Mapping of Residues in the NAD(P)H-binding Site of Proton-translocating Nicotinamide Nucleotide Transhydrogenase from Escherichia coli

A STUDY OF STRUCTURE AND FUNCTION

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Conformational changes in proton pumping transhydrogenases have been suggested to be dependent on binding of NADP(H) and the redox state of this substrate. Based on a detailed amino acid sequence analysis, it is argued that a classical βαβα βα dinucleotide binding fold is responsible for binding NADP(H). A model defining βα, ββ, βD, and βE of this domain is presented. To test this model, four single cysteine mutants (cfIα348C, cfβ390C, cfβk424C, and cfβR425C) were introduced into a functional cysteine-free transhydrogenase. Also, five cysteine mutants were constructed in the isolated domain III of Escherichia coli transhydrogenase (ecIIIH345C, ecIIIA348C, ecIIIR350C, ecIIIRD392C, and ecIIIPK424C). In addition to kinetic characterizations, effects of sulfhydryl-specific labeling with N-ethylmaleimide, 2-(4-maleimidylalanilino)naphthalene-6-sulfonic acid, and diazotized 3-aminopyridine adenine dinucleotide (phosphate) were examined.

The results are consistent with the view that, in agreement with the model, β-Ala348, β-Arg350, β-Ala390, β-Asp392, and β-Lys424 are located in or close to the NADP(H) site. More specifically, β-Ala348 succeeds βB. The remarkable reactivity of βR350C toward NNADP suggests that this residue is close to the nicotinamide moiety of NADP(H). β-Ala390 and β-Asp392 terminate or succeed βD, and are thus, together with the region following βA, creating the switch point crevice where NADP(H) binds. β-Asp392 is particularly important for the substrate affinity, but it could also have a more complex role in the coupling mechanism for transhydrogenase.

Proton-translocating nicotinamide nucleotide transhydrogenase couples the reversible stereospecific transfer of hydride equivalents from the 4A position of NADH to the 4B position of NADP+ with translocation of one proton (Ref. 1; see also Ref. 2) from the periplasmic space to the cytosol, according to the following reaction.

\[ H^+ + NADH + NADP^+ \rightleftharpoons H^+ + NAD^+ + NADPH \]

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For reviews on transhydrogenase, see Olausson et al. (2), and Jackson (3).

Membrane-bound transhydrogenase is composed of three domains. In the Escherichia coli enzyme, domain I (ecI, 1 to α404) and domain III (ecIII, β260 to β462) are exposed to the cytosol and contain the binding sites for NAD(H) and NADPH, respectively. Domain II (~α510 to β1 to ~β260) spans the membrane. Domain I (dI) from E. coli (4, 5), Rhodospirillum rubrum (rrI) (6), and bovine (7, 8) have been overexpressed, purified, and partially characterized. So far, domain II has not been expressed as a separate entity. Interestingly, dII is not required for transhydrogenation to occur (decoupled from proton translocation), as initially shown by Yamaguchi and Hatefi (7). Mixtures of recombinant dI and dIII from the same species or from different species catalyze decoupled “forward” and “reverse” reactions (cf. Reaction 1) and the so-called “cyclic reaction” (which involves the reduction of bound NADP+) by NADH, followed by the oxidation of bound NADPH by AcPyAD+ (5, 8, 9). From a recent study, it was observed that mixtures of rrI plus rrIII and rrI plus ecIII behaved similarly (10). They catalyzed high cyclic reaction rates (about the same as those observed in the complete E. coli and R. rubrum enzymes) that were limited by the transfer of hydride equivalents (10) and slow reverse reaction rates that, with excess rrI under usual assay conditions, were limited by the release of NADP+ (5, 8). With this knowledge at hand, it is now possible to use the rrI plus ecIII system to complement mutagenesis experiments performed on the complete enzyme. In addition to substrate binding affinities and hydride equivalent transfer rates, release rates of NADP+ and relative affinities between domains are properties that can be studied in mixtures of rrI and ecIII.

A three-dimensional model of the NAD(H)-binding site in E. coli transhydrogenase has been predicted (11). It adopts the
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structure of a classical $\beta$-barrel dinucleotide binding domain and constitutes approximately half of the residues in ecI. The structure of alanine dehydrogenase (1-AlaDH), a homologue of transhydrogenase domain I, has recently been solved (12). The monomeric unit of 1-AlaDH consists of two structurally similar domains, one of which adopts a classical dinucleotide binding fold responsible for NAD(H) binding, thus supporting the general features of the model (11).

Since proton translocation is very likely associated with conformational changes that affect binding and release of NADP(H) (5, 13, 14), information about the structural architecture of the NADP(H)-binding site is essential for understanding the proton pumping mechanism. It has been proposed that ecIII adopts a structure resembling the classical $\beta$-barrel fold found in e.g. glutathione and thioredoxin reductases (2).

