such that the α and β subunits of the wild-type enzyme were labeled only to a low extent. [More usual levels of labeling are shown in Figures 1 and 2 of Glavas et al. (1993).] Figure 5 shows that [^{14}C]-DCCD labels the β subunit of βH91N (lane 3) and βH91C (lane 6) mutants to the same extent as the wild-type subunit (lane 1). Similar results were obtained with βH91D (not shown). The β subunits of βH91S (lane 4) and βH91T (lane 5), and especially of β91K (lane 2) are labeled to a greater extent than the wild-type subunit. The data of Figure 6 confirm the results of Figure 5 and show that the label is located in the 25 kDa transmembrane domain of the β subunit. In the mutants βH91S, βH91T, and βH91C, the β subunit is resistant to digestion by trypsin in the presence of NADPH, so the label remains in the β subunit. It can be concluded that mutation of βHis91 profoundly affects the conformation of the transmembrane domain of the β subunit of the *E. coli* transhydrogenase.

Site of Labeling of the β Subunit by DCCD. As stated above, the site of labeling of the β subunit could not be determined by sequencing. The following indirect approach was used to determine the possible site of reaction with DCCD.

N-Cyclohexyl-*N*′-[4-(dimethylamino)naphthyl]carbodiimide (NCD-4) is a fluorescent analog of DCCD which is known to react at the same site as DCCD in some proteins (Chadwick & Thomas, 1983; Pick & Weiss, 1985; Wang & Beattie, 1993). Figure 7 compares the inhibitory effect of 0.5 mM DCCD and 0.5 mM NCD-4 on the catalytic activity of nonmutant transhydrogenase. The extent of inhibition is

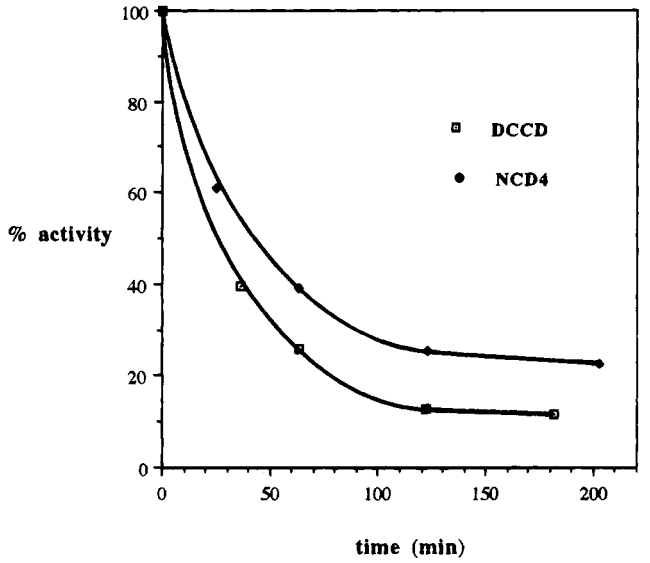

FIGURE 7: Inhibition of catalytic activities of transhydrogenase with NCD-4 and DCCD. Wild-type washed membranes (1 mg/mL) were incubated with either 0.5 mM NCD-4 or 0.5 mM DCCD. At timed intervals, aliquots were removed and catalytic activities measured as described in Materials and Methods. The values are expressed as percentage of control activity without inhibitor.

similar with both reagents. The labeled enzyme was examined by SDS−polyacrylamide gel electrophoresis. The β subunit was labeled as shown by the fluorescence of the label (Figure 8A). The label was located in the 25 kDa transmembrane domain as indicated by tryptic cleavage (Figure 8B).

In order to characterize the environment around the NCD-4 labeled group, we examined the effectiveness of a series of spin labels in quenching the fluorescence of this label. CAT-16 is a cationic amphiphilic spin label which partitions such that the polar group and the spin label are at the membrane surface. 5-DSA, 7-DSA, and 12-DSA are stearic acid molecules in which the doxyl spin label is linked at the indicated region of the chain. Thus, these spin labels probe into the lipid bilayer at different depths from the membrane surface.

Stern−Volmer or dynamic collisional quenching obeys the equation

$$I_o/I = 1 + K_D[Q]$$

where I_o and I are the fluorescence intensities in the absence and presence of quencher, [Q] is the concentration of the quencher, and K_D is the Stern−Volmer quenching constant (Lacowicz, 1983).

