Essential Glycine in the Proton Channel of
Escherichia coli Transhydrogenase*

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The nicotinamide nucleotide transhydrogenases of mitochondria and bacteria are proton pumps that couple hydride ion transfer between NAD(H) and NADP(H) bound, respectively, to extramembranous domains I and III, to proton translocation by the membrane-intercalated domain II. Previous experiments have established the involvement of three conserved domain II residues in the proton pumping function of the enzyme: His°91, Ser°139, and Asn°222, located on helices 9, 10, and 13, respectively. Eight highly conserved domain II glycines in helices 9, 10, 13, and 14 were mutated to alanine, and the mutant enzymes were assayed for hydride transfer between domains I and III and for proton translocation by domain II. One of the glycines on helix 14, Gly°252, was further mutated to Cys, Ser, Thr, and Val, expression levels of the mutant enzymes were evaluated, and each was purified and assayed. The results show that Gly°252 is essential for function and support a model for the proton channel composed of helices 9, 10, 13, and 14. Gly°252 would allow spatial proximity of His°91, Ser°139, and Asn°222 for proton conductance within the channel. Gly°252 mutants are distinguished by high levels of cyclic transhydrogenation activity in the absence of added NADP(H) and by complete lack of proton pumping activity. The purified G252A mutant has <1% proton translocation and reverse transhydrogenation activity, retains 0.9 mol of NADP(H) per domain III, and has 96% intrinsic cyclic transhydrogenase activity, which does not exceed 100% on the addition of NADP(H). These properties imply that Gly°252 mutants exhibit a native-like domain II conformation while blocking proton translocation and coupled exchange of NADP(H) in domain III.

Nicotinamide nucleotide transhydrogenases (TH)† of mitochondria and microorganisms are membrane-intercalated enzymes that couple the transfer of a hydride ion between soluble domains to translocation of a proton through the integral membrane domain.

They catalyze the direct and stereospecific transfer of hydride between the 4A position of NAD(H) and the 4B position of NADP(H). The transhydrogenation reaction is coupled to transmembrane proton translocation with a $\text{H}^+/\text{H}^-$ stoichiometry of unity (Reaction 1) (1–3).

$$\text{NADH} + \text{NADP} + \text{H}^+ \rightleftharpoons \text{NAD} + \text{NADPH} + \text{H}^+$$

Reaction 1

In bovine mitochondria, the proton motive force accelerates the forward reaction 10–12-fold and shifts the equilibrium toward product formation. Due to this activity, a function of TH is to produce NADPH for reduction of toxic H$_2$O$_2$ by glutathione reductase and glutathione peroxidase. In the reverse direction, transhydrogenation from NADP(H) to NAD results in outward proton translocation and creation of a proton motive force. Because there is essentially no difference in the reduction potential of the nicotinamide cofactors, the driving force for the reverse reaction is the difference in binding affinities for substrates (NADPH and NAD) versus products (NADH and NADP). Consequently, TH provides a system in which to study the transformation of substrate binding energy into proton translocation.

The amino acid sequences of over 50 TH enzymes are available, but only the enzymes from bovine mitochondria (4), Escherichia coli (5), and Rhodobacter capsulatus (6) have been purified. The bovine enzyme is a homodimer of monomeric mass of 109 kDa. The monomer is composed of three domains: an amino-terminal ~430-residue-long extramembranous domain I that binds NAD(H), a ~400-residue-long central domain II that is composed of 14 transmembrane a-helices, and a carboxyl-terminal ~200-residue-long extramembranous domain III that binds NADP(H) (1, 4, 7). The extramembranous domains I and III come together in the mitochondrial matrix to form the active site for hydride transfer. Bovine TH lacks significant protein mass on the cytosolic side of the mitochondrial inner membrane, except for the oligopeptide loops connecting transmembrane a-helices (8). The prokaryotic enzymes have the same overall domain structure, but the polypeptide is composed of two or three separate gene products: E. coli and R. capsulatus as a and b subunits and Rhodospirillum rubrum as $\alpha_1$, $\alpha_2$, and $\beta$ subunits (1, 4, 5, 9).