In the present report, the development of a model comprising parts of the NADP(H)-binding domain is described. The prediction was tested by site-directed mutagenesis. In this context, the availability of a functional cysteine-free transhydrogenase (ctTH) (15) has been important. Using this transhydrogenase, single cysteine mutants were introduced into strategic positions. By probing the various mutants with the sulfhydryl-specific reagents NEM and MIANS, information about the location of the mutations relative to the substrate was gained. Furthermore, the reactivities of certain cysteine mutants of ctTH and ecIII enzymes toward diazotized AADP and AAD (analogues of NADP and NAD, respectively, where the amide group has been exchanged for a diazonium moiety) were examined; the aim was to find residues that would be spatially close to the nicotinamide ring. The results of all labeling experiments are discussed in view of the predicted model.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The plasmid, here denoted pCLNH, carries the gene coding for the ctTH to which an N-terminal histidine tag has been added. This plasmid serves as the DNA template for the construction of three cysteine mutants, cfA343C, cfA390C, and cfB425C. The plasmid (here denoted pEcIII) coding for the N-terminal histidine-tagged ecIII, was used as template for the introduction of a set of three single cysteine mutants, i.e. ecIIIH945C, ecIIIH350C, and ecIIIH283C. The pEcII was constructed from the pNHs plasmid (5). The primers (MedProbe, Norway), GCGTCGAACCATGGAAGCCAG-286–462. The reaction products were sequenced to verify the correctness of all mutant ecIII constructs.

Expression and Purification of Enzymes—Domain I from R. rubrum was produced in E. coli transhydrogenase enzymes and domains were carried out essentially as described (21). However, cells were resuspended in 30 mM sodium phosphate buffer containing protease inhibitor (Complete™ EDTA-free, Boehringer Mannheim), pH 7.5; cells were disrupted using an X-press (AB Biox, Gotteborg, Sweden). In the case of the ecIII mutants, the proteins were purified as described (5). Following the solubilization and subsequent centrifugation of the ctTH enzyme and the ctTH mutants, the supernatant was added to a nickel-nitrilotriacetic acid resin (12–3 ml of resin/g of membrane pellets). The columns were washed with 10–20 column volumes of buffer A containing 0.7 M NaCl and 30 mM imidazole. In a second wash (5–10 column volumes), the salt and imidazole concentrations were decreased to 0.1 M and 10 mM, respectively. The protein was eluted by increasing the imidazole concentration to 150 mM in a single step. The transhydrogenase-containing fractions were pooled, diluted with one volume of buffer A, and applied to a 6-mL Resource Q column, pretreated with 20 mL of buffer A, 30 mL of buffer B (buffer A containing 1 M NaCl), and finally 40 mL of buffer A. The column was washed with 15 column volumes of 200 mM NaCl in buffer A. The protein was eluted by increasing the NaCl concentration from 200 to 500 mM in a narrow 5-mL gradient to yield a high concentration of the protein; the transhydrogenase-containing fractions were pooled, frozen in liquid nitrogen, and stored at –80°C.

All mutants displayed a purity greater than 90% as judged by SDS-polyacrylamide gel electrophoresis using 10–20% gradient gels (Novex, Germany) (not shown). All experiments were performed at 25°C.

Activity Assays—Transhydrogenation reactions of ctTH and ctTH mutants were assayed in ctTH buffer (50 mM sodium phosphate, 1 mM EDTA, 50 mM NaCl, 0.1 mM NADP, pH 7.0). The dhIII assay buffer (20 mM each of Mes, Mops, Ches, and Tris, 50 mM NaCl, pH 7.0) was used for the reconstituted rrI plus ecIII system. Reverse and cyclic transhydrogenase activities were measured optically as described (5, 22).

MIANS Binding Measured by Fluorescence—The negatively charged MIANS molecule becomes fluorescent when it reacts with sulfhydryl groups. Therefore, the kinetics of MIANS labeling of single cysteine mutants was monitored by a SPEX model FL1 T1 reflective spectrophotometer. Excitation and emission slits were both 4.3 nm. The excitation and emission wavelengths were set to 330 and 418 nm, respectively. Reactions were initiated by the addition of MIANS to a final concentration of 4 μM. The absorbances of the substrates or substrate analogues at 330 and 418 nm were measured, and inner filter effects were corrected for according to the method described by Kubista et al. (52). Labeling of Single Cysteine Mutants by Diazotized AADP—Diazotization of the substrate analogues AADP and AAD was performed according to the protocol developed by Fisher et al. (24), and the concentrations of the products were determined optically using an absorption coefficient of 19,600 M$^{-1}$ cm$^{-1}$ at 283 nm (24) for both substrate analogues.

The experiments designed to analyze the effects of NNADP on cyclic reaction rates catalyzed by ecIII mutants are described in the legend to Fig. 5. In the case of the ecIIIK424C mutant, 1.8 μM ecIIK424C, 860 μM NNADP, and 0.40 μM MIANS were used. Similar procedures (see figure legend of Fig. 5) were followed when the effects of NNADP on reverse reactions catalyzed by cfA343C and cfA390C were investigated. 530 μM NNADP was added to 16.7 μM cfA343C, and 530 μM NNADP was added to 4.3 μM cfA390C. 5–μL samples were withdrawn at selected time and added to 1 mL of ctTH buffer; reverse reaction rates were measured using 500 μM NADPH and 500 μM AcPyD$^+$. Trypsin Digestion—Purified, detergent-dispersed, ctTH, and ctTH mutants were diluted with ctTH buffer to a concentration of about 1.5 mg/ml. NEM (1 mM) or MIANS (1 mM) was added for 1 h with the protein before the addition of trypsin. NNADP (1 mM) or NNADP$^+$ (5 mM) was added to the protein sample 5 min prior to the initiation of proteolysis. The proteins were digested at ambient temperature with trypsin at a mass ratio of 1:30 of trypsin:transhydrogenase, with and without NEM or MIANS and in the absence and presence of NNADP or NNADP$^+$. The reactions were terminated after 45 min by the addition of soybean trypsin inhibitor, at the mass ratio 2:1 of trypsin inhibitor/trypsin. Samples were analyzed by SDS-polyacrylamide gel electrophoresis using 10–20% gradient gels.

