The Proton Channel of the Energy-transducing Nicotinamide Nucleotide Transhydrogenase of *Escherichia coli*

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**Mutsuo Yamaguchi‡, C. David Stout§, and Youssef Hatefi‡†**

From the Departments of ‡Molecular and Experimental Medicine and §Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

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The nicotinamide nucleotide transhydrogenases (TH) of mitochondria and bacteria are proton pumps that couple direct 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. To delineate the proton channel of the enzyme, 25 conserved and semiconserved prototropic amino acid residues of domain II of the *Escherichia coli* transhydrogenase were mutated, and the mutant enzymes were assayed for transhydrogenation from NADPH to an NAD analogue and for the coupled outward proton translocation. The results confirmed the previous findings of others and ourselves on the essential roles of three amino acid residues and identified another essential residue. Three of these amino acids, His-91, Ser-139, and Asn-222, occur in three separate membrane-spanning α helices of domain II of the β subunit of the enzyme. Another residue, Asp-213, is probably located in a cytosolic-side loop that connects to the α helix bearing Asn-222. It is proposed that the three helices bearing His-91, Ser-139, and Asn-222 come together, possibly with another highly conserved α helix to form a four-helix bundle proton channel and that Asp-213 serves to conduct protons between the channel and domain III where NADPH binding energy is used via protein conformation change to initiate outward proton translocation.

Nicotinamide nucleotide transhydrogenases (TH) of mitochondria and microorganisms are membrane-intercalated enzymes that catalyze the direct and stereospecific transfer of a hydride ion between the 4A position of NAD(H) and the 4B position of NADP(H). This transhydrogenation reaction is coupled to transmembrane proton translocation with a H⁺/H⁻ stoichiometry of unity as shown in Equation 1 (1–3).

\[
\text{NAD} + \text{NADP} + \text{H}^{\text{out}} = \text{NAD} + \text{NADP} + \text{H}^{\text{in}} \tag{\text{Eq. 1}}
\]

The amino acid sequences of >30 TH 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 monomer molecular mass of 109,065 Da. 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 α 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 catalytic site of the enzyme. Bovine TH does not have a protein mass on the cytosolic side of the mitochondrial inner membrane with the exception of the oligopeptide loops, which connect appropriate transmembrane α helices (8). The prokaryotic enzymes have the same general tridomain structure, but each monomer is made up of two (*E. coli* and *R. capsulatus* TH) or three (*Rhodospirillum rubrum* TH) subunits (1, 4, 5). In 1995, we discovered an interesting feature of the transhydrogenase (9). Because hydride ion transfer between NAD(H) bound to domain I and NADP(H) bound to domain III is direct, we reasoned that the respective nicotinamide moieties of these nucleotides must come within a few angstroms of each other for such direct hydride ion transfer to take place. If so, we further reasoned that the nucleotide binding regions of domains I and III must have complementary surfaces and attractive forces to allow the close approximation of the nicotinamide rings of their respective nucleotides. If these considerations were correct, then soluble domains I and III in the absence of the membrane-intercalated domain II might come together and catalyze transhydrogenation. This reasoning proved correct. We demonstrated that indeed isolated or recombinant transhydrogenase domains I and III from the same or different organisms did catalyze transhydrogenation when added to a reaction mixture in the absence of domain II (9) and that this transhydrogenation was especially efficient with recombinant *R. rubrum* domains I and III (10). The kinetics of transhydrogenation as catalyzed by recombinant domains I and III were subsequently further explored by others (11–13). The crystal structures of recombinant bovine (14) and human (15) domain III containing bound NADP, recombinant *R. rubrum* domain I containing bound NAD (16), and a recombinant *R. rubrum* domain I–III complex containing a domain I dimer with one mole of NAD bound to each monomer plus a domain III monomer with bound NADP (17) have been published. In addition, we have recently determined the crystal structure of recombinant *R. rubrum* domain I dimer in the absence and the presence of bound NADH.²

² G. S. Prasad, M. Wahlberg, V. Sridhar, Y. Sundaresan, M. Yamaguchi, Y. Hatefi, and C. D. Stout, submitted for publication.

