Mutation of Tyr^{235} in the NAD(H)-binding Subunit of the Proton-translocating Nicotinamide Nucleotide Transhydrogenase of Rhodospirillum rubrum Affects the Conformational Dynamics of a Mobile Loop and Lowers the Catalytic Activity of the Enzyme*

(Received for publication, December 1, 1995, and in revised form, February 5, 1996)

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The Tyr residue in the mobile loop region of the soluble, domain I polypeptide (called Ths) of the proton-translocating transhydrogenase from Rhodospirillum rubrum has been substituted by Asn and by Phe. The recombinant proteins were expressed at high levels in Escherichia coli and purified to homogeneity. The two well defined resonances at 6.82 and 7.12 ppm, observed in the one-dimensional proton NMR spectrum of wild-type protein, and previously attributed to the Tyr residue, were absent in both mutants. In the Tyr^{235} → Phe mutant Ths, they were replaced by two new resonances at 7.26 and 7.33 ppm, characteristic of a Phe residue. In both mutants, narrow resonances attributable to Met residues (and in the Tyr^{235} → Phe mutant, resonances attributable to Ala residues) were shifted relative to the wild type, but other features in the NMR spectra were unaffected. The conformational dynamics of the mobile loop closure in response to nucleotide binding by the protein were altered in the two mutants. The fluorescence emission from Trp^{72} was unaffected by both Tyr substitutions, and the fluorescence was still quenched by NADH. The mutant Ths proteins bound to chromatophore membranes depleted of their native Ths with undiminished affinity. In these reconstituted systems, the K_{m} values for thio-NADP^+ and NADH, during light-driven transhydrogenation, were similar to those of wild-type, but the kcat values were decreased about 2-fold. In reverse transhydrogenation, the K_{m} values for NADPH were slightly decreased in the mutants relative to wild-type, but those for acetyl pyridine adenine dinucleotide were increased about 10- and 13-fold, respectively, in the Tyr^{235} → Phe and Tyr^{235} → Asn mutants. It is concluded that Tyr^{235} may contribute to the process of nucleotide binding and that substitution of this residue prevents proper functioning of the mobile loop in catalysis.

In animal mitochondria and bacteria, transhydrogenase is driven in the direction of NADP^+ reduction by the protonmotive force generated through the action of respiratory (or photosynthetic) electron transport chains.

NADP^+ + NADH + H^+ → NADPH + NAD^+ + H^+ in

REACTION 1

Uniquely in Rhodospirillum rubrum, the NAD(H)-binding domain I of transhydrogenase exists as a separate polypeptide (1-3). This polypeptide can be expressed in large quantities in Escherichia coli and purified as a water-soluble protein (4). Like the native protein (called Ths), the recombinant form is dimeric and can restore transhydrogenation activity to everted membranes (chromatophores) of R. rubrum, which have been washed to remove native Ths. Ths binds NADH with a K_d of about 20 μM (4).

Domain I of transhydrogenase has a mobile loop straddling protease-sensitive sites (Lys^{227}-Thr^{228} and Lys^{237}-Glu^{238} in R. rubrum Ths). It is detectable by NMR, and its conformation is altered when the protein binds nucleotides (5). A Gly-Tyr-Ala motif (residues 234–236 in the R. rubrum protein) in this region is conserved in all known transhydrogenase sequences. It was proposed that 3,5 and 2,6 ring protons of Tyr in the motif give rise to resonances at 6.82 and 7.12 ppm, respectively, in the NMR spectrum of R. rubrum Ths and equivalent resonances in the spectrum of E. coli domain I protein (5). Here we test this hypothesis by examining mutants of R. rubrum Ths, in which Tyr^{235} has been substituted by Asn or Phe. Because the residue is conserved, and approaches nucleotide to within 0.5 nm in domain I-AMP complex (13), Tyr^{235} might have a role in catalysis. We examine the effect of Tyr^{235} → Asn and Tyr^{235} → Phe substitutions in Ths, on the binding of NADH, as judged by quenching of fluorescence of the lone Trp residue of the protein, on conformational dynamics of the loop during the binding of NAD(H) and analogues as determined by NMR, and on catalytic activity and Michaelis constants of mutant protein in forward (energy-linked) and reverse transhydrogenation after reconstitution with depleted R. rubrum membranes bearing domain I/III proteins of the enzyme. The results are compared with those in which the effect on reverse transhydrogenation activity of mutating the equivalent residue in E. coli enzyme was measured in membrane fractions (6).