Structural studies of TH have employed recombinant preparations of the hydrophilic nucleotide binding domains I and III. Crystal structures of the 20-kDa bovine and human domain III with NADP bound have been determined (10, 11), and crystal structures of the 80-kDa dimeric R. rubrum domain I ($\alpha_1$ subunit) in the apo form (12) with NAD (13) and with NADH (12) bound have been determined. Further, a crystal structure of the heterotrimeric complex between R. rubrum domains I and III reveals details of the interactions in the domain I-domain III interface (14). Experiments have also focused on the transhydrogenation reaction between recombinant domains I and III in the absence of domain II (9, 15, 16) and shown that hydride transfer from NADH to NADP in the forward direction is fast (17, 18). Trypsin sensitivity, chemical

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† The abbreviations used are: TH, transhydrogenase(s); AcPyAD, 3-acetylpyridine adenine dinucleotide; AcPyADP, 3-acetylpyridine adenine dinucleotide phosphate; ACMA, 9-amino-6-chloro-2-methoxyacridine.

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The mutagenesis of His91 on H9 (27, 30) translocation, H9, H10, and H13 (Fig. 1), is implicated from domain I components in crystals (24, 26, 29). Between helix 14 and domain III mediates communication between helix 14 and domain III, which is implicated from 32, 33), and Ser139 on H10 (32). The experiments also revealed a role for Glu 85 on H9 (32) and identified a requirement for acidic residues may be associated with a pKa shift coupled to NDPH induced movement in domain III. Experiments focused on residues Ser260, Ser 266 show that the hinge region between helix 14 and domain III mediates communication between domains II and III, which involves an Asp131–Arg265 salt bridge (35). Mutational study of 21 prototropic residues in H9, H10, H11, and H14 confirmed the requirement for His91 (H9) and Asn222, 222 (H13) and identified the requirement for Ser139 (H10) but did not implicate any residues on H14 (32). Based on the mutagenesis data and the high degree of sequence conservation in helix 14, we proposed that the proton channel is composed of H9, H10, H11, and H14 (32). Subsequently, residues 240–260 of helix 14 have been mutated to Cys (36). Reduced activities for the Gly245, Gly249, and Gly252 mutants on one face of the expected α-helix were interpreted in terms of helix-helix interactions within domain II, and enhanced activities for the Ser260, Ser 266 and Ser 266 mutants support an allosteric effect of helix 14 on NDPH binding in domain III (36).

The four α-helices predicted to comprise the proton channel contain eight highly conserved glycine residues, including three on H14 (Fig. 1). Amino acid replacements at Ser392 suggested that one role for glycine could be to allow water molecules within the proton channel (32). At the same time, substitutions for glycine uniquely test packing contacts within domain II. For these reasons, to test the four-helix bundle model and to evaluate the participation of H14, we have mutated each conserved glycine and assayed the mutant TH enzymes. Because Gly245 on helix 14 is uniquely implicated, additional replacements at this residue were prepared, and the mutant enzymes were purified and assayed.

**Experimental Procedures**

**Materials**—NAD, NADH, NADPH, and ATP were obtained from Calbiochem. AcPyAD, AcPyADP, and ACMA were obtained from Sigma. E. coli strain MC4100TH, whose transhydrogenase gene was replaced with the kanamycin resistance gene, was reported previously (34).

**Site-directed Mutagenesis**—The wild type transhydrogenase gene (pDC21) was digested with SalI/BamHI and the resulting DNA fragment was inserted into the SalI/BamHI site of pTZ18U. With this plasmid, site-directed mutagenesis was carried out using the reagents and protocols outlined in the Bio-Rad Muta-Gen mutagenesis kit (37). The plasmid DNA was prepared from individual colonies, and mutants were identified by DNA sequencing. The mutant DNA fragment was excised by appropriate restriction enzymes and replaced with the counterpart of pDC21. **Culture of E. coli Cells**—The E. coli strain MC4100TH was transformed with pDC21 or mutant plasmids. Each single colony was inoculated into the LB medium containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml). Cells were grown aerobically at 37 °C until the late logarithmic phase, collected by centrifugation at 8,000 rpm for 5 min (Sorvall GSA rotor), and washed with 0.9% NaCl.