Determination of Protein Concentration—The concentration of proteins was determined by BCA using bovine serum albumin as standard (25).

Determination of the NADP(H) Content in ecIII and ecIII Mutants—The concentration of NNADP$^+$ was determined by a modified Klingen-
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Fig. 1. Analysis of primary structures of dIII of transhydrogenase and selected regions of six NADP(H)-binding proteins that adopt the classical $\beta\alpha\beta\alpha$ dinucleotide binding fold. Residues in the E. coli sequence are shaded when 10 of 11 transhydrogenase sequences display an identical amino acid as judged by a multiple sequence alignment obtained with thePILEUP program within the GCG package (47). The transhydrogenases and their respective accession numbers are: E. coli (E.c.TH), P07002 ($\beta$-subunit); R. rubrum, U05294; Caenorhabditis elegans, 1945493; Eimeria tenella, L07954; Entamoeba histolytica, U13421; Hemophilus influenzae, P43010; Synechocystis sp., P73496; bovine, P11024 (for sequence modification, see Ref. 18); Mus musculus, Z49204; Homo sapiens, Z50101; Mycobacterium tuberculosis, Z92770. The boldface residues in the E. coli transhydrogenase sequence were subjected to mutagenesis in the present investigation. The result of the secondary structure and accessibility predictions by the PHD program (28, 29) is presented in the same figure. The secondary structure ($\text{PHD sec.}$) and the accessibility ($\text{PHD acc.}$) were predicted by the PHD algorithm (28, 29). H, helix; E, $\beta$-strand; L, loop; b, buried; e, exposed. Assuming that domain III contains a classical dinucleotide binding domain, a tentative assignment of secondary structure elements (Model) constituting parts of this fold is indicated below the PHD prediction. The six NADP(H)-binding proteins chosen for comparison are (accession codes from the Brookhaven Protein Data Bank appear within parenthesis) as follows. GR, glutathione reductase (1GRA); TR, trypanothione reductase (1TYP); DHB, 17-$\beta$-hydroxysteroid dehydrogenase (1A27); PGD, 6-phosphogluconate dehydrogenase (1PGN); NCPR, NADPH-cytochrome P-450 reductase (1AM0); FNR, ferredoxin reductase (2FMR). The secondary structure elements of glutathione reductase are indicated below the alignment, and the residues involved in the corresponding structures in the aligned sequences are underlined. The first $\beta\alpha\beta\alpha$ motif sequences as well as the $\beta$D regions are aligned. The Wierenga fingerprint residues are marked with asterisks for the normal locations of glycines and alanines in the glycine-rich consensus, and the positions where small hydrophobic residues should dominate are indicated by a single dagger. The positively charged amino acids that interact with the 2'-phosphate are shown in boldface type. In the sequences constituting $\beta\alpha$, the strictly conserved glycines are printed in boldface type.

Results

Structural Model of the NADP(H)-binding Domain of E. coli Transhydrogenase—Sequence similarity searches such as FASTA and BLAST (27) failed to unambiguously detect any proteins homologous to domain III of transhydrogenases. Therefore, modeling by homology was not applicable, and information about structural arrangements must rely on alternative procedures. Secondary structure and accessibility predictions in combination with multiple sequence alignments represent important tools in this context. The shaded residues in the E. coli transhydrogenase sequence shown in Fig. 1 signify positions that are conserved (36% of all residues in dIII) among transhydrogenase from 11 different sources. The result of the secondary structure and accessibility predictions by the PHD program (28, 29) is presented in the same figure. The patterns that emerge can be compared with conserved features observed in other NADP(H)-binding proteins with known structures. Among these proteins, the classical $\beta\alpha\beta\alpha$ dinucleotide binding domain is the most common structure, but alternative NADP(H)-binding folds exist (30, 31). There are several characteristics of the transhydrogenase sequences (Fig. 1), general characteristics of the transhydrogenase sequences (Fig. 1), which alter the corresponding structures in the aligned sequences are underlined. The first $\beta\alpha\beta\alpha$ motif sequences as well as the $\beta$D regions are aligned. The Wierenga fingerprint residues are marked with asterisks for the normal locations of glycines and alanines in the glycine-rich consensus, and the positions where small hydrophobic residues should dominate are indicated by a single dagger. The positively charged amino acids that interact with the 2'-phosphate are shown in boldface type. In the sequences constituting $\beta\alpha$, the strictly conserved glycines are printed in boldface type.