MATERIALS AND METHODS

In Vitro Manipulation and Analysis of DNA, and Generation of Ths Mutants—Routine operations, including agarose-gel electrophoresis, preparation of plasmid DNA, growth and handling of E. coli strains, and preparation of competent cells and transformation, were carried

* This work was supported by the Wellcome Trust and the Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: Ths, the NAD(H)-binding peripheral membrane polypeptide (domain I) of transhydrogenase from R. rubrum; AcPDAD^+, acetyl pyridine adenine dinucleotide (oxidized form); Mops, 4-morpholinepropanesulfonic acid.
Mutants in Tyr^{235} of the Mobile Loop of Transhydrogenase

RESULTS

One-dimensional Proton NMR Spectra of Tyr^{235} \rightarrow \text{Asn and Tyr}^{235} \rightarrow \text{Phe} mutants of Ths—Mutant and wild-type R. rubrum Ths, the domain I polypeptide of transhydrogenase, were expressed in E. coli, and purified. During chromatography on QA-Trisacryl, Reactive-Green-19 agarose, and ACA-44 (1,4), both mutant proteins displayed similar elution profiles to wild-type Ths, (not shown). Notably, the proteins had an apparent molecular mass of approximately 74 kDa by non-denaturing gel filtration, indicating that, like the wild type, they are dimeric. On SDS-polyacrylamide gel electrophoresis, both mutant proteins displayed similar elution profiles to wild-type Ths (Fig. 1). Evidently, resonances from other Tyr residues (at positions 120, 146, and 154) are very broad, as expected for residues in a molecule having the correlation time of an 80-kDa protein like the Ths dimer. The appearance of resonances in the Tyr^{235} \rightarrow \text{Asn mutant, between 2.6 and 2.8 ppm, corresponding to the } \beta \text{Ch}_{2} \text{ of the Asn residue introduced into the loop, was obscured by those from diethiothreitol (and its breakdown products) in the sample. However, in the Tyr}^{235} \rightarrow \text{Phe mutant, new narrow resonances were observed at 7.26 and 7.33 ppm, characteristic of ring protons of Phe. These resonances were considerably sharper than those in wild-type protein at 7.33 ppm, which were also attributed to Phe (possibly Phe^{235}).}

Unexpectedly, the two Tyr^{235} substitutions both had effects on resonances previously assigned to the CH_{3} groups of Met residues (Fig. 1). The NMR spectra of wild-type Ths at 20 °C reveal sharp resonances attributable to Met residues at 1.97 and 2.04 ppm with shoulders at 2.06 and 2.08 ppm (5,13); they are designated MetA, MetB, MetC, and MetD, respectively (Table I). In the Tyr^{235} \rightarrow \text{Asn mutant, MetB was unaffected, but MetA was shifted downfield by 0.04 ppm, and MetC was shifted 0.02 ppm downfield to overlap with MetD at 2.08 ppm. In the Tyr^{235} \rightarrow \text{Phe mutant, MetB again was unaffected, MetA was again shifted downfield (but only by 0.02 ppm), and a slight downfield shift of MetC resulted in a peak at 2.065 ppm with a shoulder at 2.08 ppm (MetD, Table I). In the Tyr^{235} \rightarrow \text{Phe mutant, but not the Tyr}^{235} \rightarrow \text{Asn mutant, the region of the } \alpha \text{Ala residues was resolved into two distinct components (each possibly comprising one or more } \alpha \text{Ala } \beta \text{Ch}_{2} \text{ doublets). Note that trypsin treatment of wild-type protein also resulted in separation of two } \alpha \text{Ala components (5); the mobile loop region includes several } \alpha \text{Ala residues.}

