The Role of Invariant Amino Acid Residues at the Hydride Transfer Site of Proton-translocating Transhydrogenase*

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Transhydrogenase couples proton translocation across a membrane to hydride transfer between NADH and NADP+. Previous x-ray structures of complexes of the nucleotide-binding components of transhydrogenase ("dI2-dIII1" complexes) indicate that the dihydronicotinamide ring of NADH can move from a distal position relative to the nicotinamide ring of NADP+ to a proximal position. The movement might be responsible for gating hydride transfer during proton translocation. We have mutated three invariant amino acids, Arg-127, Asp-135, and Ser-138, in the NAD(H)-binding site of Rhodospirillum rubrum transhydrogenase. In each mutant, turnover by the intact enzyme is strongly inhibited. Stopped-flow experiments using dI2-dIII1 complexes show that inhibition results from a block in the steps associated with hydride transfer. Mutation of Asp-127 and Ser-138 had no effect on the binding affinity of either NAD+ or NADH, but mutation of Arg-127 led to much weaker binding of NADH and slightly weaker binding of NAD+. X-ray structures of dI2-dIII1 complexes carrying the mutations showed that their effects were restricted to the locality of the bound NAD(H). The results are consistent with the suggestion that in wild-type protein movement of the Arg-127 side chain, and its hydrogen bonding to Asp-135 and Ser-138, stabilizes the dihydronicotinamide of NADH in the proximal position for hydride transfer.

The transport of substances by proteins across membranes is important in many biological processes. In some cases the transport reaction is energetically coupled to a chemical transformation step. The coupling mechanism may involve large conformational changes within the transport protein (1–3), but how the transport and the chemical transformation reactions reversibly propagate the conformational change in individual proteins is the subject of intensive research.

Transhydrogenase is found in the inner membranes of animal mitochondria and the cytoplasmic membranes of bacteria. It couples the transport of protons to the redox (hydride transfer) reaction between NAD(H) and NADP(H) as shown in Reaction 1.

\[
\text{NADH + NADP}^+ + \text{H}^+_\text{out} \leftrightarrow \text{NAD}^+ + \text{NADPH} + \text{H}^+_\text{in}
\]

**REACTION 1**

Under most physiological conditions, transhydrogenase is driven toward the reduction of NADP+ by NADH through the utilization of a transmembrane proton electrochemical gradient (Δp) generated by the respiratory (or sometimes photosynthetic) electron transport chain. In different cells and tissues, the enzyme is responsible for producing the NADPH needed for either biosynthesis or glutathione reduction (4–6). Acting together with the isocitrate dehydrogenases, transhydrogenase may also participate in the regulation of the tricarboxylic acid cycle (7).

A schematic of intact transhydrogenase is shown in Fig. 1. The dI3 component of the enzyme binds NADH; dII1 binds NADP+, and dI1 spans the membrane. Intact transhydrogenase is a "dimer" of two dI1-dIII1 "monomers" (although there is variability in the polypeptide organization in different species). A hydride transfer site is located at the interface between dI and dIII of each monomer, some distance (>30 Å) from the proton translocation pathway through dI1. It is thought that protonation/deprotonation reactions associated with translocation shift the enzyme monomers alternately between a conformation in which hydride transfer is blocked and one in which hydride transfer is allowed. A switch in proton access to the translocation machinery from one side of the membrane to the other is effected by conformational changes linked to the redox state of the bound NADP(H) (8, 9).

Mixtures of separately purified dI1 and dIII1 readily form a dI1-dIII1 complex (see Fig. 1), which provides a useful experimental system for investigating the conformational changes associated with the hydride transfer reaction (10–15). The nucleotide-binding properties of the complex and the transient state kinetics of the redox reaction have been described in some detail (13, 16). The Rhodospirillum rubrum transhydrogenase has been particularly suitable for these analyses and, furthermore, has yielded high resolution structures of isolated dI1 and dIII1 and of the dI1-dIII1 complex in different nucleotide-bound forms (17–22). In mammals, the structure of isolated dIII1 has been solved (23, 24), and in Escherichia coli the structure of isolated dI1 has been solved (25).

Experiments showed that hydride transfer between the two nucleotides bound to transhydrogenase is direct and does not involve any intermediate redox steps (26). The x-ray structures of the dI1-dIII1 complex indicate that conformational changes can occur in NAD(H) and the NAD(H)-binding pocket in the hydride transfer site (18, 20). In the different nucleotide conformations, the adenosine moiety of the nucleotide remains relatively fixed in its binding pocket, but the Nic(H) ring occupies different positions because of bond rotations in the linking pyrophosphate and ribose groups. In the "proximal" position, the NicH ring of NADH can align with the Nic- of NADP+ to effect rapid, direct hydride transfer between the respective C-4(N) atoms. However in the "distal" position, the Nic- and NicH rings are too far apart for hydride transfer.

The on-line version of this article (available at http://www.jbc.org) contains supplementary Figs. S1–54.

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* The abbreviations used are: dI, the NAD(H)-binding component of transhydrogenase; Nic-, nicotinamide (oxidized form); AcPdAD, acetyl pyridine adenine dinucleotide (oxidized form); dI1-dIII1, the NADP(H)-binding component; dI, the membrane-spanning component; dI1R127A, dI protein in which Arg-127 is replaced with Ala, etc.; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; PDB, Protein Data Bank; NAD(H), indicates both NAD+ and NADH etc.
transfer. It was proposed that the change in conformation of the NAD(H) is important in gating the hydride transfer reaction during coupling to proton translocation. Charged and polar side chains of amino acid residues close to the Nic(H) rings appear to participate in the reaction in ways that are not yet clear. The invariant Arg-127 in the “RQD loop” (residues 126–136) of *R. rubrum* dI is of particular interest because, in different x-ray structures, the orientation of its side chain changes in parallel with alterations in the position of the Nic(H) ring of bound NAD(H). These concerted movements affect sets of H-bond and van der Waals interactions between the arginine side chain, the nucleotide, and the side chains of other conserved amino acid residues at the hydride transfer site. Notable among these is the invariant Asp-135, which also contacts the Nic(H) ring in some structures, and which can interact with the invariant Ser-138. Asp-135 can also form an H-bond with invariant Gln-132; this residue can also interact with the Nic(H) ring of NAD(H) and also with the 2′-OH group of the Nic-ribose of the NADP(H) bound to dIII. The x-ray structures indicate that the side chain of Gln-132 may undergo changes in configuration, and mutation of this residue was found to inhibit hydride transfer (27). The emerging picture is of critical, local conformational changes at the hydride transfer site that are primary events in the reception/generation of the long distance conformational changes that couple the redox reaction to proton translocation (9). In this study we describe the effects of mutating Arg-127, Asp-135, and Ser-138 on nucleotide binding affinity, the kinetics of hydride transfer, and the x-ray structure of the dI⋅dII complex. It is concluded that these amino acid residues have an important role in correctly positioning the Nic(H) ring of NAD(H) for hydride transfer.