 shading procedure previously described (5). The concentration of NADPH was deduced from optical spectra of the ecIII enzyme samples using an extinction coefficient of 6200 M\(^{-1}\) cm\(^{-1}\) at 339 nm. The NADPH concentrations were compared with the corresponding BCA-determined data. After 6 h, 40-mg samples of each chamber were transferred to 1 ml of buffer plus NADPH were applied to opposite sides of the dialysis membrane. The dialysis chamber was rotated at about 60 rpm at 4 °C. After 6 h, the aliquots from each chamber were transferred to 1 ml of buffer containing 10 mM Mops, pH 7.0. The fluorescence of the sample was measured at the emission wavelength of 460 nm using an excitation wavelength of 340 nm. Oxidized glutathione and glutathione reductase were added to determine the fluorescence contribution from NADPH.
approximately 8-fold higher than that displayed by cfTH. The substrate is fitted, is situated between the C-terminal ends of βA and βD, since the switch point, creating the cleft into which the substrate is fitted, is situated between the C-terminal ends of βA and βD, these strands exhibit conserved features. The hydrophobic character of βA and the glycine-rich consensus at the switch point are well recognized, but the features of βD have been analyzed in less detail. From analysis of the six NADP(H)-binding proteins, glutathione reductase, trypanothione reductase, 17β-hydroxysteroid dehydrogenase, 6-phosphogluconate dehydrogenase, and NADPH-cytochrome P-450 reductase (see Fig. 1), it is observed that they all carry a strictly conserved glycine residue located at the C-terminal end of βD or succeeding βD, approximately where the switch point is formed; furthermore, the strand is hydrophobic, and in glutathione reductase and trypanothione reductase a well conserved aspartic acid residue is located at the N terminus of βD. The lengths of the βD strands vary. Residues β-Asp383 to β-Gly389 in E. coli transhydrogenase are suggested to constitute βD. The three β-strands (βA, βB, and βD) making up the dinucleotide binding core have now been assigned. Some NAD(P)(H)-binding proteins do not contain additional β-strands in this fold (30). In transhydrogenase, however, the stretch β418–β424 is strongly predicted as a β-strand; it is hydrophobic; its N-terminal residues are conserved, which is reasonable since they should be close to the substrate; the conserved β-Arg416 could correspond to well conserved charged residues preceding βE in several other proteins; and, finally, in the “PREDB” program, designed to predict β-strand contacts, β-Asp383 to β-Gly389 and β-Gln418 to β-Lys424 were strongly predicted to make contacts as parallel β-strands.3

The result of the prediction is summarized as a visual guide in Fig. 2. Below, on the one hand, experiments intended to test this model will be described, and, on the other hand, the model will be used with the aim to help rationalize the properties of several mutants.

**Single Cysteine Mutants Inserted into Cysteine-free E. coli Transhydrogenase**—Four single cysteine mutants, cfβA348C, cfβA390C, cfβK424C, and cfβR425C, were constructed, expressed, and purified as described under “Experimental Procedures.” These residues should, according to the prediction, be in or near the NADP(H)-binding site. Some kinetic and thermodynamic properties of the mutants are given in Table I. None of the mutated residues was essential, but cfβA348C, cfβK424C, and cfβR425C displayed markedly reduced reverse transhydrogenation activities. In addition to yielding the lowest activities, cfβR425C also gave the most elevated KmNADPH value, approximately 8-fold higher than that displayed by cfTH. The substi-

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3 T. Hubbard, personal communication.
Table I

| cTH enzyme | $K_m$ NADPH ($\mu$M) | $V_{max}$ reverse activity (U/mg) | $V_{max}$ cyclic activity (U/mg) | $K_d$ NADPH ($\mu$M) |
|------------|----------------------|---------------------------------|---------------------------------|-----------------------|
| cTH        | 14                   | 6.9                             | 100                             | 12.7                  | 100                   | 1.2 |
| cbA348C    | 23                   | 1.4                             | 20                              | 3.0                   | 24                    | 3   |
| cbA390C    | 33                   | 5.1                             | 74                              | 13.5                  | 106                   | 5.3 |
| cbK424C    | 40                   | 2.1                             | 30                              | 6.3                   | 50                    | 6.4 |
| cbR425C    | 120                  | 0.7                             | 10                              | 1.0                   | 8                     | —   |

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NADPH had the following effects on the inhibition rates. It slightly decreased the inhibition rate of cbA348C by MIANS (Fig. 3A); it displayed a strong protective effect on cbA390C from both MIANS labeling (Fig. 3B) and NEM labeling (not shown); and it also considerably lowered the MIANS inhibition rate of cbK424C (Fig. 3C); however, no protection, or even a slight stimulation, was observed for the cbR425C mutant (Fig. 3D).

As MIANS reacts with sulphhydryl groups, it becomes fluorescent (34). Therefore, binding of MIANS to the mutant can be followed fluorometrically. This provides for an alternative approach to that described above for studying the effect of substrates on MIANS binding rates. As can be seen in Fig. 3, A–D, the trends were similar to those found by the activity measurements. Thus, NADPH protected cbA390C (Fig. 3B) most effectively, but it also caused decreased binding rates for the cbA348C (Fig. 3A) and cbK424C (Fig. 3C) mutants, whereas no protection was observed for the cbR425C (Fig. 3D) mutant. Qualitatively, the NADP+ molecule displayed similar effects on MIANS binding as the reduced substrate (Fig. 3, A–D).

Trypsin Cleavage—Transhydrogenase displays a characteristic cleavage pattern by trypsin. The $\beta$-subunit is normally only susceptible to proteolysis in the presence of NADP(H) to yield a distinguishing 30-kDa fragment (35), allowing a qualitative evaluation of NADP(H)-induced conformational changes in the $\beta$-subunit (results not shown). In the absence of MIANS or NEM, this trypsin fragment was produced only upon NADP(H) binding for the four cysteine mutants described above. After preincubation of the cbA348C mutant with MIANS, the $\beta$-subunit became very sensitive to trypsin digestion even in the absence of NADP(H), indicative of a structural perturbation. Treatment with NEM had no effect on the cleavage pattern of the cbA348C mutant but rendered the $\beta$-subunit of the cbA390C mutant resistant to trypsin digestion even in the presence of NADP(H); NEM also drastically reduced the ability of NADP(H) to induce the trypic 30-kDa fragment for the cbK424C mutant. In the case of the cbR425C mutant, neither NEM nor MIANS caused an altered NADP(H)-dependent trypsin cleavage pattern.