Resonances other than those assigned to Tyr and Met (and, in the Tyr^{235} \rightarrow \text{Phe mutant, } \alpha \text{Ala}) were indistinguishable in mutant and wild-type proteins. Thus, the CH_{3} of Thr at 1.25 ppm and ring protons of Phe at 7.37 ppm (although these were obscured in the Tyr^{235} \rightarrow \text{Phe mutant), the tentative Gly CH_{3} at 3.96 ppm and Glu CH_{2} at 2.31 ppm, the resonances from unassigned amino acid residues at 7.6–7.9 ppm, fine structure superimposed on the broad methyl absorption at 0.8–1.0 ppm, and the ring-shifted methyl protons at approximately 0.17,
NMR, of amino acid residues in the mobile loop of wild-type Ths (5, 13). NMR spectra recorded in titrations of the Tyr235 mutants. 0.30, and 0.63 ppm were all essentially unchanged in both purified protein, no further additions; B, plus 30 μM NADH; C, plus 200 μM NADH; D, plus 200 μM NAD⁺.

The Effect of NAD⁺, NADH, and Analogues on NMR Spectra of Mutant Ths—The conformational dynamics, as revealed by NMR, of amino acid residues in the mobile loop of wild-type Ths during binding of NAD(H) and analogues have been discussed (5, 13). NMR spectra recorded in titrations of the Tyr235 → Asn and Tyr235 → Phe mutants with NADH and NAD⁺ are shown in Figs. 2 and 3, respectively. They reveal some differences from data for wild-type Ths.

In wild-type Ths, a two-step binding reaction is revealed in NMR spectra recorded during nucleotide titrations (5, 13). It is characterized by specific broadening of MetA at low concentrations of nucleotide, followed, at higher concentrations, by broadening of other resonances assigned to the mobile loop. In titrations with NADH the two-step reaction is barely perceptible at 20 °C, although easily resolved at 37 °C (13). In the Tyr235 → Phe mutant Ths (Fig. 3), a similar sequence of events was observed to that in wild-type. Two differences were: (a) the new Phe resonances at 7.26 and 7.33 ppm broadened during the titration in the same way that the Tyr resonances broadened in wild-type protein, and (b) of the two Ala resonances, split in the mutant, the more upfield was more sensitive to broadening by NADH. Probably because in the Tyr235 → Asn mutant the MetA resonance is displaced downfield in the absence of nucleotides (see Table I), the two-step binding reaction was clearly observed in NADH titrations even at 20 °C (Fig. 2).

Thus, 30 μM NADH led to more extensive broadening of MetA than, for example, the Ala or the Thr resonances. Higher concentrations (200 μM) did lead to broadening of the latter. The dependence of resonance broadening on NADH concentration in both mutants was similar to that with wild-type protein. NMR spectra recorded in titrations of mutant Ths with the analogue, AcPdADH, were qualitatively similar to those from NADH titrations (not shown).

Also reflecting ligand-protein interaction, linewidths of the NADH (and AcPdADH) resonances remained broad during titration against both of the mutant proteins until added nucleotide reached concentrations approaching 10⁻⁸ M (i.e. in considerable excess of protein concentration). Similar behavior was observed with wild-type protein and was suggested to result from decreased mobility of NADH in its protein-bound state and to an intermediate fast exchange (5).

In titrations of the mutant proteins with NAD⁺, the two-step reaction observed with wild-type protein (5) was again evident. Thus, moderately low concentrations of oxidized nucleotide had a specific effect on the MetA resonance, before other mobile loop resonances were broadened (Figs. 2 and 3). In the Tyr235 → Asn protein, in which the MetA resonance was displaced downfield (see above), addition of NAD⁺ led, not only to broadening, but also to a shift back upfield that was more extensive than that in wild-type Ths (Fig. 2; compare Ref. 5). Whereas the concentration dependence of resonance broadening was similar in NADH titrations for wild-type Ths and for mutant proteins, this was not the case with NAD⁺, where higher concentrations were required for both mutants to give the response observed in the wild type.