**Preparation of Membranes**—The cells (wet weight, ~5 g per 4 litters of LB medium) were suspended in 20 ml of 50 mM Tris-HCl (pH 7.8), containing 1 mM dithiothreitol and 5 mM MgCl 2, and sonicated in a Branson sonifier at output 8 and 25% pulse for 5 min. Unbroken cells were removed by centrifugation at 15,000 rpm for 10 min in a Beckman model L ultracentrifuge, and membranes were collected by centrifugation at 40,000 rpm for 45 min. Membranes were suspended in 5 ml of the buffer and homogenized.

**Enzyme Purification**—Transhydrogenase was extracted from E. coli membranes with 2% Triton X-100 in 50 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, 1 mM dithiothreitol, and 0.4 mM phenylmethylsulfonyl fluoride (TEDP buffer) at ~4 mg/ml protein concentration. The sample was centrifuged at 40,000 rpm (Beckman 42Ti rotor). The supernatant was applied to a DEAE Biegol A column (Bio-Rad) (1.0 × 12.5 cm) and washed with 8 column volumes of TEDP buffer containing 0.05% Triton X-100 and 0.125 mM NaCl. The enzyme was eluted by a linear gradient (0.125–0.4 mM) of NaCl in TEDP buffer containing 0.05% Triton X-100 (total volume 60 ml), and active fractions were combined and concentrated to 2 ml with a Centriprep YM-30 concentrator (Millipore Corp.). The enzyme was then applied to a Sepharose 4B column (Amersham Biosciences) (1.5 × 70 cm) equilibrated with TEDP buffer containing 0.05% Triton X-100 and 10% glycerol. Active fractions were combined and concentrated to 1 ml with a Centriprep YM-30 concentrator. The purified enzyme was stored at ~ 80 °C until use.

**Enzyme Assays**—Transhydrogenase activities were assayed as previously described (32, 34). Transhydrogenase activity was assayed spectrophotometrically at 375 nm in a 37 °C reaction mixture (1 ml), containing 50 mM sodium phosphate (pH 7.0), 0.2 mM NADPH, 0.2 mM AcPyAD, and 5–50 μg of protein from E. coli membranes or purified enzymes. Cyclic transhydrogenase activity was measured at 375 nm in a 37 °C reaction mixture (1 ml), containing 50 mM MES-KOH (pH 6.0), 0.2 mM NADH, 0.2 mM AcPyAD, with or without 10 μM NADPH, and 5–50 μg of protein from E. coli membranes or purified enzymes. A difference extinction coefficient of 6.1 mM -1 cm -1 for absorbance at 375 versus 430 nm was used to calculate specific activities. All assays are the average of three or four measurements.

**Proton Translocation**—Proton translocation coupled to reverse transhydrogenation catalyzed by sonicated E. coli membranes was monitored by measuring the quenching of ACMA fluorescence (32). The reaction mixture (2 ml) contained 10 mM HEPES-KOH (pH 7.4), 5 mM MgCl 2, 0.3 mM KCl, 2 μM ACMA, 0.2 mM NADPH, 0.4 mM AcPyAD, and E. coli membranes (10–100 μg of protein). ACMA fluorescence was measured at 37 °C by a Jobin Yvon Fluoromax-3 fluorescence spectrophotometer, using an excitation wavelength of 415 nm and an emission wavelength of 485 nm. The reaction was initiated by the addition of AcPyAD. Assays are the average of two or three measurements.

**Protein Determination**—Protein concentrations were determined using the BCA protein assay reagents (Pierce).

**Determination of NADPH/Contents**—NADP and NADPH were extracted separately from purified wild type and G252A mutant transhydrogenases, and their contents were determined as described by Klencka and others (38).

**Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (39) using 12% polyacrylamide gel. The gels were stained with Coomassie Brilliant Blue and destained in 10% acetic acid.
amino acid replacements at Gly252 were purified and assayed. Subsequently, a series of mutant enzymes for transmembranes were prepared and assayed, as previously described (32). Seven additional strictly or highly conserved glycines in helices H9, H10, H13, and H14 are shown in boldface type; Glu85, a residue sensitive to mutation that potentially interacts with Asp213, is also highlighted (32). The soluble domains of TH, I and III, occur in the cytosol. Domain I is linked at the NH2 terminus of helix H1 of the subunit; domain III is linked at the COOH terminus of helix H14 in the β subunit, Helix H5 is present in mitochondrial TH, where the enzyme is a single polypeptide.