**EXPERIMENTAL PROCEDURES**

Recombinant dI, its mutant, dI.Q132N, dII, and its mutant, dIII.E155W, from *R. rubrum* transhydrogenase were expressed from the plasmids pCD1 (28), pGVB1 (27), pNIC2 (29), and pJDV1 (30), respectively, in appropriate strains of *E. coli* after induction with isopropyl β-D-thiogalactoside. The proteins were purified from the harvested cells by column chromatography and stored, as described in the earlier reports.

The plasmid, pCD1, was subjected to site-directed mutagenesis using the Stratagene Quikchange kit and DNA primers supplied by AltaBioscience. The resulting new plasmids pGVB3, pGVB4, pGVB2, and pH25 encoded dLR127A, dLR127M, dLD135N, and dLS138A, respectively. The DNA of all plasmids was sequenced to confirm there were no errors introduced by the PCR. The plasmids were then used to transform cells of *E. coli* C600. The cells were grown and induced, and the dI proteins were purified and stored, as described for wild-type and for dI.Q132N. Because the mutant proteins were inactive (see “Results”), fractions were selected during column chromatography by analysis using SDS-PAGE; the enriched fractions were easily detectable from the intense, stained band (PAGE Blue-83, Fluka) at ~40 kDa. Typically, 100–200 mg of pure protein were obtained from 3.0 liters of bacterial culture. Protein concentration was determined by the microtanning procedure (31) and is expressed as dI monomers unless otherwise stated.

Before use, the dI proteins were thawed on ice and either used directly or concentrated in Vivaspin centrifugal filters (10-kDa cutoff). Similarly thawed dII and III proteins were used directly or were concentrated in 5-kDa cut-off filters, and if indicated, they were treated completely to oxidize their bound nucleotide or to replace it with NADPH (13).

*R. rubrum* was grown under photosynthetic conditions, as described (32). The cells were harvested, washed, and then disrupted in a French press in 100 mM Tris-HCl, pH 8.0, 10% (w/v) sucrose (chromatophore buffer). Everted membrane vesicles (chromatophores) were separated by differential centrifugation. The vesicles were diluted into chromatophore buffer and washed repeatedly by centrifugation until their transhydrogenation activity was lowered by >90%. This procedure removes the dI polypeptide of the transhydrogenase. The vesicles were stored at −20 °C in chromatophore buffer containing 50% (w/v) glycerol. The bacteriochlorophyll concentration of the vesicles was measured using the in vivo extinction coefficient of 140 mε cm⁻¹ at 880 nm (33).

Steady-state transhydrogenation was measured (in the reverse direction, see Reaction 1) as the reductive dehydrogenase and was purified, as described (34, 35). Control experiments were performed in parallel with [4-2H]NADP⁺ (an analogue of NAD⁺) by NADPH at 375 nm (ε = 6.1 mM⁻¹ cm⁻¹) on a Shimadzu UV-2401 spectrophotometer. "Cyclic transhydrogenation" was measured as the reduction of AcPdAD⁺ by NADPH in the presence of bound NADP⁺/NADPH.

Binding affinities between dI and dIII and between dI and NADH were determined by isothermal titration calorimetry (16) using a Microcal MCS at 20 °C and were analyzed using ORIGIN software as supplied by the manufacturer. Before use, purified proteins were dialyzed for 3 h against 20 mM Hepes, pH 8.0, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol at 4 °C and then concentrated by filtration (see above). The binding of NADH to dI proteins was followed by the fluorescence change of Trp-72 at 310 nm with 280 nm excitation on a Spex Fluoromax with 4 nm slits (28).

Stopped-flow absorbance and fluorescence changes were recorded on an Applied Photophysics DX-17MV. The mixing dead time of the instrument was 1.31 ms (26). The reduction of AcPdAD⁺ by NADPH was measured in absorbance mode at 375 nm (5 nm slits, 2 nm optical path length) during stopped-flow after 1:1 mixing. [4S-2H]NADPH (formerly described as [4B-2H]NADPH) was freshly prepared using 98% deuterated glucose (Aldrich), hexokinase, and glucose-6-phosphate dehydrogenase and was purified, as described (34, 35). Control experiments were performed in parallel with [4S-2H]NADPH that was freshly prepared (with undeuterated glucose) and purified in the same way. The reduction of NADP⁺ by NADH was measured by following the Trp fluorescence change in dI.dIII.E155W complexes on the DX-17MV. The excitation light was at 280 nm (selected with the instrument’s monochromator, 9.3 nm slits), and the emission was collected through a WG305 cut-off filter. Previous experiments have shown that fluoro-
The programs MOSFLM (37), XDS (38), and SCALA (39). The data were integrated and scaled using the program EPMR (40). The \( \text{(dI.R127A)}_{2}\text{(dIII.E155W)}_{1} \) and wild type, and initial phases were obtained by molecular replacement using CNS (42). The refinement characteristics are summarized in Table 1.

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### TABLE 1

| Data collection statistics\(^a\) | \((\text{dL.R127A})_{2}\text{dIII}\) | \((\text{dL.D135N})_{3}\text{dLLE155W}\) | \((\text{dL.S138A})_{2}\text{dIII}\) |
|---|---|---|---|
| Resolution (Å) | 47.7–2.6 (2.74–2.60) | 31.4–2.3 (2.42–2.30) | 45.9–3.2 (3.37–3.20) |
| Completeness (%) | 98.5 (92.1) | 100 (100) | 98.9 (99.2) |
| Multiplicity | 4.9 (2.8) | 4.5 (4.5) | 3.9 (3.9) |
| \(I/IP\) | 11.4 (2.2) | 5.7 (2.2) | 13.4 (4.0) |
| No. of observations | 133,821 (10,211) | 223,382 (32,580) | 70,869 (10,414) |
| No. of unique observations | 27,222 (3653) | 50,177 (7207) | 18,400 (2660) |
| \(R_{\text{merge}}\) | 11.7 (44.2) | 6.6 (34) | 7.2 (32.6) |