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* This work was supported by the National Institutes of Health, United States Public Health Service Grants GM61545 and DK08126 (to Y. H.). Synthesis of nucleotides was supported in part by the Sam and Da. The monomer is composed of 14 transmembrane α 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 catalytic site of the enzyme. Bovine TH does not have a protein mass on the cytosolic side of the mitochondrial inner membrane with the exception of the oligopeptide loops, which connect appropriate transmembrane α helices (8). The prokaryotic enzymes have the same general tridomain structure, but each monomer is made up of two (*E. coli* and *R. capsulatus* TH) or three (*Rhodospirillum rubrum* TH) subunits (1, 4, 5). In 1995, we discovered an interesting feature of the transhydrogenase (9). Because hydride ion transfer between NAD(H) bound to domain I and NADP(H) bound to domain III is direct, we reasoned that the respective nicotinamide moieties of these nucleotides must come within a few angstroms of each other for such direct hydride ion transfer to take place. If so, we further reasoned that the nucleotide binding regions of domains I and III must have complementary surfaces and attractive forces to allow the close approximation of the nicotinamide rings of their respective nucleotides. If these considerations were correct, then soluble domains I and III in the absence of the membrane-intercalated domain II might come together and catalyze transhydrogenation. This reasoning proved correct. We demonstrated that indeed isolated or recombinant transhydrogenase domains I and III from the same or different organisms did catalyze transhydrogenation when added to a reaction mixture in the absence of domain II (9) and that this transhydrogenation was especially efficient with recombinant *R. rubrum* domains I and III (10). The kinetics of transhydrogenation as catalyzed by recombinant domains I and III were subsequently further explored by others (11–13). The crystal structures of recombinant bovine (14) and human (15) domain III containing bound NADP, recombinant *R. rubrum* domain I containing bound NAD (16), and a recombinant *R. rubrum* domain I–III complex containing a domain I dimer with one mole of NAD bound to each monomer plus a domain III monomer with bound NADP (17) have been published. In addition, we have recently determined the crystal structure of recombinant *R. rubrum* domain I dimer in the absence and the presence of bound NADH.²

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Nicotinamide Nucleotide Transhydrogenase

Experimental Procedures

Materials—NAD, NADH, NADPH, and ATP were obtained from Calbiochem. AcPyAD, AcPyADP, and 9-amino-6-chloro-2-methoxyacridine (ACMA) were obtained from Sigma. E. coli strain MC4100TH, whose transhydrogenase gene was replaced with the kanamycin resistance gene, was reported previously (18).

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 derivative, site-directed mutagenesis was carried out using the reagents and protocols outlined in the Bio-Rad Mutagenesis kit (19). 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, ~0.65 g/liter LB medium) were suspended in 20 ml of 50 mM Tris-HCl (pH 7.8), containing 1 mM dithiothreitol and 5 mM MgCl₂, 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 1.3 ml of the same buffer and homogenized.

Enzyme Assays—Reverse 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 E. coli membranes (5–50 μg of protein). Cyclic transhydrogenase activity was measured in a 37 °C reaction mixture (1 ml) containing 50 mM MES-KOH (pH 6.0), 0.2 mM NADH, 0.2 mM AcPyAD, 10 mM NADPH, and E. coli membranes (5–50 μg of protein). ATP-dependent forward transhydrogenase activity was measured in a reaction mixture (1 ml) containing 50 mM Tris- H₂SO₄ (pH 7.8), 5 mM MgCl₂, 2 mM dithiothreitol, 0.3 mM KCl, 0.2 mM NADH, and 0.2 mM AcPyADP. This reaction was started by the addition of E. coli membranes (20–50 μg of protein), and 2–3 min later, 5 μl of 0.2 mM ATP was added. The reduction of AcPyADP before and after ATP addition was monitored at 375 nm. An extinction coefficient of 6.1 mM cm⁻¹ was used to calculate the rates.

Proton Translocation—Proton translocation coupled to reverse transhydrogenation catalyzed by E. coli membranes was monitored by measuring the quenching of ACMA fluorescence. The reaction mixture (2 ml) contained 10 mM HEPEs-KOH (pH 7.4), 5 mM MgCl₂, 0.3 mM KCl, 2 μM ACMA, 0.2 mM NADPH, 0.4 mM AcPyAD, and E. coli membranes (0–100 μg of protein). ACMA fluorescence was measured at 37 °C by a SLM photon-counting fluorescence spectrophotometer using an excitation wavelength of 415 nm and an emission wavelength of 485 nm. The reaction was started by the addition of AcPyAD. Proton translocation coupled to ATP hydrolysis was not monitored similarly in the above reaction mixture with the exception that NADPH and AcPyAD were not included and the reaction was started by the addition of 200 μM ATP or 50 μM NADH.

Protein Determination—Protein concentration was determined using the BCA protein assay reagents (Pierce).