RESULTS

This paper addresses the role of conserved glycines in domain II of transhydrogenase. Previous mutagenesis studies have shown that there are eight conserved glycines in the β subunit of domain II of E. coli TH which provide residues essential for the proton translocation activity of the enzyme (Fig. 1). Based on these data and sequence conservation of residues in helix 14, we proposed that the proton conducting channel is comprised of helices H9, H10, H13, and H14 (32). The model presumes that the essential residues are associated in order to share a common function. It further assumes a four-helix bundle because of the occurrence of this motif in protein structures. With respect to glycine, eight residues in helices H9, H10, H13 and H14 are highly conserved: Gly95, Gly138, Gly226, Gly233, Gly245, and Gly252 are strictly conserved in 51 available TH sequences; Gly252 is Ala or Ser in three species; and Gly249 is Ala in one species.

TH mutants were constructed and expressed, and E. coli membranes were prepared and assayed, as previously described (32). Seven additional strictly or highly conserved glycines in helices H9, H10, H13 and H14 are highly conserved: Gly95, Gly138, Gly226, Gly233, Gly245, and Gly252 are strictly conserved in 51 available TH sequences; Gly252 is Ala or Ser in three species; and Gly249 is Ala in one species.

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Three assays were used to evaluate TH activity (Reaction 1). Reverse transhydrogenation from NADPH (0.2 mM) in domain III to the NAD analogue, AcPyAD, in domain I measures hydride transfer but is linked to proton translocation and is a measure of domain II function (34). Cyclic transhydrogenation measures hydride transfer between domains I and III. It entails hydride transfer from NADPH (10 μM) in domain III to AcPyAD in domain I; in the presence of excess NADH, the AcPyADH formed exchanges rapidly, and "forward" transhydrogenation from NADH back to NADPH allows another cycle of hydride transfer to occur (9, 40, 41). The third assay measures the conductance of protons through domain II in "inside-out" vesicles. In this case, reverse transhydrogenation from NADPH to AcPyAD causes [H+] to increase inside the vesicles, which is detected by fluorescence quenching (42). All three assays were used to evaluate TH mutants in suspended membranes; detergent-purified TH native and mutant enzymes were assayed for reverse and cyclic transhydrogenation.

The results of replacement of the eight conserved Gly → Ala mutants are given in Table I. In comparison with wild type TH, one of these mutants, G252A, exhibits virtually no activity in the reverse transhydrogenase and proton translocation assays. The other mutants display at least 30% proton translocation activity or 50% reverse transhydrogenation activity, where the errors in the individual assays are <±10%. In addition, the Gly252 mutant is unique in that it retains essentially full cyclic transhydrogenation activity in the absence of added NADPH, whereas the other mutants regain this activity only upon the addition of NADPH (Table I). The requirement for Gly252 for proton pumping activity is consistent with its strict sequence conservation. Participation of Gly252 supports the proposed four-helix model by identifying an essential residue on helix H14 (Fig. 1). The reduced activities of the G95A mutant on H9, the G138A mutant on H10, and the G226A and G233A mutants on H13 are also consistent with the participation of these glycines in the proton channel (Table I).

In order to test the requirement for Gly252 and probe its function in proton translocation, a series of amino acid replacements were prepared and assayed (Tables II and III). The replacements introduced the prototropic amino acids, Cys, Ser, and Thr, and a hydrophobic amino acid, Val. The additional mutants, prepared in suspended E. coli membranes, were assayed as for the wild type enzyme and the G252A mutant (Table II). Subsequently, the five mutants and the wild type enzyme were purified and assayed for reverse and cyclic transhydrogenation activity (Table III). On average, the errors in these experiments were <8% of reverse activity and <5% of cyclic activity for both the membrane-suspended and purified enzymes.