In marked contrast to the considerable broadening of NADH resonances in the presence of either wild-type (5, 13) or mutant Ths (above), NAD⁺ resonances became evident in the wild-type titration spectra even at quite low concentrations, consistent with a higher Kd value for oxidized nucleotide and faster exchange. In titrations with Tyr235 → Asn and Tyr235 → Phe mutants (Figs. 2D and 3D), NAD⁺ proton resonances were detectable at even lower concentrations of nucleotide, providing another indication of its weaker binding.

As with wild-type Ths, NMR spectra of the mutants titrated with AcPdAD⁺ and with 5′-AMP were similar to those with NAD⁺ (not shown). Notably, they revealed a two-step binding process; the MetA resonance was affected at lower concentrations of nucleotide than the other loop resonances. As with NAD⁺, higher concentrations of both AcPdAD⁺ and 5′-AMP were required with both mutant proteins to produce an equivalent broadening of the narrow resonances, and again, nucleotide resonances were resolved at lower concentrations in the mutant than in wild-type titrations.

Fluorescence Properties of the Tyr235 → Asn and Tyr235 → Phe Mutants—Fluorescence from the sole Trp residue at position 72 in wild-type Ths has an extremely small Stokes shift, and a monoexponential decay with a long lifetime, indicating that it lies in a rigid, non-polar environment (4). The Tyr235 → Asn and the Tyr235 → Phe mutants of Ths have similar emission spectra, establishing that the environment of the Trp in the protein is unchanged by amino acid substitutions (data not shown).

As in wild-type protein, fluorescence from Trp72 in the Tyr235
The quenching of fluorescence of Trp72 in mutant Ths by nucleotides. Experiments were carried out in a medium containing 10 mM Tris-HCl, pH 8.0, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol. The inner-filtering effect of the nucleotides were corrected, as described. ●, wild-type Ths; ○, AcPdADH; △, NAD⁺; ■, 0.6 μM Tyr₂³⁵→Asn mutant; □, 0.6 μM Tyr₂³⁵→Phe mutant.

Fig. 4. The quenching of fluorescence of Trp72 in mutant Ths by nucleotides. Experiments were carried out in a medium containing 10 mM Tris-HCl, pH 8.0, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol. The inner-filtering effect of the nucleotides were corrected, as described. ●, wild-type Ths; ○, AcPdADH; △, NAD⁺; A, 0.6 μM Tyr₂³⁵→Asn mutant; B, 0.6 μM Tyr₂³⁵→Phe mutant.

Fig. 5. Reconstitution of depleted membranes with wild-type and mutant Ths. Chromatophores from a strain of R. rubrum that overexpresses wild-type transhydrogenase were washed with concentrated salt to remove their native Ths (see "Materials and Methods"). The washed membranes were resuspended (to a final concentration of 0.6 μM bacteriochlorophyll) in 100 mM Mops, pH 7.2, 50 mM KCl, 2 mM MgCl₂, 1 μM carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone. Ths was added to give the final concentration shown. The reduction of AcPdAD⁺ was measured at 375–450 nm. [ ], wild-type Ths; ○, the Tyr₂³⁵→Asn mutant; ●, the Tyr₂³⁵→Phe mutant.

only observed enzyme activity of Ths when it is associated with domain I/III components of transhydrogenase.

The ability of wild-type and mutant Ths to reconstitute reverse transhydrogenation activity to R. rubrum membranes depleted of native Ths is compared in Fig. 5. Depleted membranes were prepared by salt washing chromatophores isolated from a strain of R. rubrum that overexpresses wild-type transhydrogenase (see "Materials and Methods"). Experiments were performed with close-to-saturating concentrations of nucleotide substrates. Rates of reverse transhydrogenation with the Tyr₂³⁵→Asn and Tyr₂³⁵→Phe mutants of Ths were about 18% and 44%, respectively, of wild-type protein, but docking affinities revealed by double-reciprocal plots (data not shown) were undiminished.