Results

Meuller and Rydstrom (20) have shown that the membrane-intercalated domain II of the two-subunit E. coli TH monomer is composed of 13 transmembrane α helices, 4 helices at the COOH terminus of the α subunit and 9 helices at the NH₂ terminus of the β subunit. The domain II of the single subunit bovine TH monomer is composed of 14 continuous transmembrane α helices (1, 4). Mueller and Rydstrom (20) showed that the E. coli TH lacks the segment corresponding to the fifth bovine α helix and recommended that for easy interspecies comparison, the transmembrane α helices of E. coli TH be designated as shown in Fig. 1 (i.e., the α subunit ending with helix 4 and the β subunit starting with helix 6).

For reasons that are detailed under “Discussion” (also for review see Ref. 1), we considered that the proton channel of TH is probably located in the segment of the β subunit between transmembrane α helices 9 and 14. Therefore, a systematic mutational study was undertaken in which the effects of mutations of prototropic residues were investigated, mainly in this segment of domain II of the E. coli TH. The mutant E. coli membranes were assayed for the following activities: reverse transhydrogenation from NADPH to the NAD analogue AcPyAD; cyclic transhydrogenation in the presence of NADPH from NADH to AcPyAD (10, 21, 22); proton translocation coupled to reverse transhydrogenation; and proton translocation coupled to ATP hydrolysis and/or NADH oxidation. The latter

Fig. 1. Domain II of E. coli TH. The essential residues βHis-91, βSer-139, βAsp-213, and βAsn-222 are highlighted in black circles. βGlu-85 is in a black square, and the conserved glycine residues of helices 9, 10, 13, and 14 are shown in bold. For details, see “Results.” This figure was adapted from that in Ref. 20.
 assays were considered important controls, because they checked membrane integrity where TH mutations appeared to have inhibited transhydrogenation-coupled proton translocation. The results obtained have been separated into two tables. Table I shows the results of the mutations of 21 conserved and semiconserved (with the exception of βLys-149) prototropic residues, which appeared to be nonessential. As seen in this table, certain mutations had large effects on one or more of the activities tested. In the absence of information regarding the overall structure of the TH molecule, it is difficult to rationalize the effects of these mutations on the TH activities shown in Table I. However, certain possibilities can be conceived regarding the results of mutations of βGlu-85, which are considered under “Discussion.”

Table II shows the results of mutations of four other prototropic residues of the β subunit domain II. As was shown previously by others and confirmed here, βHis-91 (23) and βAsn-222 (24) appeared to be essential residues. One reason for repeating and extending the mutational studies of others was that they had not stated whether their E. coli membrane preparations containing the TH mutants incapable of transhydrogenation-coupled proton translocation were otherwise intact and capable of forming a proton electrochemical potential coupled to ATP hydrolysis and/or respiration. Although not shown in Tables I and II, we carried out this important membrane integrity test in all of the cases where proton translocation rates and extents coupled to reverse transhydrogenation were nil or drastically diminished. The results showed that all the E. coli membrane preparations containing TH mutants with impaired proton translocation activity were capable of rapid proton translocation coupled to ATP hydrolysis and NADH oxidation. Another reason was that certain literature reports showed substantial proton translocation activity for mutants that exhibited negligible reverse TH activity (see below).

As seen in Table II, the mutation of βHis-91 appeared to be tolerated to various low extents for reverse and cyclic transhydrogenation but not for proton translocation. Among the βHis-91 mutations, the replacement of this residue with Asn did not alter the reverse TH activity but greatly diminished cyclic and proton translocation activities. Literature data on the activities of βH91N mutation are mixed. The reverse TH activity reported for this mutation ranges from 80 (25–27) to 1% reverse TH activity, and 30 and 25% proton pumping activity (28). As seen in Table II, our βH91K and βH91R mutants exhibit relative to the wild type, respectively, 2 and 1% reverse TH activity, and 30 and 25% proton pumping activity (28). As seen in Table II, our βH91K and βH91R mutants were not only impaired for reverse transhydrogenation, they were also incapable of proton translocation. It has been reported that βH91K and βH91R mutants exhibit relative to the wild type, respectively, 2 and 1% reverse TH activity, and 30 and 25% proton pumping activity (28). As seen in Table II, our βH91K and βH91R mutants were not only impaired for reverse transhydrogenation, they were also incapable of proton translocation. In the TH of certain microorganisms (e.g. Mycobacteria), the residue corresponding to βH91 is Asn. In these organisms, the amino acid residues following Asn are also different. In E. coli, the sequence starting at βHis-91 is HSFGVL, whereas in Mycobacterium leprae and Mycobacterium tuberculosis, the sequence is NGVGGGT. Whether the TH in these organisms is incapable of proton translocation or more probably the nearby residue changes make it possible for Asn to perform the same function as βHis-91 of E. coli TH remains to be seen (see also Ref. 29).