Fig. 2 shows SDS-polyacrylamide gel electrophoresis of membrane preparations in which wild type TH and the Gly252 mutant enzymes were overexpressed, demonstrating that the markedly reduced activity for the mutants is not due to inability of the cells to express the mutant polypeptides. SDS-polyacrylamide gel electrophoresis of purified E. coli TH and the Gly252 mutants shows that the wild type enzyme and the five
Gly252 mutants are essentially pure (Fig. 3). Although the level of expression of the G252C and G252S mutants is somewhat reduced (Fig. 2), this does not significantly affect the specific activity of these mutants relative to the others (Table III). Purification in detergent increases the specific activity of the wild type and mutant enzymes to a similar extent (5.1–9.0-fold in the cyclic assay with added NADPH), which is similar to the 9.1-fold increase in reverse transhydrogenation activity for the wild type enzyme (Tables II and III). Hence, the mutants behave like the wild type with respect to purification in Triton X-100, and the mutants behave similarly to each other. This indicates that the use of detergent has not adversely affected the properties of the wild type and mutant enzymes with respect to the E. coli membranes.

The Gly252 mutants are all severely impaired for proton translocation in vesicles (Table II) and, upon purification, are impaired for reverse transhydrogenation, which entails proton translocation (Table III). Substitution of prototropic residues in the G252C, G252S, and G252T mutants does not restore activity. This is unlike the situation in which Ser139 replacements to Cys, Thr, or Gly restored 33–77% activity (in the latter case, the S139G mutant may permit bound water to mimic the serine side chain). At the same time, the G252A mutant is as inactive as the G252V mutant with its bulkier hydrophobic side chain, so there is no correlation with respect to side chain volume (Table III). Together, the data indicate a strict requirement for glycine. The simplest explanation is that Gly252 is required for the properties of the wild type and mutant enzymes with respect to the E. coli membranes.

While evaluating Gly → Ala mutants, we observed that the G252A mutant is unique in that it was not necessary to add NADPH to stimulate cyclic transhydrogenation (Table I). In this regard, the activity of the wild type enzyme and seven

| TH       | Helix | Reverse TH activity | Cyclic TH activity | Proton translocation |
|----------|-------|---------------------|--------------------|----------------------|
|          |       | \( \mu \text{mol}^{-1} \text{mg}^{-1} \) | % | \( \mu \text{mol}^{-1} \text{mg}^{-1} \) | % | | % |
| Wild type|       | 1.95 ± 0.25         | 100                | 0.42 ± 0.10          | 8.73 ± 0.35 | 38.4 | 100 |
| G95A     | H10   | 1.11 ± 0.02         | 56                 | 0.65 ± 0.14          | 3.93 ± 0.20 | 16.9 | 44  |
| G95A     | H10   | 1.97 ± 0.10         | 101                | 0.24 ± 0.04          | 6.15 ± 0.27 | 38.8 | 101 |
| G95A     | H10   | 1.11 ± 0.08         | 57                 | 0.20 ± 0.01          | 2.94 ± 0.14 | 12.3 | 32  |
| G226A    | H14   | 0.97 ± 0.05         | 50                 | 0.69 ± 0.02          | 6.14 ± 0.10 | 11.5 | 30  |
| G233A    | H14   | 0.96 ± 0.02         | 49                 | 0.21 ± 0.01          | 2.68 ± 0.07 | 11.5 | 30  |
| G245A    | H14   | 2.03 ± 0.20         | 104                | 0.57 ± 0.04          | 5.09 ± 0.58 | 38.4 | 100 |
| G245A    | H14   | 1.55 ± 0.03         | 79                 | 0.69 ± 0.12          | 6.23 ± 0.23 | 29.5 | 77  |
| G252A    | H14   | 0.051 ± 0.004       | 2.6                | 8.35 ± 0.25          | 3.35 ± 0.20 | 0    | 0    |

A Cyclic transhydrogenation activity was measured with and without the addition of 10 \( \mu \)M NADPH. 
B Reverse transhydrogenation units are \( \mu \)mol of AcPyAD reduced by NADH/min/mg of protein measured at 375 nm (see ‘Experimental Procedures’). Values are averages of three or four experiments.
C Units are \( \mu \)mol of AcPyAD reduced by NADH/min/mg of protein measured at 375 nm. Values are averages of three or four experiments.
D Proton translocation coupled to reverse transhydrogenation measured by fluorescence quenching of ACMA at 485 nm. Units are the initial rate (\( \Delta F/\text{mg} \)) per mg of protein upon the addition of AcPyAD. Values are averages of duplicate or triplicate experiments.