**Refinement statistics**

- **Wilson B/average B**: 41.3/50.3, 43.1/53.1, 75.5/106
- **No. of observations**: 133,821 (10,211), 223,382 (32,580), 70,869 (10,414)
- **Resolution (Å)**: 47.7–2.6 (2.74–2.60), 31.4–2.3 (2.42–2.30), 45.9–3.2 (3.37–3.20)
- **No. of non-hydrogen atoms/waters**: 6601/56, 7171/382, 6766/2
- **r.m.s.d.** (Å/angle (degrees)): 0.007/1.163, 0.007/1.184, 0.007/1.227
- **Ramachandran\(^b\) (%)**: 88.4, 11.5, 0.1, 0.0, 93.2, 6.8, 0.0, 0.0, 89.0, 10.8, 0.1, 0.1
- **R/\(R_{\text{free}}\)**: 0.25/0.30, 0.20/0.24, 0.21/0.27

**Assembly of Mutant dL Proteins into Intact Transhydrogenase and dLdIII Complexes and Measurements of Hydride Transfer Rates**—Arg-127 in the dL component of \( R.\) rubrum transhydrogenase was mutated to Ala (dL.R127A) and to Met (dL.R127M); Asp-135 was mutated to Asn (dL.D135N) and Ser-138 to Ala (dL.S138A). All the mutant dL proteins were produced at high levels in appropriately induced cells of \( E.\) coli, and they displayed similar elution profiles to wild-type dL during purification by column chromatography.

The dL component of transhydrogenase can be removed from everted membrane vesicles of \( R.\) rubrum by repeated centrifugation washing (32, 43). The separated supernatant and membrane fractions are devoid of transhydrogenase activity, but addition of either native or recombinant wild-type dL to the depleted membranes leads to complete recovery (28) (Fig. 2A). None of the mutant dL proteins gave a significant increase in the transhydrogenation rate upon addition to dL-depleted membranes. When transhydrogenation was partially restored to depleted membranes with a low concentration of wild-type dL, the subsequent addition of any of the mutant proteins led to inhibition. Fig. 2A shows illustrative data for dL.D135N; data for other mutants are shown in supplemental Figs. S1A and S2A. These results indicate that the dL mutants can bind to exposed dL/dIII sites on the cytoplasmic face of the bacterial membranes but that these amino acid substitutions compromise the activity of the resultant enzyme.

In dLdIII complexes, the steady-state rates of forward and reverse transhydrogenation are extremely slow and therefore difficult to measure accurately; they are profoundly limited by the rates of product NADPH (or NADP\(^+\)) release (12). However, steady-state "cyclic" transhydrogenation catalyzed by complexes made from wild-type dL and dIII is fast. The cyclic reaction involves reduction of NADP\(^+\) by NADH, followed by oxidation of NADPH by AcPdAD\(^+\), and does not require release of either NADP\(^+\) or NADPH from the dII-binding site (44). The dL-dIII complexes are made simply by mixing the two proteins. Mixtures of any of the double mutants were unable to catalyze the cyclic reaction at significant rates. However, the addition of any of the mutant dL proteins to wild-type complex inhibited the rate of reaction. Fig. 2B presents data for dL.D135N, and data for other mutants are shown in supplemental Figs. S1B and S2B. The results indicate that the mutant proteins can displace the wild-type dL from binding sites on dIII (to give an inactive complex) and again establishes their structural integrity.

The binding reactions between dL mutants and wild-type dL were also investigated by isothermal titration calorimetry. The heat changes recorded during additions of mutant dL (dL.R127A, dL.R127M, dL.D135N and dL.S138A) to a solution of dIII were very similar to those observed with wild-type protein (16), see supplemental Fig. S3. In all cases the heat changes indicated a binding stoichiometry of approximately two dL polypeptides to one dIII, reflecting the composition of the complex.
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binding affinity was high for all the mutant dl-dIII complexes and in each case was indistinguishable from the wild-type complex, with $K_d$ values ≤ 60 nM; the lower limit is set by the high concentration of proteins required to observe measurable heat changes.

Wild-type dl-dIII complexes catalyze a single turnover burst of rapid hydride transfer before the steady-state rate slows due to the limiting dissociation of either NADPH (forward reaction) or NADP$^+$ (reverse reaction). The apparent first-order rate constant ($k_{app}$) for the conveniently measured burst of reaction between AcPdAD$^+$ and NADPH in the stopped-flow spectrophotometer is $\sim$600 s$^{-1}$ (26). At saturating concentrations of AcPdAD$^+$ (13), $k_{app}$ is partly determined by a conformational change following binding and partly by the hydride transfer rate itself (27, 45). At protein concentrations that ensured virtually complete formation of complexes (see above), $k_{app}$ was greatly decreased by amino acid substitution of Arg-127, Asp-135, or Ser-138. The experimental trace for (dl.S138A)$_2$dIII complexes is shown in Fig. 3A; the traces for wild-type complexes and (dl.D135N)$_2$dIII complexes are shown in the supplemental Fig. S4. Inhibition was most pronounced in (dl.R127A)$_2$dIII and (dl.R127M)$_2$dIII complexes. Here the rate of reaction was not significantly greater than nonenzymic reduction of AcPdAD$^+$ by NADPH, and therefore, no single turnover burst of reaction was discernible (data not shown). For reasons that will become clear below, the experiments with these two complexes were performed at high (5 mM) AcPdAD$^+$ concentrations (30-fold greater than was required to give half-maximal $k_{app}$ values for the burst in wild-type complexes). Given the signal/noise ratio on the stopped-flow trace, it was concluded that these mutations of Arg-127 inhibit hydride transfer (and/or any associated conformational change consequent upon AcPdAD$^+$ binding) by $\geq$10$^3$. Experiments with (dl.D135N)$_2$dIII and (dl.S138A)$_2$dIII complexes did reveal a single turnover burst of hydride transfer from NADPH to AcPdAD$^+$, but one that was greatly inhibited relative to the wild-type complexes. The $k_{app}$ at close-to-saturating AcPdAD$^+$ concentrations was 0.04 and 23 s$^{-1}$ for (dl.D135N)$_2$dIII and (dl.S138A)$_2$dIII, respectively, which represents a decrease of about 10$^4$- and 26-fold relative to wild type. The dependence of $k_{app}$ for the burst on the concentration of AcPdAD$^+$ indicated that half-maximal values were obtained at higher AcPdAD$^+$ concentrations ($\approx$0.5 and 1.0 mM for (dl.D135N)$_2$dIII and (dl.S138A)$_2$dIII, respectively) than with the wild-type complex ($\approx$0.15 mM (13)), data not shown. If the binding of AcPdAD$^+$ is fast relative to hydride transfer and associated conformational changes, these values reflect the equilibrium binding constant ($K_d$) of