However, it should be mentioned that in these organisms the

| Mutant | Reverse TH activity | Cyclic TH activity | Proton translocation |
|--------|---------------------|--------------------|----------------------|
|        | %                   | %                  | %                    |
| R31A   | 19                  | 8                  | 8                    |
| R31E   | >100                | 61                 | >100                 |
| R31L   | 41                  | 15                 | 46                   |
| N34T   | >100                | 92                 | >100                 |
| K76Q   | 99                  | 95                 | ≥100                 |
| K77A   | 9                   | 4                  | 11                   |
| K77L   | 13                  | 4                  | 19                   |
| K77Q   | 49                  | 46                 | 62                   |
| E85A   | 9                   | 19                 | 9                    |
| E85C   | 4                   | 1                  | 8                    |
| E85K   | 15                  | 4                  | 14                   |
| E85L   | 8                   | 5                  | 7                    |
| E85Q   | 3                   | 2                  | 4                    |
| E85S   | 22                  | 6                  | 20                   |
| S92A   | >100                | 95                 | >100                 |
| E124A  | 28                  | 68                 | 41                   |
| E124K  | 46                  | 30                 | 36                   |
| T135A  | 73                  | 52                 | 31                   |
| T135C  | 66                  | 13                 | 16                   |
| T135D  | 71                  | 36                 | 28                   |
| T135K  | 5                   | 100                | 1                    |
| T135L  | 33                  | 77                 | 9                    |
| T135S  | 23                  | 17                 | 12                   |
| Mutant | Reverse TH activity | Cyclic TH activity | Proton translocation |
|--------|---------------------|--------------------|----------------------|
|        | %                   | %                  | %                    |
| T137A  | ≥100                | 70                 | ≥100                 |
| K145A  | 44                  | 21                 | 50                   |
| K145E  | 46                  | 57                 | 67                   |
| K145L  | 19                  | 23                 | 2                    |
| K149Q  | 49                  | 58                 | 67                   |
| K153A  | 17                  | 16                 | 19                   |
| K153Q  | 50                  | 60                 | 67                   |
| R160A  | 45                  | 18                 | 13                   |
| R160L  | 97                  | 40                 | 68                   |
| N164T  | >100                | >100               | ≥100                 |
| S219A  | 100                 | >100               | >100                 |
| S223A  | >100                | >100               | >100                 |
| Y224F  | 83                  | >100               | 95                   |
| S225A  | 87                  | 88                 | 57                   |
| S250A  | >100                | >100               | >100                 |
| S251A  | >100                | >100               | 77                   |
| S256A  | 89                  | 90                 | 95                   |
| Y257F  | >100                | >100               | >100                 |
residues corresponding to the other three essential residues shown in Table II are all conserved. As seen in Tables I and II, cyclic TH activity is generally close to reverse TH activity with the exception of the case of βH91K mutation (Table II) where reverse TH activity is greatly diminished but cyclic activity is very high. This may be related to the finding of Bragg and Hou (28) that βH91K mutants contain bound NADP(H), which would favor the cyclic reaction (1, 10, 21, 22).

Another domain II residue previously shown to affect TH catalytic and proton translocation activities when mutated is βAsn-222 (24). Our extensive mutational data in support of the essential role of βAsn-222 are shown in Table II. It is seen that in contrast to all of the other βAsn-222 mutations, which completely inhibited proton translocation, βN222C and βN222D exhibited a low level of proton translocation activity (see also Ref. 24). This observation is not inconsistent with our proposed mechanism of proton translocation by TH (see “Discussion”).

We had shown earlier that βAsp-213, the only conserved dicarboxylic acid residue in the entire domain II of TH, appears to play a role in proton translocation (18). Its mutation to Asn and Ile resulted in parallel losses, respectively, of 70 and 90% reverse transhydrogenation and proton translocation activities. The results of additional mutations of this residue shown in Table II support our previous findings. It is seen that the mutation of βAsp-213 to Glu was partially tolerated, but other mutations greatly inhibited catalytic activity and nearly completely abolished proton translocation activity. Our results regarding the mutation of βAsp-213 to Lys and Arg differ from those of others. Bragg and Hou (28) have reported that as compared with wild type, βD213K exhibited 7% reverse and 49% proton translocation activities and βD213R showed 5% reverse and 28% proton translocation activities. Bragg and Hou (28) do not explain how it is possible for the exergonic reverse transhydrogenation to be so much lower than the rate of the coupled endergonic proton translocation (see above for data on βH91K and βH91R mutants). We appreciate that the different monitoring methods used (absorbance versus fluorescence) can result in small differences between the estimated rates of hydride ion transfer and proton translocation, especially when the results are calculated as percent of wild type activities. However, in our hands, βH91K and βH91R were incapable of proton translocation (see above and Table II), and βD213K and βD213R exhibited marginal rates of proton translocation commensurate with their very low reverse transhydrogenation activities.