| TH       | Reverse TH activity | Cyclic TH activity | Proton translocation |
|----------|---------------------|--------------------|----------------------|
|          | \( \mu \text{mol}^{-1} \text{mg}^{-1} \) | % | \( \mu \text{mol}^{-1} \text{mg}^{-1} \) | % |
| Wild type| 1.95 ± 0.25         | 100                | 0.42 ± 0.10          | 8.73 ± 0.35 | 38.4 | 100 |
| G252A    | 0.051 ± 0.003       | 2.6                | 8.35 ± 0.25          | 3.35 ± 0.20 | 0    | 0    |
| G252C    | 0.037 ± 0.004       | 1.9                | 7.30 ± 0.20          | 7.40 ± 0.20 | 0.6  | 1.6  |
| G252S    | 0.046 ± 0.005       | 2.4                | 6.89 ± 0.02          | 7.27 ± 0.11 | 0.4  | 1.0  |
| G252T    | 0.044 ± 0.010       | 2.3                | 8.12 ± 0.38          | 8.83 ± 0.60 | 0    | 0    |
| G252V    | 0.048 ± 0.004       | 2.5                | 1.82 ± 0.02          | 1.94 ± 0.03 | 0    | 0    |

A Volumes calculated for the van der Waals surface of the amino acid in a polypeptide relative to glycine (60.1 Å³) at pH 7.
mutants increased ~10–20-fold with the addition of NADPH, whereas the G252A mutant displayed maximal activity. Consequently, we assayed the wild type and G252A mutant enzymes for NADP(H) content. Purified wild type enzyme contained no NADP(H) and had ~1% cyclic activity without the addition of NADPH (Table III). On the other hand, the purified G252A mutant enzyme contained 0.8 mol of NADP and 0.1 mol of NADPH per αβ subunit assembly, based on duplicate measurements, and displayed 96% of cyclic activity without the addition of NADPH (Table III). The presence of 0.9 mol of NADP(H) per domain III binding site accounts for the high cyclic activity for the G252A mutant and is consistent with the intrinsic activity of the other Gly252 mutants in membrane preparations (92–98% versus added NADPH) (Table II) and upon purification (51–94% versus added NADPH) (Table III). These data indicate that mutation at Gly252 results in accumulation of NADP(H) in the enzyme.

DISCUSSION

The Proton Channel of TH—Transhydrogenase is a proton pump that couples proton transfer through the membrane to hydride transfer between soluble domains with a stoichiometry of [H⁺]/[H⁻] = 1. In the forward reaction, the proton motive force is used to reduce NADP; in the reverse reaction, the binding energy for NADPH versus NADP is used to pump a proton outward (Reaction 1) (1–3). We have used mutagenesis to further define the membrane-intercalated proton channel. Based on our previous results and the work of others, we proposed that α-helices H9, H10, H13, and H14 comprise a four-helix bundle in which residues His91, Ser139, and Asn222 participate with bound water molecules in a hydrogen-bonded network for proton conductance. To test this model, we have mutated eight highly conserved glycine residues in these transmembrane helices (Fig. 1). One of these, Gly252, on helix 14 is required for activity, confirming the participation of this helix in the proton channel. Replacements at residue 252 suggest that glycine allows close packing of all four essential residues within the proton channel (Fig. 4). However, the domain II structure is not known, and it is also possible that the role of Gly252 is to allow flexibility or to accommodate a bend or twist in the helix. Helical deformation and displacement is observed...
as a feature of proton translocation in bacteriorhodopsin (43, 44).

Glycine Residues in Helix 14 — Three of the mutated glycine residues occur on helix 14, Gly245, Gly249, and Gly252 (Fig. 1). Because helix 14 is linked at its C terminus to domain III, it may participate in communicating conformational change between domains II and III. Experiments focused on β subunit residues 260–266 indicate that this occurs with the hinge region (35); recently, helix 14 residues 240–260 have been mutated to Cys (36). In particular, the G245C, G249C, and G252C mutants had significantly reduced reverse transhydrogenation activity (24%, 40%, and <5%, respectively). Further, the G245L, G249L, and G252L mutants had reverse activities of 22, 70, and 7%, respectively (36). These data are consistent with residues 245, 249, and 252 occurring the same face of helix 14 and indicate that disruption of helical packing within domain II affects proton pumping (36).