Dissociation Constants for NADPH—The results of the catalytic activity assays and the proteolysis experiments suggest that MIANS and NEM binding to the cbA390C and cbK424C mutants may influence NADP(H) binding. Therefore, measurements of the dissociation constants of NADPH ($K_a$ NADPH) in the absence and presence of bound NEM were performed by equilibrium dialysis (see “Experimental Procedures” and Ref. 26). The data are presented in Fig. 4, and the resulting $K_a$ values are included in Table I. The values of $K_a$ NADPH in the absence of NEM for the cbA390C and the cbK424C mutants were approximately 5 and 6 $\mu$M (11.5 $\mu$M in a second preparation of the cbK424C mutant), respectively; no bound NADPH was detected for either of these mutants in the presence of NEM in the concentration range of NADPH tested (see Fig. 4), suggesting that NEM caused a decrease of their binding affinities for NADPH by at least 2 orders of magnitude. The cTH enzyme displayed an affinity for NADPH (1–2 $\mu$M) that was unaffected by NEM (Table I and Fig. 4); this value is similar to that found for wild-type transhydrogenase.

Probing the NADP(H)-binding Site by Single Cysteine Site-directed Mutagenesis in Domain III of E. coli Transhydrogenase—Five single cysteine mutants of ecIII, ecIIIH345C, ecIIIA348C, ecIIIR350C, ecIIID392C, and ecIIIK424C, were expressed and purified according to the scheme described under “Experimental Procedures.”

A characteristic feature of the isolated dIII from various species is that they have high affinities for the NADP(H) substrates, which was manifested by the fact that normally almost 100% of the protein molecules contain bound substrate, the majority of which was in their oxidized state (5, 8, 9). In order to determine if the mutations introduced in ecIII affected the affinities for NADP(H), the substrate contents were measured in all mutants (see “Experimental Procedures”). From the results shown in Table II, it was noticed that the mutants behaved differently from the nonmutant ecIII. First, a smaller fraction of the ecIII mutants carried substrates. Second, the fraction of reduced substrate was greater. The most striking observation, however, was that the purified ecIIID392C mutant did not contain any measurable amounts of bound substrates. Certainly, this particular mutation must have led to strongly decreased affinities for NADP(H). Protein-protein titrations, keeping the ecIII mutant concentration fixed and varying the rrI concentration, were performed for each mutant, both for the cyclic reaction and for the reverse reaction (not shown). From the titration curves, maximal rates were estimated, as well as the concentration of rrI required to reach half of the maximal rates. In the case of the cyclic reaction, this rrI concentration is dependent on the affinity between the two domains, whereas in the reverse reaction it is determined by the relative kinetics of the component mechanistic steps, including dI and dIII association and dissociation (8, 10). The results from the protein-protein titrations are summarized in Table III.

Reverse Reaction Catalyzed by rrI plus ecIII and rrI plus ecIII Mutant Mixtures—Every mutant exhibited elevated reverse transhydrogenation rates compared with the ecIII reference, the most pronounced effect demonstrated by the ecIIID392C mutant (approximately 14 times the rate catalyzed by rrI plus ecIII) and the least effect displayed by the ecIIIH345C and ecIIIR350C mutants (approximately 2.5 times the rate of the wild-type control). The rates of release of NADP+ must, at a minimum, be as fast as the turnover of the reverse reaction

4 J. Rydström, X., Hu, and J. B. Jackson, unpublished results.
Therefore, as an approximation, the mutants may be ranked according to effects on NADP release rates as follows: ecIIID392C, ecIIIA348C, ecIIIK424C, ecIIIH345C, ecIIIR350C. The ratios of the rrI concentrations required to reach half-maximal rates to the fixed mutant concentrations, did not vary extensively between the various mutants but were all high compared with the ratio displayed by the native ecIII (Table III). Possibly, a high ratio may correlate with a high rate of reverse transhydrogenation.

**Cyclic Reaction Catalyzed by rrI plus ecIII and rrI plus ecIII Mutant Mixtures**—All of the single mutants catalyzed relatively high rates of cyclic transhydrogenation, ranging from about 20% for the ecIIID392C mutant to about 70% for the ecIIIA348C mutant as compared with wild-type ecIII. This indicated that the abilities of these mutants to transfer hydride equivalents, which have been shown to be rate-limiting for the cyclic reaction under the present conditions (10), have not been.

hydrogenase in the absence and presence of NADP(H), monitored by fluorescence. To 10.0 ml of cfTH buffer, the mutant was added to a final concentration as indicated below. In the experiment with NADPH, 500 μM of the substrate was added at this point. One ml of this mixture was withdrawn before the addition of MIANS, and the rate was measured to obtain the activity at time 0. This initial activity was normalized to a relative activity of 1. At the indicated times, 1-ml aliquots were withdrawn for activity measurements. The concentrations of AcPyAD and NADPH were both 500 μM.