The finding that βSer-139 appears to be essential is new and agrees with our previous suggestion regarding the possible role of conserved hydroxylated residues, which are prevalent in the distal helices of domain II (1). The situation with βSer-139 is particularly interesting. As seen in Table II, this residue could not tolerate the mutation to Ala, Asp, His, Lys, Leu, or Asn. In all of these cases, proton translocation was completely abolished. However, the replacement of the hydroxymethyl group of βSer-139 with the hydroxyethyl of threonine or the thiomethyl of cysteine was tolerated. These results as well as the permissible βS139G mutation are understandable in terms of our concept regarding the mechanism of proton translocation by TH, which is described under “Discussion.”

**DISCUSSION**

**The Proton Channel of TH**—The reason for concentrating our domain II mutagenesis studies on the segment of this domain downstream of helix 8 was that this region contains a highly conserved sequence of amino acids, especially around βHis-91 in helix 9, around βSer-139 in helix 10, in the extramembranous loop connecting helices 12 and 13, and throughout the remainder of domain II and approximately 15 residues in the extramembranous region beyond helix 14. This high degree of sequence conservation is rare among the hydrophobic domains of other proteins and was therefore suggestive of functional significance. The data reported in Tables I and II show that among 25 conserved and semiconserved prototropic residues of domain II of the _E. coli_ TH, which were mostly located in helices 9–14, four residues, namely, βHis-91, βSer-139, βAsp-213, and βAsn-222, appear to be essential for proton translocation. βAsp-213 is considered to be located outside the membrane in a cytosolic-side loop that connects transmembrane helices 12 and 13, whereas βHis-91, βSer-139, and βAsn-222 are located, respectively, in helices 9, 10, and 13 (Fig. 1). We propose that in the native structure of TH, α helices 9, 10, and 13 come together possibly with the highly conserved helix 14 to form a four-helix bundle in which residues βHis-91, βSer-139, and βAsn-222 are necessary for proton translocation. On the other hand, the important role of the absolutely conserved hydroxyethyl group of βSer-139 is new and agrees with our previous suggestion regarding the possible role of conserved hydroxylated residues, which are prevalent in the distal helices of domain II (1). The situation with βSer-139 is particularly interesting. As seen in Table II, this residue could not tolerate the mutation to Ala, Asp, His, Lys, Leu, or Asn. In all of these cases, proton translocation was completely abolished. However, the replacement of the hydroxymethyl group of βSer-139 with the hydroxyethyl of threonine or the thiomethyl of cysteine was tolerated. These results as well as the permissible βS139G mutation are understandable in terms of our concept regarding the mechanism of proton translocation by TH, which is described under “Discussion.”

**Table II**

Catalytic properties of _E. coli_ TH mutated at amino acid residues βHis-91, βSer-139, βAsp-213, and βAsn-222

| Mutant | Reverse TH activity | Cyclic TH activity | Proton translocation |
|--------|---------------------|--------------------|----------------------|
| H91A   | 7                   | 10                 | 0                    |
| H91C   | 24                  | 11                 | 0                    |
| H91D   | 25                  | 12                 | 0                    |
| H91E   | 15                  |                    |                      |
| H91G   | 27                  | 15                 | 0                    |
| H91I   | 28                  | 6                  | 2                    |
| H91K   | 5                   | 120                | 1                    |
| H91L   | 18                  | 11                 | 2                    |
| H91M   | 16                  | 3                  | -1                   |
| H91N   | 86                  |                    |                      |
| H91R   | 7                   | 8                  | 0                    |
| H91S   | 10                  | 6                  | <1                   |
| H91T   | 17                  | 6                  | <1                   |
| H91V   | 17                  | 3                  | 0                    |
| H91W   | 3                   | 3                  | 0                    |
| H91Y   | 7                   | 8                  | 1                    |
| D213A  | 13                  | 14                 | 4                    |
| D213E  | 20                  | 28                 | 18                   |
| D213G  | 2                   | 3                  | 0                    |
| D213K  | 4                   | 18                 | 5                    |
| D213L  | 4                   | 6                  | 3                    |
| D213R  | 5                   | 33                 | 6                    |
|        |                     |                    |                      |