![FIGURE 2. Reconstitution of R. rubrum transhydrogenase with wild-type dl and with dl.D135N. A, reconstitution of dl-depleted R. rubrum membranes. The membranes were resuspended to 10$^{-5}$ bacteria/bacteriophyll in 50 mM Mops, 50 mM KCl, and 2 mM MgCl$_2$, pH 7.2, at 25 °C. The dl component was added, and the suspension was then supplemented with 200 nM NADPH and 200 nM AcPdAD. The steady-state rate of AcPdAD$^+$ reduction was determined as described under "Experimental Procedures." The titration was performed with wild-type dl ( ), with dl.D135N ( ), and with dl.D135N in the presence of 25 nM wild-type dl ( ). B, reconstitution of dl.dIII complexes. Cyclic transhydrogenation was measured at 25 °C in 50 mM Mops, 50 mM KCl, and 2 mM MgCl$_2$, pH7.2, supplemented with 200 nM NADH and 200 nM AcPdAD$^+$ as described under "Experimental Procedures." The dIII concentration was 38 nM in all experiments. The titration was performed with wild-type dl ( ), dl.D135N ( ), and dl.D135N in the presence of 50 nM wild-type dl ( ).](Image)

![FIGURE 3. Stopped-flow kinetics of hydride transfer in complexes of dl.S138A and dIII. Experiments were performed in 20 mM Na-Hepes, 10 mM (NH$_4$)$_2$SO$_4$, and 1 mM dithiothreitol, pH 8.0 at 20 °C, as described under "Experimental Procedures." A, reduction of AcPdAD$^+$ by NADPH. The experiment was performed in the absorbance mode. The first syringe contained 2.4 mM AcPdAD$^+$, and the second contained 100 nM dl.S138A and 50 nM dIII in either its [4$^3$-H]NADPH form or its [4$^5$-H]NADPH form. The traces are the average of six recordings at 375 nm after 1:1 mixing. B, reduction of NADP$^+$ by NADH. The experiment was performed in the fluorescence mode. The first syringe contained 200 nM NADH and the second contained 50 nM dl.S138A and 25 nM dl.E155W. The trace is the average of six recordings after 1:1 mixing.](Image)
the analogue nucleotide; the condition is more likely to be met in complexes with the mutant dl protein than in those with wild-type dl (and see Ref. 45).

Sets of experiments were performed with dl\textsubscript{2}dIII\textsubscript{1}, (dl.D135N)\textsubscript{2}dIII\textsubscript{1}, and (dl.S138A)dIII\textsubscript{1}, complexes in which the single turnover burst of AcPdAD\textsuperscript{−} reduction with [45\textsuperscript{−}H]NADPH and [45\textsuperscript{−}H]NADPH was compared (all at 20 °C). In good agreement with Ref. 13, the deuterium isotope effect on \( k_{\text{app}} \) was 2.3 for the wild-type protein. However, in contrast to the results with (dl.Q132N)dIII\textsubscript{1}, in which the isotope effect was eliminated (27), AcPdAD\textsuperscript{−} reduction by [45\textsuperscript{−}H]NADPH was still 2.0- and 2.7-fold greater than by [45\textsuperscript{−}H]NADPH with (dl.D135N),dIII\textsubscript{1}, and (dl.S138A)dIII\textsubscript{1}, complexes, respectively. Fig. 3A shows experimental traces for (dl.S138A)dIII\textsubscript{1}, complexes. The data for wild-type complexes and for (dl.D135N)dIII\textsubscript{1}, are shown in supplemental Fig. S4A and S4B, respectively.

The mutant dIII protein, dIII.E155W, has essentially wild-type kinetics and nucleotide-binding properties (30). When NAD\textsuperscript{+} is bound to the protein, the fluorescence of Trp-155 is high; when NADPH is bound, the fluorescence is low. Usefully therefore, the rate of hydride transfer from NADH to NADPH on (dl.dIII.E155W)\textsubscript{1}, complexes can be measured from the fluorescence change (15, 36). In stopped-flow experiments the fastest measurable rate constant (\( k_{\text{app}} \approx 800 \text{ s}^{-1} \)), limited by the apparatus dead time, was achieved at 50–100 \( \mu \text{M} \) NADH (although this is not saturating (45)). In experiments with complexes constructed from dIII.E155W and either dl.D135N or dl.S138A, the fluorescence change accompanying NADPH\textsuperscript{−} reduction by NADH was greatly slowed relative to similar complexes made with wild-type dl. At 100 \( \mu \text{M} \) NADH and saturating NADPH\textsuperscript{−}, \( k_{\text{app}} \) was 0.1 \( \text{ s}^{-1} \) for (dl.D135N),dIII.E155W\textsubscript{1}, and 6.6 \( \text{ s}^{-1} \) for (dl.S138A),dIII.E155W\textsubscript{1}; see Fig. 3B for experiments on the latter protein. These values represent decreases relative to wild-type dl of about 10\textsuperscript{5}- and 10\textsuperscript{7}-fold, respectively, for similar solution conditions. Note that experiments with higher concentrations of NADPH were prohibited because of the increasing inner-filtering effect of the nucleotide. The Trp fluorescence change accompanying NADPH\textsuperscript{−} reduction by NADH in (dl.R127A),dIII.E155W\textsubscript{1}, and (dl.R127M),dIII.E155W\textsubscript{1}, complexes was extremely slow (\( k_{\text{app}} \approx 0.005 \text{ s}^{-1} \)); a fluorescence change with similar rate constant was observed upon mixing NADH with dIII.E155W loaded with NADPH\textsuperscript{−} in the absence of dl. Again we conclude that mutations of Arg-127 have a very strong inhibitory effect on hydride transfer (>10\textsuperscript{5}).

\( \text{NAD}^+ \) and NADH Binding to Mutant dl Proteins and dl.dIII\textsubscript{1} Complexes—Three sets of experiments were performed to determine the NAD(H)-binding constants of wild-type and mutant dl and the corresponding dl,dIII\textsubscript{1} complexes. Depending on constraints imposed by the methodology, some data are reliable for low and others for high binding constants.