Figure 9A shows the effect of the spin labels on the fluorescence of the NCD-4 labeled transhydrogenase. These Stern−Volmer plots showed that the order of efficiency of quenching was 5-DSA > 7-DSA = CAT-16 > 12-DSA > CAT-1. The data for CAT-1 are not shown. This polar spin label gave extremely low levels of quenching at concentrations up to 1 mM. The curved nature of the Stern−Volmer plots suggest that a fraction of the fluorophore was inaccessible to the quencher. Therefore, the data were replotted according to the equation

$$I_o/(I_o - I) = 1/(f_a K[Q]) + 1/f_a$$

where K is the quenching constant of the accessible fraction and f_a is the fraction of the initial fluorescence accessible to quencher (Lacowicz, 1983). Straight lines were obtained (Figure 9B). A value of 59% was obtained for f_a. The K values were 0.091 μM (5-DSA), 0.049 μM (7-DSA), 0.033 μM (CAT-16), and 0.024 μM (12-DSA) giving the same order of quenching as in Figure 9A. The carboxyl group of 5-DSA is ionized and anchors the molecule at the membrane surface allowing the hydrocarbon chain to intercalate into the membrane lipid. The paramagnetic nitroxide group is located at a distance of about 6.25 Å from the membrane surface (Wang & Beattie, 1993). Thus, it is likely that NCD-4, and by analogy DCCD, labels a group at about 6 Å from the membrane surface. Since only the site of labeling in the 25 kDa region of the β subunit is within the membrane, the sites of labeling of the α subunit being extramembraneous, it is likely that only this site is responding to the spin label quenchers.

DISCUSSION

The predicted structure for the pyridine nucleotide transhydrogenase shows 10 transmembrane α-helices (Figure 1) (Holmberg et al., 1994). Four of these helices are found at the C-terminal region of the α subunit. The remaining six helices constitute the N-terminal domain of the β subunit. There is a notable lack of conserved protonatable residues in these regions which might be predicted to be involved in a proton pumping mechanism. Previously, we have mutated these conserved residues and have shown that only βHis91 is important for the activity of the transhydrogenase. We have now shown using a further set of mutants that this residue is likely involved in the proton pumping by the transhydrogenase. Thus, the mutant βH91N retains 80% of hydride transfer activity but only 7% of proton pumping activity. There are several possible explanations for the retention of some pumping activity by this mutant. It is difficult to determine the potential contribution to net proton pumping by the chromosomally encoded normal transhydrogenase in plasmid-containing strains, although it is almost undetectable in detergent-washed vesicles of nontransformed JM109 (Figure 2). It is also possible that other nearby residues could contribute to proton translocation even after mutation of βHis91. Thus, inhibition of proton pumping by mutation of Asp61 mutants of subunit c of the *E. coli* F_1F_o ATPase was relieved by substitution of Asp for Ala24 in an adjacent helix (Zhang & Fillingame, 1990). Furthermore, replacement of Asp96, a residue involved in the bacteriorhodopsin proton pump (Khorana, 1993), inhibited proton translocation by only 90% (Mogi et al., 1988). Finally, the quenching of quinacrine used to assay proton translocation is logarithmic rather than linear. Thus, although there is retention of some proton pumping activity, it is likely much lower on a linear scale than the presence of 7% residual quenching might indicate.

It is particularly interesting that changes at βHis91 can affect the conformation of the β subunit. We have previously shown that a conformational change in this subunit is induced by binding of $NADP^+$ or NADPH, one of the pyridine nucleotide substrates of the transhydrogenase (Tong et al., 1991). The conformational change was detected by the exposure of the peptide bond at βArg265 to cleavage by trypsin. Three types of response to trypsin were detected in