| Mutant | Reverse TH activity | Cyclic TH activity | Proton translocation |
|--------|---------------------|--------------------|----------------------|
| S139A  | 6                   | 12                 | 0                    |
| S139C  | 34                  | 63                 | 33                   |
| S139D  | 2                   | 4                  | 0                    |
| S139E  | 85                  | 21                 | 41                   |
| S139F  | 5                   | 2                  | 0                    |
| S139K  | 2                   | 4                  | 0                    |
| S139L  | 4                   | 6                  | 0                    |
| S139N  | 3                   | 3                  | 0                    |
| S139T  | 96                  | 77                 | 71                   |
| N222A  | 18                  | 17                 | 0                    |
| N222C  | 58                  | 59                 | 11                   |
| N222D  | 21                  | 23                 | 13                   |
| N222G  | 2                   | 2                  | 0                    |
| N222H  | 35                  | 18                 | 0                    |
| N222K  | 11                  | 15                 | 0                    |
| N222L  | 52                  | 28                 | 1                    |
| N222Q  | 15                  | 16                 | 0                    |
| N222R  | 2                   | 9                  | 0                    |
| N222S  | 3                   | 1                  | 0                    |
| N222Y  | 3                   | 7                  | 0                    |
and βAsn-222 participate together with bound water molecules in a hydrogen-bonded network for proton conductance (see also Ref. 29). The participation of bound water in proton conductance has been demonstrated in bacteriorhodopsin (30–32) and appears very likely in cytochrome c oxidase (33–35). In Fig. 1, βHis-91, βSer-139, and βAsn-222 are shown at approximately the same depth from either side of α helices 9, 10, and 13. However, it should be pointed out that the correct structure of domain II is not known, and it is possible that these essential residues are located in such positions in their respective α helices that together with bound water they span the membrane from the cytosolic to the periplasmic side. Furthermore, it may be noted that dispersed throughout the proposed four-helix bundle are eight conserved glycines in the 33 available TH sequences. In the E. coli TH (see Fig. 1) these conserved glycines are βGly-85 in helix 9, βGly-132 (Ala in Eimeria tenella) and βGly-138 in helix 10, βGly-226 and βGly-233 in helix 13, and βGly-245, βGly-249 (Ala in E. tenella), and βGly-252 in helix 14. Together these conserved glycines may be required for the formation of a channel for bound water within the proposed four-helix bundle.

A proton channel as proposed here would agree with the mutational data reported in Table II. Thus, the permissible mutations βS139T and βS139C are understandable, because the hydroxethyl group of threonine and the thiomethyl group of cysteine could easily be considered to perform the same role as the hydroxymethyl of serine in the hydrogen bond network described above. The low proton translocation activities of βN222D and βN222C could also be rationalized in terms of the ability of Asp and Cys to participate via hydrogen bonding in proton conductance. Furthermore, the permissible βS139G mutation is not as surprising as it seems. One can conceive of the vacancy created by the absence of the hydroxymethyl of serine to be filled with a molecule of water and its participation in proton conductance through the hydrogen bond network. As seen in Table I, the mutations of βGlu-85 resulted in variable low activities. It is difficult to consider βGlu-85 as an essential residue, because in the TH of many organisms, βGlu-85 is replaced with Gln and because all of the βGlu-85 mutations shown in Table I were tolerated to variable small extents. However, the location of βGlu-85 is of interest (a) because of its proximity to βHis-91 in the α helical structure of helix 9, and (b) because in the proposed helix bundle, βGlu-85 may come close to βAsp-213 and assist in efficient proton conductance from βAsp-213 into the proton channel (see below). We have checked the possibility that in the E. coli TH βGlu-85 may take part together with βAsp-213 in proton conductance between domains II and III by mutating both residues to Ala. The resulting βE85A/βD213A double mutant showed only 1% reverse TH activity as compared with wild type and was completely incapable of proton translocation.

The TH Energy Coupling Mechanism—As seen in Equation 1, reverse transhydrogenation from NADPH to NAD is coupled to outward proton translocation. Because the difference in the reduction potentials of NADPH/NADP and NADH/NAD couples is negligible (ΔE0′ ~ 5 mV) and all of the substrates and products are located on the same side of the membrane, the driving force for outward proton translocation in the above reaction would have to be the difference in the chemical potentials of substrates and products, which in an enzymatic reaction means the difference in the binding energies of reactants and products. It further means that the enzyme would have to utilize this binding energy for outward proton translocation by undergoing a substrate-promoted conformation change. In forward transhydrogenation, the protonmotive force would then be expected to alter enzyme conformation and substrate/product affinities, resulting in facilitation of the forward reaction (1, 4).