Concentrations of the mutants were as follows: 120 nM cfβA348C (A), 43 nM cfβA390C (B), 71 nM cfβK424C (C), 215 nM cfβR425C (D). The experiments with MIANS (open squares), MIANS and NADPH (open circles), and the controls performed in the presence of NADPH and methanol (open triangles) were performed on the same day for each mutant. The fluorescence measurements were performed as described under “Experimental Procedures.” Concentrations of MIANS, NADPH, and NADP were 4 μM, 100 μM, and 1.0 mM, respectively. The concentrations of mutants and descriptions of the traces are as follows. A, 0.31 μM cfβA348C; upper trace, MIANS; middle trace, MIANS plus NADP; lower trace, MIANS plus NADP. B, 0.35 μM cfβA390C; upper trace, MIANS; middle trace, MIANS plus NADP; lower trace, MIANS plus NADP. C, 0.29 μM cfβK424C; upper trace, MIANS; middle trace, MIANS plus NADP; lower trace, MIANS plus NADP. D, 0.32 μM cfβR425C; upper trace, MIANS; middle trace, MIANS plus NADP; lower trace, MIANS plus NADP. I, relative fluorescence intensity.
dramatically affected. Three protein-protein titrations using various fixed concentrations of ecIII and variable rrI were performed in order to provide for an adequate analysis of the affinities between rrI and the intact ecIII and between rrI and the ecIII mutants. By comparing the half-saturating rrI concentration for a particular mutant with the appropriate (similar fixed concentration of ecIII as mutant) corresponding rrI concentration for nonmutant ecIII (Table III), it was possible to determine if the interdomain affinity had changed. Significant decreased affinities were detected only for the ecIIIH345C and the ecIIIID392C mutants (Table III).

The Effect of Diazotized AAD(P) on Single Cysteine Mutants—Several NAD(P)-binding proteins have been shown to be irreversibly inhibited by diazotized AAD(P) at neutral pH, and the conclusions were that cysteines close to the nicotinamide moiety had been modified (24, 36–40). As expected, AADP was a competitive inhibitor with respect to the NADP(H)-binding site in E. coli transhydrogenase with a $K_i$ of about 100 $\mu$M, and AAD at a concentration of 1 mM did not compete with NADPH binding (not shown). Since the investigated mutants were targeted toward the NADP(H)-binding site, they were subjected to diazotized AADP (NNADP) treatment. To test if the effects observed were specific, each mutant was also treated with the same concentration of diazotized AAD (NNAD). Cyclic transhydrogenation activities catalyzed by the rrI plus ecIII system were measured for ecIII mutants, whereas reverse transhydrogenation activities were analyzed for cfTH mutants. The results of these experiments are presented in Fig. 5. Neither the ecIIIH345C (Fig. 5A), nor the ecIIIK424C (not shown) mutant was specifically inhibited by NNADP; for the latter mutant, both NNAD and NNADP caused an initial 25% drop in activity, whereafter the activity remained essentially constant. The ecIIIH345C mutant was significantly inhibited by both NNADP (about 93% inhibition after 2 h) and NNAD (about 65% inhibition after 2 h) (Fig. 5B), but with some preference for the NNADP molecule. The corresponding mutant in the intact enzyme, i.e. cfβA348C, was much less affected by similar concentrations of the reactive substrates (about 20 and 30% inhibition by NNAD and NNADP, respectively) (not shown). Possibly, this could be a consequence of the much lower affinity of the intact enzyme for NADP(H) relative to that of the isolated ecIII. The ecIIIR350C mutant reacted rapidly with even very low concentrations of NNADP, and at these low concentrations hardly any reactivity with NNAD was observed (Fig. 5C). Also, the ecIIIID392C mutant reacted preferentially with NNADP, and the cyclic reaction rate was decreased by about 85% after 1 h, compared with about 48% in the presence of NNAD (Fig. 5D). The cfβA390C mutant in the intact enzyme was not affected much by either NNADP or NNAD (approximately 20% inhibition after 3 h for both reagents) (not shown).

The results suggest that β-Ala$^{348}$, β-Arg$^{350}$, and β-Asp$^{392}$ are located in, or very near, the NADP(H)-binding site, since the corresponding cysteine mutants displayed a greater reactivity toward the NNADP than toward the NNAD molecule.

**Table II**

The content of substrates in the isolated transhydrogenase domain III mutants

The concentrations of NADP$^+$ were determined by a modified Klinenberg procedure, and the concentrations of NADPH were estimated from optical spectra (see "Experimental Procedures"). The $\lambda_{max}$ values correspond to the wavelength at which maximal absorption was observed for the interval 290–450 nm in optical spectra.

| ecIII enzyme | NADPH | NADP$^+$ | $\lambda_{max}$ |
|--------------|-------|----------|----------------|
| ecIII        | 5     | 87       | 267            |
| ecIIIH345C   | 25    | 44       | 267            |
| ecIIIA348C   | 60    | 6        | 267            |
| ecIIIR350C   | 30    | 24       | 267            |
| ecIIIH392C   | 0     | 77       |                |
| ecIIIK424C   | 15    | 22       | 267            |

**Table III**

A summary of protein-protein titrations for the cyclic and reverse reactions catalyzed by rrI mixed with various ecIII enzymes