To preface the first set of experiments, it is pointed out that Trp-72 in wild-type dl has unusual photochemical characteristics indicating an exceptionally rigid local environment for the residue (28, 46). The fluorescence of Trp-72 is quenched by energy transfer when NADPH binds to the protein. The \( K_v \) value (\(<-20 \mu \text{M}) estimated from the data is at the high end of a reliable determination because the inner-filtering effect becomes unmanageable large at nucleotide concentrations above \(<-50 \mu \text{M}). The fluorescence excitation and emission spectra of all four of the new dl mutants were similar to those of wild-type protein, showing that the rigid structure of the domain housing Trp-72 (designated dl.1 (18)) was unaffected by the amino acid substitutions in or around the RQD loop (data not shown). In dl.D135N and dl.S138A, the Trp-72 fluorescence was quenched by NADH in the same concentration range as in wild-type dl. Approximate \( K_v \) values are given in Table 2. In contrast, the addition of 50 \( \mu \text{M} \) NADH to dl.R127A and to dl.R127M failed to cause significant fluorescence quenching, perhaps indicating high \( K_v \) values.

Isothermal titration calorimetry returns more reliable \( K_v \) values for dl in the 20 \( \mu \text{M} \) range (16). Heat changes associated with the addition of NADPH indicated that the \( K_v \) value for the nucleotide was similar in wild-type dl and in dl.D135N and dl.S138A (Table 2). In all three proteins, the binding stoichiometry was \( \sim1 \) mol of NADPH per mol of dl monomer. However, in titrations of dl.R127A and dl.R127M with NADH, the incremental heat changes were much smaller. The binding sites of these two mutants (at 190 \( \mu \text{M} \) protein monomers) were not saturated even up to 700 \( \mu \text{M} \) added nucleotide, and therefore the \( K_v \) value and stoichiometry could not be unambiguously determined from the data. If it is assumed that each monomer has one NADH-binding site, the \( K_v \) value is calculated to be in the region of 700 \( \mu \text{M} \) for both dl.R127A and dl.R127M (Table 2).

Isothermal titration calorimetry experiments showed that NADH binds to NADPH-loaded, wild-type dl,dIII\textsubscript{1} complexes at two non-equivalent sites (16). One site has a stoichiometry of 0.96 NADPH per complex and a \( K_v \) of \(<17 \mu \text{M). The other has a much higher \( K_v \) value, probably around 300 \( \mu \text{M). We attributed the first to dl.A) and the second to dl.B); see Fig. 1 for component nomenclature. Therefore, the association of dIII (with its bound NADPH) is thought to lower the NADH affinity of the adjacent dl but to have little effect on the nucleotide-binding affinity of the nonadjacent dl. Very similar behavior was observed with complexes formed from NADPH-loaded wild-type dl and either dl.D135N or dl.S138A (Table 3).

The Trp-72 fluorescence quenching and the calorimetry protocols do not give reliable binding constants either for dl proteins and NAD\textsuperscript{+} or for the Arg-127 mutants and NADH because the \( K_v \) values are rather high (>200 \( \mu \text{M} \) nucleotide). Thus, we performed a final set of binding experiments using NMR spectroscopy. In NMR spectra of isolated wild-type dl, the \( ^1\text{H} \) resonances derived from a mobile loop (amino acid residues 220 to 240) are relatively sharp against much broader resonances from other regions of the molecule (47–50). The sharper resonances undergo chemical shift changes and broaden when NAD(H) binds to dl, indicating that the loop closes down on the surface of the molecule. In the absence of nucleotide, the \( ^1\text{H} \) spectra of dl.R127A,

### Table 2

| Protein | Trp fluorescence | Calorimetry | NMR |
|---------|------------------|-------------|-----|
| Wild-type dl | 18\textsuperscript{a} | 18 (n = 1.0)\textsuperscript{b} | <100\textsuperscript{c} |
| dl.R127A | b.ND\textsuperscript{b} | 700\textsuperscript{d} | 800\textsuperscript{d} |
| dl.R127M | b.ND\textsuperscript{b} | 700\textsuperscript{d} | 800\textsuperscript{d} |
| dl.Q132N | 20\textsuperscript{e} | 25 (n = 0.7)\textsuperscript{f} | <100\textsuperscript{g} |
| dl.D135N | 20\textsuperscript{e} | 16 (n = 0.9)\textsuperscript{f} | <100\textsuperscript{g} |
| dl.S138A | 23\textsuperscript{e} | 24 (n = 0.7)\textsuperscript{f} | <100\textsuperscript{g} |

\( ^{a} \) Measurements were carried out as described (28) with 1 \( \mu \text{M} \) protein in the buffer system described under “Experimental Procedures.”

\( ^{b} \) Measurements were carried out as described (16) between 50 and 200 \( \mu \text{M} \) protein in the buffer system described under “Experimental Procedures.”

\( ^{c} \) Stoichiometries (mol of NADH mol\textsuperscript{−1} dl monomer) are shown in parentheses.

\( ^{d} \) Measurements were carried out as described (49) with 300–600 \( \mu \text{M} \) protein in the buffer system described under “Experimental Procedures.”

\( ^{e} \) See Ref. 28.

\( ^{f} \) See Ref. 16.

\( ^{g} \) See Ref. 49.

\( ^{h} \) \( K_v \) values were too low to be estimated accurately but were consistent with the values calculated from Trp fluorescence and calorimetry experiments (see text).

\( ^{i} \) b.ND indicates binding was not detectable. No Trp fluorescence quenching was observed after addition of up to 50 \( \mu \text{M} \) NADH.

\( ^{j} \) Data are from this work.