Dependences of the rate of reverse transhydrogenation on the concentration of AcPdAD⁺ (saturating NADPH) in the reconstituted systems of depleted chromatophores, and either wild-type or mutant Ths, are shown in Fig. 6A. Double-reciprocal plots (not shown) yielded Kₘ values for AcPdAD⁺ of approximately 800, 600, and 60 μM in the Tyr₂³⁵→Asn and Tyr₂³⁵→Phe mutants and wild type, respectively.

Because the Kₘ values for AcPdAD⁺ in the mutants were high, it was not practicable to carry out experiments with saturating concentrations of this nucleotide. Thus, Fig. 6B shows the dependence of reverse transhydrogenation rate on NADPH concentration at 1.1 mM AcPdAD⁺. Double-reciprocal plots (not shown) gave an approximate apparent Kₘ for NADPH of 15 μM for the Tyr₂³⁵→Asn mutant, 15 μM for the Tyr₂³⁵→Phe mutant, and 30 μM for wild-type Ths.

Rates of light-driven reduction of thio-NAD⁺ by NADH (forward transhydrogenation) in depleted chromatophores reconstituted, either with mutant or wild-type Ths, were also investigated (Fig. 7). The light drives photosynthetic electron transport, generating a proton electrochemical gradient, which leads to enhanced proton flux through transhydrogenase in its physiological (forward) direction. Depleted membranes were prepared from wild-type chromatophores washed under mild conditions to remove Ths whilst preserving coupling activity. Because the overexpressing strain could not be used, the level of accuracy was lower than in Fig. 6. Fig. 7 shows that maxi-

The Effect of Substituting Tyr₂³⁵ of Ths with Asn and with Phe on Catalytic Properties of Reconstituted Transhydrogenase Complex—Isolated Ths does not catalyze transhydrogenation between NADPH and AcPdAD⁺ (1, 4) or between NADH and AcPdAD⁺ (data not shown). The suggestion (19) that reduction of AcPdAD⁺ by NADH in intact transhydrogenase takes place between the domain I polypeptides is therefore unlikely. Other explanations for this reaction are possible (20, 21). We have...
mutual rates of light-driven forward transhydrogenation in reconstituted systems were about 2-fold lower for both mutants than for wild-type Ths. Dependences of rates of forward transhydrogenation on nucleotide concentrations (Fig. 7, A and B) show that in both mutants the $K_m$ values for thio-NADP$^+$ and for NADH were not significantly different from wild-type $K_m$ values of approximately 5 and 4 mM, respectively.

Fig. 8 shows that mutant Ths proteins displaced the wild-type protein from its binding site on chromatophores. Addition of wild-type Ths to chromatophores led to a small increase in the rate of reverse transhydrogenation, presumably because some domain I protein was lost from the membranes during preparation (1, 4). However, addition of either the Tyr235<sup>3</sup>Asn or the Tyr235<sup>3</sup>Phe mutant Ths resulted in substantial loss of activity, indicating that association-dissociation of domain I with domains II/III of transhydrogenase can occur on the time scale of the experiment.

**DISCUSSION**

By substituting Tyr<sup>235</sup> of wild-type Ths with Phe and Asn, we have tested our prediction that $^1$H NMR signals at 6.82 and 7.12 ppm in the wild-type protein are attributable to that residue. Complete loss of those resonances from the spectra of both mutants unambiguously confirms the assignment. New, well-defined resonances at 7.26 and 7.33 ppm in the Tyr226<sup>3</sup>Phe mutant are characteristic of Phe ring protons, and further indicate that the Phe has adopted the mobile nature of the original Tyr. The emergence of new resonances in the Tyr226<sup>3</sup>Asn mutant was masked by dithiothreitol present in the sample.
Substitution of Tyr\textsuperscript{235} for Asn also led to changes in resonances assigned to Met residues, notably a marked downfield shift of MetA and a smaller shift in MetC. The assignment of these residues is not yet possible, but is pertinent because the behavior of the MetA resonance reflects events at an intermediate stage of nucleotide binding (Refs. 5 and 13; see below). It was suggested (5) that MetA might derive from Met\textsuperscript{239}, and experiments are now in progress to test this. Substitution of Tyr\textsuperscript{235} for Phe also led to shifts of the MetA and MetC resonances and to separation of Ala resonances. The fact that the amino acid residue at position 235 influences the NMR-detectable Met and Ala residues indicates that there is structural organization in the loop even in the absence of nucleotides, but the nature of the interactions is not understood. Evidently protons of the Ala, MetA, and MetC residues can sample more than one environment on the NMR time scale; the effect of the Tyr\textsuperscript{235} substitution might be to alter the exchange rate between different conformations, or it might result in changes in the chemical shift of the Met and Ala resonances in one of the conformational states, e.g. by altering positions of charged or aromatic groups relative to methyl groups of the amino acid residues.