Our early studies provided support for the above expectations. We first obtained evidence in submitochondrial particles that TH utilizes the proton motive force for cyclic conformation change in forward transhydrogenation (1, 36) and demonstrated a proton motive force-promoted substrate affinity increase of ~9–10 kJ/mol during hydride ion transfer from NADH to the NADP analogue AcFyADP (1, 4, 37, 38). Finally, using purified bovine TH, we showed for the first time that the binding of NADPH but not of NADH, NAD, or NADP, made two distant peptide bonds (Lys410–Thr411 at the junction of domains I and II and Arg602–Leu603 in the domain II extramembranous loop connecting α helices 6 and 7) susceptible to trypsin hydrolysis (4, 39). These results indicated that NADPH binding at domain III alters the conformation of this domain and its position relative to domains I and II. This finding was subsequently supported by data for the E. coli enzyme (40).

We have now shown that the conserved βAsp-213 of E. coli TH, which is probably located in the domain II extramembranous loop connecting α helices 12 and 13, is necessary for efficient proton translocation coupled to reverse transhydrogenation (Table II). This finding provides for a mechanism
whereby the NADPH-promoted conformation change of domain III can be linked to the proton channel of the enzyme in domain II (see above). It has been suggested by others that the conserved βAsp-392 at the NADPH binding site may be involved in proton translocation (41). One can certainly conceive of the possibility that the NADPH-promoted conformation change of domain III makes it possible for βAsp-392 to release a proton, which would somehow be conveyed to the domain II proton channel via βAsp-213 (Fig. 2). As mentioned above, the NADPH-promoted conformation change of domain III appears to move this domain away from the top of domain II as suggested by our previous work showing that Arg602Leu bond in the loop between helices 6 and 7 becomes susceptible to trypsin attack (39). This movement of domain III away from domain II could make the environment of βAsp-213 more hydrophilic and lower its pKa, resulting in proton release into the nearby proton channel. Using the catalytically active cysteine-free mutant of E. coli TH as the parent enzyme, Althage et al. (42) have converted βAsp-213 and βArg-265 separately and together to Cys in this otherwise cysteine-free protein. They have shown that both βD213C and βR265C mutants react with a fluorescent maleimide and that the presence of NADP(H) considerably increases the accessibility of the Cys residues to the maleimide derivative. βArg-265 is located five residues beyond helix 14 at the junction between domains II and III. When the fluorescent maleimide was added to the double mutant βD213C/βR265C, it reacted poorly, suggesting to the authors that the two Cys residues have formed a disulfide bond.

The authors infer from these data that in the native TH βAsp-213 and βArg-265, which they consider is not an essential residue, form a salt bridge. If so, this interaction would interfere with our proposed role of βAsp-213 in proton conductance. However, Althage et al. (42) state that their double mutant TH contained no bound nucleotides and have shown as recounted above that the presence of NADP(H) greatly increases the accessibility of βD213C and βR265C to modification by the fluorescent maleimide. Therefore, it seems probable that in the functioning enzyme substrate-promoted conformation changes, which increase the solvent exposure of these residues, would also prevent their coming together to form a salt bridge and allow βAsp-213 to function in proton conductance. Returning to the proposed mechanism, we would expect that in forward transhydrogenation, incoming protons would follow the path described above (i.e. through the hydrogen-bonded channel of domain II to βAsp-213 and domain III), resulting in affinity increase for NADP, which we have earlier shown (1, 4, 37, 38), and probably in affinity decrease for NADPH as suggested by kinetic data.