\( ^{k} \) Calculations are from the data assuming \( n = 1. \)

\( ^{l} \) See Ref. 27.
dI.D135N, and dI.S138A closely resemble those of wild-type dI; the chemical shifts of the mobile loop resonances are not significantly changed by the single amino acid substitutions (parts of the spectra are shown in Fig. 4 for dI.D135N). These data show that, in the absence of nucleotide, the mutations do not affect the dynamics of the mobile loop and do not have large effects on the overall fold of the protein in solution. In dI.R127M, the spectrum is very similar to wild type but an additional peak, attributable to the introduced Met-127, is evident in the S-CH₃ region (Fig. 5A). The addition of NADH to dI.D135N and to dI.S138A led to a broadening of the mobile loop resonances very similar to that observed in experiments with wild-type protein; Fig. 4A shows illustrative data for dI.D135N. The $K_d$ value for NADH binding cannot be accurately measured from the change in chemical shift of individual resonances, because the system is in intermediate exchange on the NMR time scale and band broadening makes the titration end points difficult to determine (47). Nevertheless, data taken from the chemical shift changes of Met-239 (e.g. Fig. 4A) are consistent with the $K_d$ of ~20 μM that we calculated from the Trp fluorescence and calorimetry (Table 2). The effects of NADH on the NMR spectra of dI.R127A and dI.R127M were substantially different from those observed on the spectrum of wild-type protein; Fig. 5A shows illustrative data for dI.R127M. Much higher concentrations of NADH were required for alterations in chemical shift and band broadening. Probably because of the weaker nucleotide binding and therefore faster exchange between bound and free forms (most likely because of a greater rate constant for the dissociation step), the band broadening was less pronounced, and changes in chemical shift were more easily measured. The end point of the titration was therefore more reliably determined and, usefully, this gave a more accurate estimation of the $K_d$ value for NADH binding to dI.R127A and dI.R127M (see Table 2) than was possible from either the fluorescence

**TABLE 3**

Binding affinities of dl.dIII complexes for NADH determined by isothermal titration calorimetry

| Protein                  | $K_d$ | $K_d$ |
|-------------------------|-------|-------|
| dl.dIII                 | 17    | 300   |
| (dl.Q132N) dIII         | 15    | 200   |
| (dl.D135N) dIII         | 25    | 500   |
| (dl.S138A) dIII         | 29    | 300   |

| a Data are from Ref. 16. |
| b Data are from Ref. 27. |
| c Data are from this work. |

**A dI.D135N**

**B dI.D135N**

**FIGURE 4.** The effect of NAD(H) on the ^1^H NMR spectrum of dI.D135N. Spectra were recorded with 520 μM dl at the NAD(H) concentration indicated, as described under "Experimental Procedures"; only the chemical shift range from 1.5 to 2.5 ppm is shown. The S-CH₃ resonances of Met-226 and Met-239, which are both located in the mobile loop of dl, are assigned on the basis of mutagenesis experiments (49, 54). The heavy arrows give an indication of the progress of the Met-239 resonance throughout the titrations. A, NADH titration; B, NAD⁺ titration.

**FIGURE 5.** The effect of NADH on the ^1^H NMR spectrum of dI.R127M (A) and NAD⁺ on the ^1^H NMR spectrum of dI.R127A (B). See Fig. 4; the concentration of dI.R127M was 350 μM, and the concentration of dI.R127A was 600 μM.
or calorimetry experiments. Note that the Met-127 resonance was also broadened during the NADH titration, indicating a change in the environment of this residue upon NADH binding (Fig. 5A).

Somewhat analogously, NAD⁺ and wild-type dI are in relatively fast exchange. Thus, the titration end point of the Met-127 resonance was also broadened during the NADH titration, indicating a change in the environment of this residue upon NADH binding (Fig. 5A).

Crystal Structures of Complexes Composed of Mutant dI and Wild-type dIII—By using procedures described previously for wild-type dI-dIII complexes, we succeeded in solving crystal structures of (dI.R127A)₂dIII₁, (dI.D135N)₂dIII₁, and (dI.S138A)₂dIII₁ in its NAD⁺/NADP⁺ form (PDB 2FR8), (dI.D135N)₂(dIII.E155W)₁ in its NAD⁺/NADP⁺ form (PDB 2F8S), and (dI.S138A)₂dIII₁ in its NADH/NADPH form (PDB 2F8R), see Table 1. Like the (dI.Q132N)₂dIII₁ complex in its NAD⁺/NADP⁺ form described in a previous report, all the mutant proteins have essentially the wild-type fold, confirming that the amino acid substitutions have little effect either on the structural integrity of the dI protein or on the interaction between dI and dIII. All the complexes are asymmetric, having two dI polypeptides (A and B, see Fig. 1) and one dIII polypeptide; this is in spite of the fact that one structure (PDB 2F8R) has different cell dimensions. In each structure, the dIII component interacts more extensively with dI(B) than with dI(A). NADP(H), which is invariably in good electron density, always occupies a similar conformation at the C-terminal edge of the β-sheet of dIII. NADH binds next to this in dI(B) to form a hydride transfer site. It also binds to the unpartnered d(A), and in the intact enzyme, this presumably forms the second hydride transfer site of the transhydrogenase dimer.

In wild-type dI₂dIII₁ structures, whether with bound NADH and NADPH (PDB, 1U2D (20)) or bound NAD⁺ and NADPH⁺ (PDB, 1HZZ (18)), the electron density for NAD(H) is better defined in dI(A) than in dI(B). In dI(A), the Nic(H) of the NAD(H) adopts a distal conformation (20). In dI(B), the Nic(H) density is always weaker than that of the adenosine, implying disorder in this part of the nucleotide. However in PDB 1U2D, the density of the NicH in dI(B) is sufficient to show that the ring adopts a proximal position relative to that of the NicH of the NADPH (20). It was suggested that movement of the NicH of the NAD(H) between the distal and proximal conformations (through rotations of the pyrophosphate and ribose-phosphate bonds) is probably responsible for the greater disorder seen in this part of the nucleotide in the dI(B) subunit.

The variations in the NAD(H) electron densities observed in the wild-type dI₂dIII₁ complexes are also evident in the mutant proteins but are more pronounced. In the dI(A) polypeptide of (dI.R127A)₂dIII₁, and especially in that of (dI.D135N)₂(dIII.E155W)₁, the electron density of the adenosine part of the nucleotide is as strong and clearly defined as the neighboring polypeptide (suggesting tight-binding and order) but that of the Nic⁺ moiety is weak. This indicates that, in contrast to the wild-type protein, the Nic⁺ is somewhat disordered even in dI(A). In (dI.D135N)₂(dIII.E155W)₁, the Nic⁺ density in dI(A) is clear enough to establish that the nucleotide is in a distal position and that its carboxamide group is syn to the N-ribose. A similar situation was evident in dI(A) of wild-type dI₂dIII₁ complexes crystallized at high NAD⁺ concentrations (20) and in one of the four polypeptides in the asymmetric unit of isolated dI-NAD⁺ (17). In the dI(B) polypeptides of (dI.D135N)₂(dIII.E155W)₁ and (dI.R127A)₂dIII₁, the NAD⁺ density is weak along the entire length of the nucleotide, and we cannot draw conclusions about its conformation. The (dI.S138A)₂dIII₁ complex (whose hydride transfer rate, it should be recalled, is less strongly inhibited than in the other mutants) is more revealing. The NADH in dI(A) of (dI.S138A)₂dIII₁ is in strong electron density, and as in the equivalent wild-type complex (20), this density suggests a distal nucleotide conformation with its carboxamide group anti to the N-ribose. The adenosine and pyrophosphate of the NADH in dI(B) are also in quite good density and indicate a similar structure to that of the wild-type complex. However, the density of the N-ribose and NicH, although rather weak, is clear enough to indicate that the NicH adopts a predominantly distal conformation (Fig. 6), and this contrasts with the proximal conformation found in the wild type.