The values are estimations derived from protein-protein titration curves where the concentration of rrI was varied (not shown). The assays were carried out in dIdIII buffer (see "Experimental Procedures"). The $V_{max}$ values correspond to the fixed ecIII enzyme concentrations used in the various protein-protein titrations. The $[rI]/[ecIII]$ values correspond to the concentrations of rrI required to reach half-maximal rates for the cyclic and reverse reactions.

| ecIII enzyme | $V_{max}$ reverse activity | [ecIII enzyme] | [rrI]/[ecIII enzyme] | $V_{max}$ cyclic activity | [ecIII enzyme] | [rrI]/[ecIII enzyme] |
|--------------|---------------------------|----------------|----------------------|---------------------------|----------------|----------------------|
|              | mol min$^{-1}$ (mol ecIII enzyme)$^{-1}$ | nM       | nM       | mol min$^{-1}$ (mol ecIII enzyme)$^{-1}$ | nM       | nM       |
| ecIII        | 4                          | 4900      | 20       | 0.004                     | 4900      | 4         |
| ecIII        | 3                          | 1300      | 60       | 0.046                     | 1250      | 39        |
| ecIIIH345C   | 32                         | 900       | 90       | 0.130                     | 3300      | 7         |
| ecIIIA348C   | 5                          | 1240      | 100      | 0.081                     | 2000      | 8         |
| ecIIIR350C   | 10                         | 470       | 100      | 0.213                     | 900       | 43        |
| ecIIIH392C   | 75                         | 730       | 60       | 0.082                     | 2200      | 9         |
| ecIIIK424C   | 17                         | 730       | 60       | 0.082                     | 2200      | 9         |
gested to reflect structural alterations. The decreased binding rates of MIANS in the presence of NADP(H) suggest that β-Ala348 is physically either in or near the substrate binding site or that the conformational changes known to be induced by substrate binding (14, 35) result in a shielding effect.

The pronounced ability of NADP(H) to protect the cfβA390C mutant from reacting with MIANS (Fig. 3B) and NEM (not shown) and the fact that reaction with NEM yielded an enzyme derivative virtually unable to bind the NADPH substrate support the predicted location of β-Ala390 at the end of the NADP(H)-binding site. Necessarily, β-Asp392, which has been shown to be crucial for reverse transhydrogenation (44), must also be in or very near the NADP(H) site.

The α-Lys424 and α-Arg425 residues are conserved among the known transhydrogenase sequences (Fig. 1). Both amino acids are charged, and the former is a possible candidate for direct, and the latter for indirect, participation in vectorial protonation events. Furthermore, it was earlier proposed that the two residues could be located close to the nicotinamide moiety of the NADP(H) molecule and by their positively charged nature possibly stabilize the binding of reduced substrate relative to the oxidized species (5). Therefore, these sites were found to be interesting targets for mutagenesis. For similar reasons as described for the cfβA390C mutant, the present results (see Fig. 3C) are clearly consistent with the view that α-Lys424 is located within a few Å of the NADP(H) substrate. In a recent study, it was found that replacing α-Lys424 with an arginine strongly repressed NADP(H) binding (45). It is suggested that this effect and the NEM effect on the cfβK424C mutant both are of steric nature. In contrast, except for the high K<sub>M</sub><sub>NADPH</sub> value for βR425C, there was no evidence that α-Arg425 interacted directly with NADP(H) (Fig. 3D), although it should be close to the binding site according to the model. A likely explanation for this is that the side chain of α-Arg425 does not point toward the substrate. Thus, even a large molecule can react with the thiol group of the cfβR425C mutant without dramatically disturbing the ability of NADP(H) to bind and induce the conformational rearrangements that upon trypsin digestion yield the characteristic 30-kDa fragment. However, all mutants of α-Arg425 that have been characterized (Ref. 45; see Table I), exhibited a substantial loss of transhydrogenase activities. Although not essential, this residue may still be important for the catalytic mechanism of the enzyme.

A second set of cysteine mutations was introduced into ecIII, which in the nonmutant state is devoid of cysteines. As described in the Introduction, the rrI plus ecIII hybrid system offers a possibility to study properties such as substrate affinities, hydride equivalent transfer rates, substrate release rates, and domain I-domain III interactions in a semiquantitative manner. Mutants in domain III of the intact enzyme that were either difficult to study for various reasons (see below) or found to be particularly interesting were expressed as corresponding

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**FIG. 5.** The effects of NNAD(P) on cyclic reaction rates catalyzed by various ecIII cysteine mutants. The ecIII cysteine mutants were mixed with the NNAD(P) and diluted with dIdIII buffer to yield the mutant and the NNAD(P) concentrations indicated below. Incubations were carried out at room temperature. From the incubation mixtures, samples were withdrawn at the given times for activity measurements. Substrate concentrations were as follows: 100 μM NADP<sup>+</sup>, 400 μM AcPyAD<sup>1</sup>, and 300 μM NADH. The ecIIID392C mutant was preincubated for 10 min with rrI and NADP<sup>+</sup> prior to the measurements in order to achieve maximal activity. The concentrations of the mutants and NNAD(P) substrate analogues in the incubation mixtures and the rrI concentration added to the assay solution were as follows: 2.6 μM ecIIIH345C, 860 μM NNAD(P), and 1.2 μM rrI (A); 6.9 μM ecIIIA348C, 860 μM NNAD(P), and 0.79 μM rrI (B); 2.2 μM ecIII392C, 11 μM NNAD(P), and 0.79 μM rrI (C); 4.7 μM ecIIID392C, 110 μM NNAD(P), and 0.79 μM rrI (D). The AAD experiments are denoted by open circles, and the AADP experiments are shown by open squares. The initial activities were normalized to 1.
cysteine mutants in ecIII. The βHis345C mutant was previously shown to poorly assemble into membranes (42, 43) and was here found to be difficult to purify (not shown). None of the various β-Asp392 mutants analyzed so far catalyzed reverse transhydrogenation (44). Although not essential, βR350C seems to be an important residue for catalysis, but its role is unclear (43). Together with these three residues, β-Ala348 and β-Lys424 were also mutated for further characterization of the NADP(H) site.