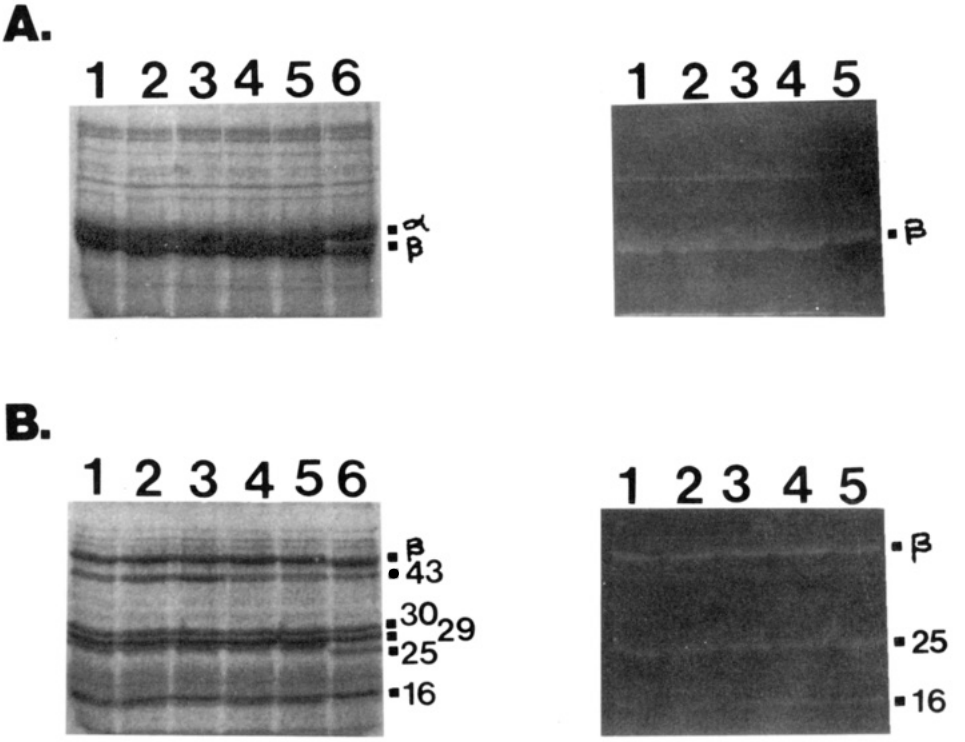

FIGURE 8: NCD-4 labeling of undigested (A) or trypsin-digested (B) transhydrogenase. Washed membranes (1 mg/mL in buffer A) were labeled with 0.5 mM NCD-4 in the absence (lane 1) or presence of 0.5 mM NAD^+ (lane 2), NADH (lane 3), $NADP^+$ (lane 4), or NADPH (lane 5) for 5 h at room temperature. Undigested samples were examined by SDS−polyacrylamide gel electrophoresis on 8% gels (A). Samples were also digested with trypsin for 30 min at a trypsin/transhydrogenase ratio of 1:100 (w/w) in the presence of 0.5 mM NADPH. Digestions were stopped with twice the trypsin weight of soybean trypsin inhibitor, and samples were then applied to 12% SDS−polyacrylamide gels (B). The gels were photographed under 254 nm UV light before staining and destaining as usual. Stained gel, left. Unstained gel photographed under UV light, right. Lane 6, unlabeled transhydrogenase. The positions of the subunits and fragments (in kDa) are indicated.

the βHis91 mutants (Figure 4). Thus, βH91C, βH91S, and βH91T did not undergo the NADP(H)-induced conformational change. By contrast, the βH91K mutant adopted the trypsin-sensitive conformation even in the absence of added NADP(H) or other pyridine nucleotides. However, we have recently shown that bound $NADP^+$ is present in this mutant enzyme (N. A. Glavas and P. D. Bragg, unpublished data). Last, the βH91N mutant, like the wild-type enzyme, underwent the conformational change in the presence of NADPH. This last result supports the view that βH91 is a proton translocating group and that the lack of proton pumping is not a consequence of the blocking of a substrate binding-induced conformational change necessary for proton pumping.

There is relatively little information on the role of specific amino acid residues in proton pumping enzymes. Well-characterized systems are bacteriorhodopsin, F_1F_o ATPase, reaction center of *Rhodobacter sphaeroides*, and cytochrome *bo*. In these systems, aspartic acid residues are believed to play a primary role in the proton pathway (Henderson, 1990; Khorana, 1993; Fillingame, 1990; Paddock et al., 1994; Thomas et al., 1993). However, there is some evidence that His245 of subunit a of the F_1F_o ATPase and His257 of the tetracycline carrier of *E. coli* are involved in proton translocation (Cain & Simoni, 1988; Yamaguchi et al., 1991). Since acidic residues in proton pumping systems are targets for covalent modification by DCCD, modification of the transmembrane region of the β subunit by [^{14}C]DCCD is of interest. Mutation at βHis91 clearly affected the ease with which DCCD modified the target group. This was particularly evident in the increased rate of modification in the βH91K mutant. As discussed above, the enzyme in this mutant already seems to be in the conformation normally induced by $NADP^+$ or NADPH since the enzyme contains bound $NADP^+$. It is of interest that addition of these pyridine nucleotides also increase the rate of DCCD modification of the β subunit of the wild-type enzyme [see Figure 1 of Glavas et al. (1993)].