The Glu-to-Trp mutation in the dIII component of (dI.D135N)₂(dIII.E155W)₁, known to have only a minimal effect on the functional properties of the protein (see above), was clearly evident in the x-ray structure; as expected, it barely affected the fold of the protein.

DISCUSSION

The equivalents of Arg-127, Glu-132, Asp-135, and Ser-138 in the RQD loop of the dI component of R. rubrum transhydrogenase are invariant in the available ~160 sequences of the enzyme. In crystal structures, all of these residues are in close proximity to the NMN(H) moiety of bound NAD(H), suggesting a role in the events associated with hydride transfer. Residues in the RQD loop make contacts with the dIII component and therefore might also participate in the docking of dI with dIII. However, the competition between the mutant and wild-type dI polypeptides in steady-state transhydrogenation reactions (Figs. 2A and B, and supplemental Figs. S1 and S2) and the isothermal calorimetry titrations (supplemental Fig. S3) did not reveal differences in binding affinities either with dI₂dIII₁ in membranes or with isolated dI₂dIII₁. Moreover, the x-ray structures of dI₂dIII₁ complexes carrying the mutations show that neither the interaction surface between dI and dIII nor the structure around the NAD(H) site in dI₂dIII₁ are significantly altered relative to wild-type protein. Therefore, the effects that we observe can probably be ascribed entirely to local events in the NAD(H)-binding pocket of the hydride transfer site.

The distal/proximal switch of the NAD(H) conformation in the intact transhydrogenase is important in gating the hydride transfer reaction during proton translocation (20). The Nic(H) rings of NAD(H) and NADP(H) are highly reactive, and thus hydride transfer will be facile when their C-4(N) atoms are apposed. It is therefore necessary to keep the NicH ring of NADH apart from the Nic⁺ ring of NADP⁺ during the formation of the Michaelis complex of the enzyme and the nucleotide substrates. Otherwise, the redox reaction would take place in the absence of proton translocation. Arg-127, Glu-132, Asp-135, and Ser-138 appear to behave as an integrated unit with a role in the operation of this switch.

The substitution of Arg-127 with either Ala or Met strongly inhibited the rate of transhydrogenation and altered the nucleotide-binding properties of the dI protein. When dI.R127A and dI.R127M were reconstituted into intact enzyme in membranes, transhydrogenation rates were negligible. In dI₂dIII₁ complexes, the κ₉₅ values for forward and reverse
hydride transfer were >10⁵-fold lower than in the wild-type protein even when, according to $K_D$ values, the NAD(H) site was substantially occupied. We had initially suspected that the positively charged guanidinium group of Arg-127 might be responsible for destabilizing NAD⁺ binding ($K_D$ for isolated dl ≈300 μM (49, 51)) relative to NADH binding ($K_D$ ≈ 20 μM (28, 51)). Under physiological conditions, this property is important because the substrate NAD(H) has to bind from a solution (the mitochondrial matrix or bacterial cytoplasm) in which its concentration is low and the product NAD⁺ has to be released (into the same solution) for NADH binding than on NAD⁺ binding. Why the Arg-127 mutations have a larger effect on NADH binding than on NAD⁺ binding remains unexplained. Structural changes at the site, perhaps resulting from the loss of coulombic interactions between the Arg-127 and the nucleotide pyrophosphate, may be important.