All ecIII mutants displayed decreased affinities for NADP(H), which were reflected by the enhanced rates of reverse transhydrogenation (see below) and by the lower amounts of bound substrates (see “Results”). Possibly, the increased levels of reduced substrate bound to the purified mutant were also a consequence of increased release rates of NADP+. Note also that greater levels of rrf were required to reach half-maximal rates for the ecIII mutants than for the nonmutant ecIII and that the increased reverse reaction rates seemed to be correlated with this fact. It has been reported previously that for both rrf plus rriII (8) and rri plus ecIII mixtures (10), rrf can perform several rounds of the following cycle before NADP+ is released from ecIII: binding of NAD+, association with ecIII, transfer of the hydride equivalent from NADP on ecIII to NADP+ bound to rriII, dissociation from ecIII, and release of NADH. Thus, the less time NADP+ spends on the ecIII enzyme, the fewer ecIII each rrf can productively visit during the time of one turnover of ecIII. In summary, all mutants affected the binding site to various degrees, as anticipated from the model and in accordance with the results obtained for the cTH mutants.

The two mutant hybrid mixtures rrf plus ecIIIH345C and rri plus ecIIIID392C also catalyzed significantly lower cyclic reaction rates than the reference rrf plus ecIII mixture, about 25 and 18%, respectively (Table III). Since the cyclic reaction is at least partly limited by hydride equivalent transfer (10), which is also direct (46), it is possible that these mutations caused an unsuitable orientation of the substrates to give an efficient catalysis. All other mutants displayed only minor effects.

Of all the ecIII mutants, ecIIIID392C displayed the most dramatic difference from wild-type ecIII behavior. Most, if not all, of its properties can be explained by its decreased affinity for substrates, e.g. the fact that it was purified in the absence of bound substrates (Table II), the high rates of reverse transhydrogenation (Table III), and the 10-min incubation with NADP(H) required to achieve maximal rates. However, once the NADP(H) substrate was bound, it was able to transfer hydride equivalents to NADH bound to rriI. Also, it was interesting to note that the pH dependence of the reverse reaction catalyzed by rri plus ecIIIID392C was distinctly different from the pH profiles displayed by the wild-type rri plus ecIII and the other two mutant mixtures analyzed, i.e. rri plus ecIIIH345C and ecIIIRD395C (not shown). It has been hypothesized that βD392 could be directly involved in vectorial proton transport (44). The present results do not exclude this possibility.

The coupling between proton movement from the periplasmic side to the cytosolic side, on the one hand, and the production of NADPH and NAD+ from NADP+ and NADH, on the other hand, are probably governed by conformational changes. Binding of NADP(H) to dIII certainly gives rise to structural rearrangements (35). Furthermore, the conformation of ecIII has been reported to be dependent on whether the bound NADP(H) is oxidized or reduced (5). One or several residues that mediate this redox-dependent conformational change should therefore be located in close proximity to the nicotinamide ring of NADP(H). In an attempt to localize such residues, the cysteine-specific reactive NAD(P) analogues, diazotized AADP (36), were used to probe several cysteine mutants. Diazotized AADP caused a more rapid inhibition of the ecIIIAD348C, ecIIIIRD392C, and ecIIIIRD392C mutants than did diazotized AAD (see Fig. 5), supporting the possibility that these residues are located in or near the NADP(H)-binding site. Interestingly, ecIIIRD395C displayed a pronounced reactivity toward diazotized AADP. Judging from the model presented in Fig. 2, this result was surprising. One possibility is that β-Arg350 is not involved in the stabilization of the 2*-phosphate group as postulated (Ref. 2; Fig. 2 and see “Results”) and thus is misplaced in the model. If β-Arg350 is very close to the nicotinamide ring of NADP(H), it could have an important function in mediating a putative conformational change triggered by the redox state of the NADP(H) substrate (5). A second possibility is that the diazotized AADP molecule binds to the cysteine mutant as it approaches the site. A third, but less likely, possibility is that NADP(H) does not bind to ecIII as depicted in Fig. 2, which is the common mode of NADP(H) binding to classical ββαβα dinucleotide binding domains. These possibilities are presently being investigated.

In conclusion, theoretical predictions and a cysteine scanning approach proved to be a fruitful combination in order to yield a low resolution structure of the NADP(H)-binding site in energy-transducing transhydrogenase. The results suggest that dIII adopts a classical NAD(P)(H) binding fold, where the residues β-Ala348, β-Arg350, β-Ala390, β-Ala392, and β-Lys424 are in or near the NADP(H) site. β-Asp392 is a critical residue for appropriate substrate binding, and β-Arg350 may be close to the nicotinamide ring of NADP(H).

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