By comparison, the G245A, G249A, and G252A mutants have 104, 79, and <3% reverse transhydrogenation activity, respectively (Table I), and the purified G252A and G252C mutants have ~1 and ~4% activity (Table III). The results for the purified G252C mutant are the same in both studies. With regard to helix-helix association in domain II, the data for residues 245 and 249 are in accord with a helix packing effect, in that the smaller Ala replacements are more active than the larger Cys and Leu replacements. However, the reverse transhydrogenation and proton translocation activities of the Gly252 mutants are consistently much lower, not only for the Ala, Cys, and Leu replacements, but also for Ser, Thr, and Val replacements (Tables II and III). One might expect the larger side chains to affect activity to a greater extent. However, not only are all six replacements essentially inactive, but there is no correlation with side chain volume (Table III). In other words, Ala and Ser appear to affect a structurally precise arrangement of His91, Ser139, and Asn222 as severely as larger amino acids. These data argue for a unique requirement for glycine at position 252 on helix 14 (Fig. 4).

Effects on Cyclic Transhydrogenation — This reaction involves hydride transfer between domains I and III and can proceed in the absence of domain II (9, 15–18). This activity depends upon NADPH binding in domain III, whereas in intact TH it is influenced by domain II (30, 32–35), consistent with a mechanism in which NADPH binding induces conformational change as a basis for enzyme function (Reaction 1) (1–4, 19–23). With respect to cyclic transhydrogenation, the domain II Gly → Ala mutants exhibited 31–96% activity in the presence of added NADPH (Table I). In cysteine-free TH, the Gly → Leu mutations at positions 245, 249, and 252 had comparable activities with added NADPH (52, 48, and 13%); the Gly → Cys mutants had lower activity (<5, <5, and 16%), but this could be restored with addition of R. rubrum domain I (36). These domain II replacements do not exhibit a clear effect on cyclic transhydrogenation.

In striking contrast, Gly252 mutants are distinguished by substantial cyclic transhydrogenation activity in the absence of added NADPH. The purified G252A mutant retains nearly stoichiometrically bound NADPH (0.9 mol per domain III), whereas no NADPH is bound in the wild type enzyme following purification. The G252C, G252S, G252T, and G252V mutants also display high intrinsic cyclic activity, presumably also due to bound NADPH (Table II); variability following purification may reflect differences in stability (Table III). Because retention of bound NADPH is correlated with complete loss of proton pumping activity, this implies that the Gly252 mutants are unable to undergo a conformational change that otherwise would couple domains II and III and promote release of NADPH. Hence, the data for the Gly252 mutants are consistent with a mechanism in which the status of the proton channel in domain II affects the conformation of domain III.

Native and Nonnative Conformations — A number of domain II mutants have been characterized that are “uncoupled” (i.e. in which cyclic activity is stimulated independent of proton pumping). In some mutants, cyclic activity is generally >140%, proton pumping is 6–30%, and 0.0–0.40 mol of NADP(H) is bound per domain III (30, 33, 45). In contrast, both reverse and cyclic activities exceeding that of wild type is observed for the S250A, S251A, S256A, and Y237F mutants (32). Greatly enhanced reverse and cyclic activities were observed for the S250C, S251C, and S256C mutants, consistent with intrinsically bound NADP(H) (36). $K_m$ for NADP(H) and the pH dependence of the cyclic reaction were also affected in these mutants (30, 33, 35, 36). These properties may be due to nonnative conformations induced by mutagenesis.

In the Gly252 mutants, TH is also uncoupled, but proton translocation is completely abrogated while NADP(H) remains tightly bound. Consequently, reverse activity and proton pumping are nil, but cyclic transhydrogenation activity between domains I and III proceeds normally due to the intrinsically bound NADP(H). We suggest that the Gly252 mutants, especially G252A and G252S, impede proton conductance in a conformationally benign manner (i.e. without disturbing helix-helix packing in domain II). This would be possible if the glycine replacement disrupted the precise interaction of the His91, Ser139, and Asn222 side chains. Hence, for G252A, proton pumping and reverse transhydrogenation are <1%, intrinsic cyclic activity is ~100% (but importantly not >100%), and ~1.0 mol of NADP(H) is bound per domain III. These properties suggest that TH is arrested in a native-like conformation, unable to translocate a proton and unable to release NADP(H) but able to undergo cyclic transhydrogenation due to the bound NADP(H).