The change from Tyr\textsuperscript{235} to Asn or to Phe in Th\textsubscript{H} is not accompanied by gross changes in molecular structure: the protein retains its ability to form dimers and to dock with the domain II/III components of transhydrogenase, the short-wavelength emission of Trp\textsubscript{72} is preserved, and, on the basis of NMR spectra, the protein fold and environments of amino acids in the mobile loop (with the exception of MetA, MetC, and Ala residues) are unaffected. Thus, effects of the mutations on nucleotide binding and catalytic properties of the enzyme are likely to be a direct consequence of altered properties of the loop.

For both mutants, higher concentrations of NAD\textsuperscript{+} than for wild-type Th\textsubscript{H} were required to broaden resonances ascribed to the mobile loop. This might mean either (a) that the \( K_m \) for NAD\textsuperscript{+} is increased by the amino acid substitution, or (b) that differences in exchange rate(s) between Th\textsubscript{H}, Th\textsubscript{H}–NAD\textsuperscript{+}, and Th\textsubscript{H}–NAD\textsuperscript{−} (see (5)) alter linewidths without affecting the affinity for nucleotide. Similar observations and interpretations apply also to AcPdAD\textsuperscript{+} and 5′-AMP in wild-type and mutants. The very large \( K_m \) for AcPdAD\textsuperscript{+} of both the mutant proteins, relative to the wild-type, during reverse transhydrogenation (after reconstitution with Th\textsubscript{H}–depleted membranes) might be another indication of increased \( K_m \) for oxidized nucleotide. Thus it is possible that Tyr\textsuperscript{235} contributes to the binding affinity of Th\textsubscript{H} for NAD\textsuperscript{+}, AcPdAD\textsuperscript{+}, and 5′-AMP. This is consistent with the observation that, in the two-dimensional "H NMR spectrum of wild-type 5′-AMP-Th\textsubscript{H} complex, NOE interactions were detected between Tyr\textsuperscript{235} and bound nucleotide (13).

It cannot be determined with confidence whether or not the Tyr\textsuperscript{235} → Asn or Tyr\textsuperscript{235} → Phe mutations had a significant effect on binding of reduced nucleotides by Th\textsubscript{H}. There were no clear differences between the mutant and the wild-type proteins in either the \( K_m \) values for NAD\textsuperscript{+} in forward transhydrogenation (Fig. 7) or the dependences on NADH of the protein-NMR spectra (Figs. 2 and 3), but neither of these gives an unambiguous indication of the \( K_m \). The quenching of Trp\textsubscript{72} fluorescence by reduced nucleotides (Fig. 4) must also be interpreted with care; because of inner filtering, \( K_m \) becomes more subject to error as its value increases beyond 10–20 \( \mu \text{M} \). However, it is reasonable to conclude that substituting Tyr\textsuperscript{235} with either Asn or Phe does not have a large effect on the affinity of Th\textsubscript{H} for NADH or AcPdADH. The fact that, in NADH titrations, the new Phe resonances in the Tyr\textsuperscript{235} → Phe mutant broadened in a similar way to Tyr resonances in wild-type Th\textsubscript{H} indicates that the residue can participate in mobile loop closure either with, or without, the 4-OH group. There were minor differences in the NMR spectra recorded in NADH titrations that arose from the fact that alteration of Tyr\textsuperscript{235} caused shifts in Met and Ala resonances (see above), but evidently the perturbations were not enough greatly to affect binding affinity or loop closure.