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REFERENCES

1. Hatefi, Y., and Yamaguchi, M. (1996) *FASEB J.* 10, 444–452
2. Jackson, B. J., Peake, S. J., and White, S. A. (1999) *FEBS Lett.* 464, 1–8
3. Bizouarn, T., Fjellstrom, O., Meuller, J., Axelsson, M., Bergkvist, A., Johansson, C., Karlsson, B. G., and Rydstrom, J. (2000) *Biochim. Biophys. Acta* 1457, 211–228
4. Hatefi, Y., and Yamaguchi, M. (1992) in *Molecular Mechanisms in Bioenergetics* (Ernster, L., ed) pp. 265–281, Elsevier Science Publishers B.V., Amsterdam
5. Clarke, D. M., and Bragg, P. D. (1985) *Eur. J. Biochem.* 149, 513–523
6. Lever, T. M., Palmer, T., Cunningham, I. J., Cotton, N. P. J., and Jackson, J. B. (1999) *Eur. J. Biochem.* 266, 247–255
7. Yamaguchi, M., and Hatefi, Y. (1993) *J. Biol. Chem.* 268, 17871–17877
8. Yamaguchi, M., and Hatefi, Y. (1991) *J. Biol. Chem.* 266, 5728–5735
9. Yamaguchi, M., and Hatefi, Y. (1997) *Biochim. Biophys. Acta* 1318, 225–234
10. Venning, J. D., Grimley, R. L., Bizouarn, T., Cotton, N. P. J., and Jackson, J. B. (1997) *J. Biol. Chem.* 272, 27535–27538
11. Venning, J. D., Bizouarn, T., Cotton, N. P. J., Quirk, P. G., and Jackson, J. B. (1998) *Eur. J. Biochem.* 257, 202–209
12. Pinheiro, T. J. T., Venning, J. D., and Jackson, J. B. (2001) *J. Biol. Chem.* 276, 44709–44716
13. Prasad, G. S., Sridhar, V., Yamaguchi, M., Hatefi, Y., and Stout, C. D. (1999) *Nat. Struct. Biol.* 6, 1126–1131
14. White, S. A., Peake, S. J., McSweeney, S., Leonard, G., Cotton, N. P. J., and Jackson, J. B. (2000) *Structure* 8, 1–12
15. Buckley, P. A., Jackson, J. B., Schneider, T., White, S. A., Rice, D. W., and Baker, P. J. (2000) *Structure* 8, 809–815
16. Cotton, N. P. J., White, S. A., Peake, S. J., McSweeney, S., and Jackson, J. B. (2001) *Structure* 9, 165–176
17. Yamaguchi, M., and Hatefi, Y. (1995) *J. Biol. Chem.* 270, 16653–16659
18. Rinaldi, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* 153, 367–382
19. Meuller, J., and Rydstrom, J. (1999) *J. Biol. Chem.* 274, 19072–19080
20. Fisher, R. R., and Earl, S. R. (1982) in *The Pyridine Nucleotide Coenzymes* (Evrose, J., Anderson, B., and You, K.-S., eds) pp. 279–324, Academic Press, Orlando, FL
21. Hutton, M., Day, J. M., Bizouarn, T., and Jackson, J. B. (1994) *Eur. J. Biochem.* 225, 447–453
22. Olausson, T., Fjellstrom, O., Meuller, J., and Rydstrom, J. (1995) *Biochim. Biophys. Acta* 1231, 1–19
23. Bragg, P. D. (1998) *Arch. Biochem. Biophys.* 363, 182–190
24. Glavas, N. A., Hou, C., and Bragg, P. D. (1995) *Biochemistry* 34, 7694–7702
25. Bragg, P. D., and Hou, C. (1996) *Eur. J. Biochem.* 241, 611–618
26. Bragg, P. D. (1998) *Biochim. Biophys. Acta* 1365, 88–104
27. Bragg, P. D., and Hou, C. (2001) *Arch. Biochem. Biophys.* 388, 299–307
28. Bizouarn, T., Meuller, J., Axelsson, M., and Rydstrom, J. (2000) *Biochim. Biophys. Acta* 1459, 284–290
29. Lanyi, J. K. (1999) *Int. Rev. Cytol.* 187, 161–202
30. Maeda, A., Balashov, S. P., Lugenburg, J., Verhoeven, M. A., Herzfeld, J., Baleng, M., Gen, R. B., Tomson, F. L., and Ehrey, T. G. (2002) *Biochemistry* 41, 3803–3809
31. Wikstrom, M., Morgan, J. E., and Verkhovsky, M. I. (1998) *J. Bioenerg. Biomembr.* 30, 139–145
32. Zaslavsky, D., and Gennis, R. B. (2000) *Biochim. Biophys. Acta* 1458, 164–179
33. Wikstrom, M. (2000) *Biochim. Biophys. Acta* 1458, 188–198
34. Hatefi, Y., Phelps, D. C., and Galante, Y. M. (1980) *J. Biol. Chem.* 255, 9526–9529
35. Galante, Y. M., Lee, Y., and Hatefi, Y. (1980) *J. Biol. Chem.* 255, 9641–9646
36. Hatefi, Y., Yagi, T., Phelps, D. C., Wong, S.-Y., Vik, S. B., and Galante, Y. M. (1982) *Proc. Natl. Acad. Sci. U. S. A.* 79, 1758–1760
37. Yamaguchi, M., Wakabayashi, S., and Hatefi, Y. (1990) *Biochemistry* 29, 4136–4143
38. Tong, R. C. W., Glavas, N. A., and Bragg, P. D. (1991) *Biochim. Biophys. Acta* 1080, 19–28
39. Hu, X., Zhang, J., Fjellstrom, O., Bizouarn, T., and Rydstrom, J. (1999) *Biochemistry* 38, 1652–1658
40. Althage, M., Bizouarn, T., and Rydstrom, J., (2001) *Biochemistry* 40, 9968–9976
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Mutsuo Yamaguchi, C. David Stout and Youssef Hatefi

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