The substitution of Asp-135 with Asn had similar effects on enzyme properties to the substitution of Ser-138 with Ala. Transhydrogenase rates in the intact enzyme were strongly inhibited by the mutations. In both cases, the large decrease of the $k_{cat}/K_m$ values for forward and reverse hydride transfer in dI₂dIII₁ complexes shows that this is probably the result of a compromised redox reaction. Despite this (and in contrast to results with the Arg-127 mutants), the binding affinities for NAD⁺ and NADH were unaffected (Table 1). The effects of the Asp-135 and Ser-138 mutations are similar to those produced by substitution of Gln-132 with Asn (27), but there is one clear difference. In (dI.Q132N)₂dIII₁ complexes, the deuterium isotope effect on the single turnover burst of AcPDAD⁺ reduction by NADPH, evident in the stopped-flow experiments with wild-type protein ($k_{cat}/k_{cat}^{H} \sim 2$ at 20 °C), is lost (27), but in (dI.D135N)₂dIII₁ and (dI.S138A)₂dIII₁, it is retained. It may be assumed that only the hydride transfer step will be subject to a significant kinetic isotope effect. Therefore, the loss of the effect in (dI.Q132N)₂dIII₁ complexes was taken as evidence that hydride transfer is preceded by a conformational change that is inhibited by the substitution of Gln-132 with Asn (27). This conformational change is possibly associated with the shift of the nucleotide from the distal to the proximal position indicated by the x-ray structures of wild-type dl₂dIII₁ complexes (see above). The retention of the isotope effect in (dI.D135N)₂dIII₁ and (dI.S138A)₂dIII₁ indicates that the D135N and S138A mutations lead to a decrease in rate of either the hydride transfer step alone or of both hydride transfer and the preceding conformational change. The x-ray structure of (dI.S138A)₂dIII₁ crystallized in the presence of reduced nucleotides (PDB, 2FRD) provides some useful observations in this context. In the structure, the electron density for the NADH in dl(B) is quite good (Fig. 6C). It is strong in the adenosine/pyrophosphate/N-ribose regions, indicating substantial site occupancy, but weaker for the NicH, implying some disorder in this part of the ligand. However, the electron density of the NicH is strong enough to suggest that the nucleotide adopts a predominantly distal conformation (the distance from C-4(N) of the NADH to C-4(N) of NADPH is ~5.5 Å). This is in marked contrast to dl(B) in the wild-type dl₂dIII₁ complex (PDB 1U2D, see Fig. 6B) where proximal NADH is favored and the 127 side chain is located within the cleft that separates the two domains (dl.1 and dl.2) of the dl protein. In apodl, the side chain either protrudes into the solvent or lies along the cleft opening (21). Upon NAD(H) binding, the Arg-127 side chain is drawn more deeply into the cleft, and its guanidinium group makes contact with the nucleotide pyrophosphate and N-ribose groups (17, 21). In different structures, the Nic(H) ring of the bound NAD(H) takes up either the distal or the proximal position relative to the Nic(H) ring of the NADP(H) in dI; the position correlates with a movement of dl.1 relative to dl.2 (20). In the proximal position (where the redox-active C-4(N) atoms of the NADH and NADP⁺ align for productive hydride transfer), the movement of the Nic(H) ring of the NAD(H) leaves space for the Arg-127 side chain to penetrate even deeper into the cleft. Here, the guanidinium group of the arginine forms a new H-bond with the side chains of Asp-135 which, in turn, H-bonds to Ser-138 and Gln-132. Thus, we suggest that a role of Arg-127 is to stabilize the proximal position of the NicH ring of NADH and favor hydride transfer to the closely aligned Nic⁺ ring of NADP⁺. When the guanidinium group is removed by mutation, the proximal position cannot be stabilized, and the hydride transfer reaction is severely compromised. Why the Arg-127 mutations have a larger effect on NADH binding than on NAD⁺ binding remains unexplained. Structural changes at the site, perhaps resulting from the loss of coulombic interactions between the Arg-127 and the nucleotide pyrophosphate, may be important.
C-4(N)–C-4(N) distance is 3.6 Å (20). Two other differences with the wild-type complex are revealing. First, the side chain of Gln-132 extends outwards from dl(B) of 2FRD (Fig. 6C), as it does in dl(A) of 1HZZ (not shown) and dl(A) of 1U2D (Fig. 6A); most significantly, in both of these dl(A) polypeptides, the NicH of the bound nucleotide is also in a distal conformation (see below). In contrast, the Gln-132 side chain in dl(B) of 1U2D folds back behind the NicH ring where its amide group can make an H-bond with Asp-135 contributing to the H-bond network and helping to maintain the nucleotide in the proximal position. The second difference is that the side chain of Arg-127 in dl(B) of 1U2D (Fig. 6B) is in good electron density, but the same residue in 2FRD is in weak electron density implying disorder (Fig. 6C). Together, these observations suggest that substitution of Ser-138 with Ala leads to a failure in the H-bond organization that is necessary to correctly position the NicH of NADH in the proximal position adjacent to the Nic+ ring of NADP+ for hydride transfer. Thus with an Ala residue at position 138, there can be no H-bond with Asp-135 (see Fig. 6B); therefore, the H-bonds between Asp-135 and Arg-127 and between Asp-135 and Gln-132 are weakened. This results in a destabilization of the Arg-127 and Gln-132 side chain conformations and the distal positioning of the NicH ring. A similar explanation can be invoked when Asp-135 is substituted with Asn. The kinetic isotope effects suggest that the mutations must also affect the hydride transfer step itself. This could result from a change either in the C-4(N)–C-4(N) distance or in the orientation of the NicH and Nic+ rings in the (weakly stabilized) proximal conformation, although electron densities in the hydride transfer site are not sufficient to confirm this. It might also be expected that altered coulombic interactions caused by the mutations could contribute to the hydride transfer inhibition. However, it is notable that, for both the D135N and the S138A mutants, the k_{app} values for hydride transfer were suppressed by similar factors in the forward and in the reverse directions. In the case of the two Arg-127 mutations, inhibition of hydride transfer was so strong in both directions that a similar conclusion could not be drawn.

In wild-type dl(dII) structures to date, the electron densities for NAD(H) in dl(A) clearly indicate a distal conformation, which suggested to us that the nucleotide-binding pocket in this polypeptide approximates that in the Michaelis complex of the enzyme (18, 20, 22). Because dl(A) does not have a partner dII (e.g. Fig. 1), it was proposed that only when dl (in the intact enzyme) interacts with a dII component in its “occluded state” can the bound NAD(H) shift into its proximal conformation to permit hydride transfer (see Ref. 9 for more discussion). This is an essential gating step in the turnover of the enzyme. In dlQ132N)2dIII1 (27) and dlS138A)2dIII1 (this work), the electron density for NAD(H) in dl(A) reveals a distal conformation, as in wild-type. However, in dlR127A)dII1 and dlD135N)dII1E155W1), the electron density for the Nic(H) ring is substantially weaker than that of the adenosine and pyrophosphate regions. This disorder in the Nic(H) suggests destabilization of the distal conformation of bound NAD(H) by the R127A and D135N mutations but not by the Q132N and S138A mutations. Consistent with this, Arg-127 and Asp-135 both interact with bound NAD(H) in all wild-type dl(A) polypeptides, but Gln-132 and Ser-138 do not.

It was noted that the dl.1 domain is positioned differently relative to the dl.2 domain in the A and the B polypeptides of wild-type complexes (18, 20). This was taken to indicate a movement of dl.1 relative to dl.2 that results from the binding of dII to dl(B). It was suggested that this movement could be responsible for initiating events that lead to the adoption of the proximal position of the dihydronicotinamide ring of bound NADH ready for hydride transfer to NADP+. In all the structures of the mutants in the RQD loop (PDBs 1NM5, 2FR8, 2FSV, and 2FRD), we observe a similar apparent movement of dl.1 relative to dl.2 in the A and the B polypeptides. This shows that the side chains of Arg-127, Gln-132, Asp-135, and Ser-138 are not required for the movement. Rather, the results indicate that the side chains of these invariant residues may serve to mediate between the inter-domain movement and the events at the hydride transfer site.

The main conclusion of this work is that amino acid residues in the RQD loop are centrally involved in the conformational change of bound NAD(H) between its distal and proximal forms. Mutations in the loop very sensitively affect the steps associated with hydride transfer. The complex changes in the H-bond and coulombic interactions between the Nic(H) of the NAD(H), the nucleotide pyrophosphate, and the invariant Arg-127, Gln-132, Asp-135, and Ser-138 (as well as Tyr-235 in the “mobile loop” (52)) in the distal-to-proximal shift (Fig. 6) reveal the probable intricacy of the motions involved in switching. A crucial question, still to be answered even in broad terms, is how conformational changes in dIII, driven by proton transport through dlII, are relayed to the nucleotide-binding pocket of dlI. In this context, the larger scale conformational changes deduced from a new crystal form of the R. rubrum dlI dlII, complex (22) and from a molecular dynamics simulation of the isolated E. coli dl (25) require further consideration.

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