Thus, the ease of modification of the β subunit by DCCD is a further probe of the conformational change occurring during the reaction cycle of the transhydrogenase. The group in the transmembrane region of the β subunit that is modified by DCCD has not been unambiguously identified due to technical problems in the protein sequencing of this very hydrophobic region. However, quenching of the fluorescence of NCD-4 labeled β subunit by 5-doxylstearic acid suggests that the modified group is about 6 Å below the membrane surface. There are only two acidic residues, βGlu85 and βGlu124, which are within the transmembrane region. They are not conserved within the transhydrogenase family (Holmberg et al., 1994). Both residues are at the appropriate distance from the membrane surface indicated by the spin probe. However, it is unlikely that any labeled βGlu124 would be detected by the spin probes since these were added to *everted* membrane vesicles. Any label at βGlu124 would be inaccessible to the probes. It is of interest that βGlu85 is in the same transmembrane helix as βHis91. Presumably, the conformational changes induced by NADP(H) and affecting the state of protonation of βHis91, or by the βH91K mutation, would also affect the accessibility of βGlu85 to modification by DCCD.

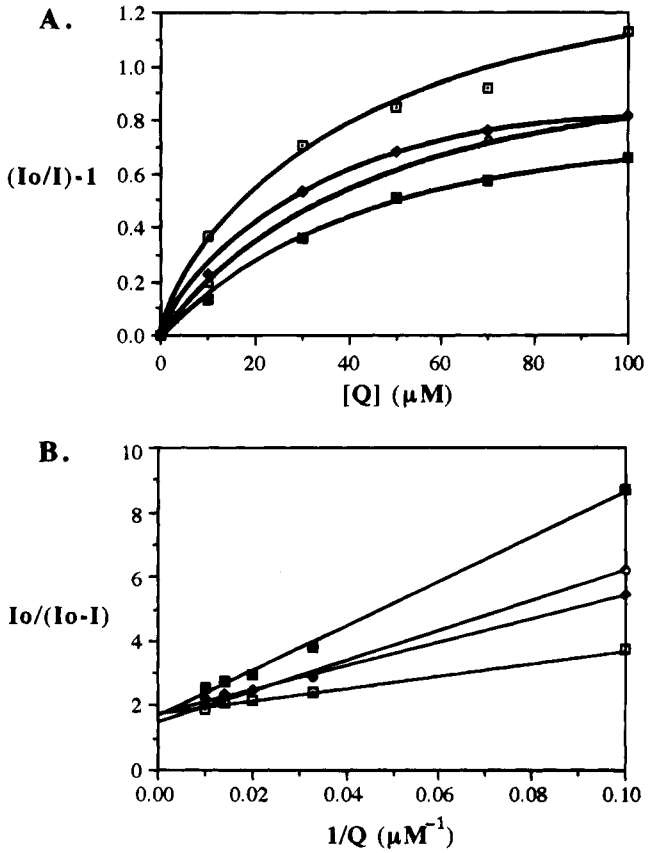

FIGURE 9: Interaction of NCD-4 labeled transhydrogenase with spin labels. Wild-type washed membranes (4 mg/mL) were labeled with 4 mM NCD-4 overnight at 4 °C. Excess NCD-4 was removed through G50 Sephadex. Fluorescence intensity of 200 μg of labeled membrane was measured in the absence (I_o) or presence (I) of varying concentrations of the following spin labels: 5-DSA (□), 7-DSA (◆), 12-DSA (■), and CAT-16 (○) as outlined in Materials and Methods. (A) Stern–Volmer plot; (B) data replotted according to the relationship $I_o/(I_o - I) = 1/(f_a K[Q]) + 1/f_a$.

In conclusion, βHis91 is situated in a critical region of the transhydrogenase molecule which is likely to respond to conformational changes induced by binding of substrate. It is probable that βHis91, as a component of the transmembrane proton pump, undergoes reversible protonation/deprotonation coupled to hydride transfer between the pyridine nucleotide substrates.

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