Based on similar properties, a native-like conformation in an uncoupled state might also occur in the M259C, R265C, and R265A mutants. In these mutants, the ability of helix 14 or the linker region to transmit conformational change could be abridged without disrupting the arrangement of the domains (35, 36). Nevertheless, the G252A mutant is unique by having ~100% cyclic transhydrogenation activity in the complete absence of proton pumping, reflected in retention of NADP(H).

Four-helix Model — Structural studies support the model for the TH proton channel. In Neurospora plasma membrane H⁺-ATPase, four α-helices are organized around the H⁺ binding site and define the proton channel (46); in M2 influenza A virus M2, an α-helical homotetramer forms a proton channel (47). In E. coli aquaglyceroporin GlpF a water channel spanning the membrane is formed by a helical barrel and two internal loops (48, 49). This structure highlights roles for Asn and Gly; two Asn side chains direct the polarity of hydrogen bonds, and interactions with H₂O molecules involve three glycines. Internally ordered networks of H₂O molecules have been observed in the structures of bacteriorhodopsin (43, 44, 50) and cytochrome c oxidase (51–53); their participation in proton conductance has been demonstrated in bacteriorhodopsin (54, 55) and is likely in cytochrome c oxidase (56, 57). In E. coli TH, cysteine replacements on the face of helix H9 suggest the presence of an aqueous cavity (58). Occupancy of H₂O within the TH proton channel has been inferred from properties of the S139G, S139C, and S139T mutants (32).

Alternative NGXGG Sequence — In addition to Asn222 and Gly252, Asn and Gly are associated in an alternative domain II sequence in TH. The residues preceding Gly252 display two patterns; in 44 species, His91 and Ser92 are conserved (i.e. ...
His<sup>91</sup>-Ser<sup>92</sup>-Xaa-Xaa-Gly<sup>95</sup>), but in seven species, the alternative conserved motif is Asn<sup>91</sup>-Gly<sup>92</sup>-Xaa-Xaa-Gly<sup>95</sup> (Fig. 1). Other conserved segments in the β subunit do not exhibit such a marked variation. The shorter His<sup>91</sup> side chain may not alter the packing of side chains, but it could accommodate an additional H<sub>2</sub>O molecule. Perhaps Gly<sup>92</sup> also accommodates an H<sub>2</sub>O molecule in place of serine (32). Gly<sup>94</sup> could allow solvent access in place of Val<sup>94</sup>/Ile<sup>94</sup>, whereas Gly<sup>95</sup> is present in both motifs. These considerations suggest that the NGG motif allows additional solvent within the proton channel, implying that a specifically ordered H<sub>2</sub>O network could function in place of His<sup>91</sup>. The NGGG motif also places two conserved asparagines in the proposed proton channel, Asn<sup>91</sup> and Asn<sup>92</sup>, which occur in the GlpF, but in that case the asparagines function to stabilize the packing of side chains, but it could accommodate an additional H<sub>2</sub>O molecule. Perhaps Gly<sup>92</sup> also accommodates a H<sub>2</sub>O molecule in place of serine (32). Gly<sup>94</sup> could allow solvent access in place of Val<sup>94</sup>/Ile<sup>94</sup>, whereas Gly<sup>95</sup> is present in both motifs. These considerations suggest that the NGXGG motif allows additional solvent within the proton channel, implying that a specifically ordered H<sub>2</sub>O network could function in place of His<sup>91</sup>. The NGGG motif also places two conserved asparagines in the proposed proton channel, Asn<sup>91</sup> and Asn<sup>92</sup>, which occur in the GlpF, but in that case the asparagines function to stabilize the packing of side chains, but it could accommodate an additional H<sub>2</sub>O molecule. Perhaps Gly<sup>92</sup> also accommodates a H<sub>2</sub>O molecule in place of serine (32). Gly<sup>94</sup> could allow solvent access in place of Val<sup>94</sup>/Ile<sup>94</sup>, whereas Gly<sup>95</sup> is present in both motifs. These considerations suggest that the NGXGG motif allows additional solvent within the proton channel, implying that a specifically ordered H<sub>2</sub>O network could function in place of His<sup>91</sup>.