Mutation of Tyr\textsuperscript{235} to either Asn or Phe led to decreases in \( K_m \) for both forward and reverse transhydrogenation, when the Th\textsubscript{H} was reconstituted with depleted membranes (Figs. 6 and 7). The loop clearly has a role in catalysis in addition to its fine-tuning effect on the binding affinity of the protein for nucleotide. Whether this is in the hydride transfer reaction, or in conformational coupling with domain II/III components of transhydrogenase, is not known. Although the Tyr\textsuperscript{235} → Phe mutant Th\textsubscript{H} had a substantially decreased activity, the Tyr\textsuperscript{235} → Asn protein was considerably more inhibited, particularly in reverse transhydrogenation. This might indicate that both the aromatic ring and the 4-OH group of Tyr\textsuperscript{235} are important in the conformational dynamics of the loop in catalysis by wild-type protein.

The equivalent of Tyr\textsuperscript{235} in bovine transhydrogenase (Tyr\textsuperscript{245}) is sensitive to modification by 5′-[\text{fluoresulfonyl}l]benzoyl]adenosine (22). Modified enzyme had reduced catalytic activity; NADH protected against modification. On the basis of this, the equivalent residue in E. coli transhydrogenase (Tyr\textsuperscript{226}) was substituted with His, Leu, Phe, and Asn (6). In crude membrane fractions isolated from bacteria carrying the mutation, specific activities (mg\textsuperscript{-1} membrane protein) of AcPdAD\textsuperscript{+} reduction by NADPH were 33% (or 51% in another strain), 38%, 45% and 42%, respectively, lower than rates in membrane fractions prepared from bacteria carrying wild-type transhydrogenase gene; \( K_m \) values for AcPdAD\textsuperscript{+} for the His, Leu, and Phe mutant membranes were, respectively, 3-, 19-, and 3-fold larger than those in wild-type membranes. Comparison between mutant and wild-type transhydrogenase in that study is complicated by uncertainty about the level of expression of the enzyme; the transhydrogenase content of the bacterial membranes was assessed from their appearance on SDS-polyacrylamide gels. Nevertheless, results of those experiments are broadly consistent with results reported here. In our experiments the statistical significance is assured because we used Th\textsubscript{H} purified to homogeneity, and the same preparation of membranes for reconstitutions with both wild-type and mutant proteins. Because the domain I protein of E. coli does not exist as a discrete polypeptide, this strategy is unavailable in that system. In our experiments, reverse transhydrogenation activities of Tyr\textsuperscript{235} mutants of R. rubrum transhydrogenase were more inhibited, relative to wild-type, than Tyr\textsuperscript{226} mutants of E. coli transhydrogenase, and \( K_m \) values of the mutants were increased by a much larger factor. There might also be real species differences between the two enzymes. The GYA motif of the loop (5) is conserved in known transhydrogenase sequences, but there is only low homology among other residues in the region; there are no other invariant amino acid residues, although small and charged residues preponderate. There is often greater variation in amino acid sequence of surface loops because individual residues make only a small contribution to the global structure. It is likely in transhydrogenase that, during closure, multiple contacts are made between the loop and the rest of the protein or bound nucleotide, and therefore, in individual species, single amino acid substitutions in the loop might have greater or lesser effects on catalysis. Uniquely in the R. rubrum enzyme effects of mutations on nucleotide binding, interaction between domains, conformational dynamics of the mobile loop, and the Michaelis parameters can be assessed separately. The present
experiments establish that Tyr$^{235}$ is important in the dynamics of mobile loop closure, and that substitution of the residue profoundly affects the Michaelis parameters, thus, for the first time, establishing a pivotal role for loop closure in catalysis.

Acknowledgments—We are grateful to Drs. John Hobman and Ken Jakeman for their help in the mutagenesis procedure, and for bacterial strains and plasmids, and to Dr. Barry A. Levine for continued advice and encouragement on the NMR spectroscopy.

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*J. Biol. Chem.* 1996, 271:10109-10115.  
doi: 10.1074/jbc.271.17.10109

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