SUBSTITUTION OF TYROSINE-146 IN THE dI COMPONENT OF PROTON-
TRANSLOCATING TRANSHYDROGENASE LEADS TO REVERSIBLE
DISSOCIATION OF THE ACTIVE DIMER INTO INACTIVE MONOMERS.
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Transhydrogenase couples the redox reaction between NADH and NADP+ to proton translocation across a membrane. The protein has three components; dI binds NADH, dIII binds NADP+ and dII spans the membrane. Transhydrogenase is a "dimer" of two dI-dII-dIII "monomers"; X-ray structures suggested that the two catalytic sites alternate during turnover. Invariant Tyr146 in recombinant dI of Rhodospirillum rubrum transhydrogenase was substituted with Phe and Ala (proteins designated dI.Y146F and dI.Y146A, respectively). Analytical ultracentrifuge (AUC) experiments and differential scanning calorimetry show that dI.Y146A more readily dissociates into monomers than wild-type dI. AUC and Trp fluorescence experiments indicate that the dI.Y146A monomers bind NADH much more weakly than dimers. Wild-type dI and dI.Y146F reconstituted activity to dI-depleted membranes with similar characteristics. However, dI.Y146A reconstituted activity in its dimeric form but not in its monomeric form, this despite monomers retaining their native fold and binding to the dI-depleted membranes. It is suggested that transhydrogenase reconstituted with monomers of dI.Y146A is catalytically compromised, at least partly as a consequence of the lowered affinity for NADH, and this results from lost interactions between the nucleotide-binding site and the protein's β-hairpin upon dissociation of the dI dimer. The importance of these interactions and their coupling to dI domain rotation in the mechanism of action of transhydrogenase is emphasised. Two peaks in the 1H-NMR spectrum of wild-type dI are broadened in dI.Y146A and are tentatively assigned to S-methyl groups of Met resonances in the β-hairpin, consistent with the segmental mobility of this feature in the structure.

Transhydrogenase is located in the inner mitochondrial membrane of animal cells and in the cytoplasmic membrane of bacteria. It catalyses the reduction of NADP+ by NADH coupled to the translocation of protons across the membrane.

\[
\text{NADH} + \text{NADP}^+ + \text{H}^+ \rightarrow \text{NAD}^+ + \text{NADPH} + \text{H}^+ \quad \text{equation 1}
\]

The enzyme provides NADPH for biosynthesis reactions (1;2) and for glutathione reduction in the protection of cells against oxidative stress (3-5). A role for transhydrogenase has also been implicated in glucose-stimulated insulin secretion by pancreatic β-cells (6).

Transhydrogenase has three components. The dI component, which binds NADH, and the dII component, which binds NADP+, protrude from the membrane, and the dIII component spans the membrane. Conformational changes link the redox reaction at the dI/dIII interface with proton translocation through dII (for recent reviews, see (7;8)). The intact enzyme is a "dimer" of two dI-dII-dIII "monomers" (9-12), although the disposition of dI, dII and dIII along polypeptides is somewhat variable between species (13). Solution studies (14;15) and X-ray structures (16-20) reveal the profound asymmetry of a complex formed from mixtures of dI and dIII (the so-called dI:dIII1 complex) of the Rhodospirillum rubrum enzyme. This was taken to suggest that transhydrogenase operates by an "alternating-sites" mechanism in which the two dI-dII-dIII monomers run 180° out-of-
phase during catalytic turnover. Consistent with this, earlier work had indicated half-of-the-sites inhibition of bovine transhydrogenase by covalent modifiers (21;22).

The alternating-sites mechanism requires that events in the two dI-dII-dIII monomers are coordinated; there must be a coupling of conformational changes across the dimer interface. In this report, we describe the effects of substituting invariant Tyr146, a residue which is centrally located in the dimer interface between the two dI components of *R. rubrum* transhydrogenase (see Fig.1). Tyr146 lies on helix α6, one of the two helices that link domains dI.1 and dI.2; the side chain of the residue makes H-bond contact with the carbonyl group of Phe160 at the junction between the C-terminus of α6 and the N-terminus of the "β-hairpin" in the symmetry-related partner polypeptide. The X-ray structures suggest that, during alternation of the sites, the β-hairpin shifts position and dI.1 rotates against dI.2; the pivot for rotation is in α6 close to Tyr146, and in α11 (20). The rotation causes a change in the conformation of the "RQD" loop, and this shifts the position of the nicotinamide ring of NADH which thus gates the hydride-transfer reaction. In all conformationally-coupled pumps like transhydrogenase, Ca<sup>2+</sup>-ATPase, F<sub>F</sub>,F<sub>I</sub>-ATPase and the ABC transporters, gating steps are necessary to prevent slip, e.g. translocation in the absence of the chemical reaction.

**EXPERIMENTAL PROCEDURES**

Wild-type dI and wild-type dIII from *R. rubrum* transhydrogenase were expressed from the plasmids, pCD1 (23) and pNIC2 (24), respectively, in appropriate strains of *E. coli* after induction with isopropyl β-D-thiogalactoside, as described in the earlier reports. The dI was purified on three consecutive chromatography columns, Q-Sepharose Fast Flow (GE Healthcare), Butyl Toyopearl (Tosohaas) and Q-Sepharose High Performance (GE Healthcare), essentially as described (23;25). The dII was purified sequentially on Q-Sepharose Fast Flow, Q-Sepharose High Performance and Butyl Toyopearl, essentially as described (26). The plasmid pCD1 was subjected to site-directed mutagenesis using the Stratagene Quickchange kit and DNA primers supplied by AltaBioscience. The resulting new plasmids, pUMO1 and pGVBS, encoded dI.Y146F and dI.Y146A, respectively. The plasmid encoding dIE7 was constructed by PCR using pCD1 as a template, and primers that included a coding sequence for the 7-Glu tag. A Ndel-KpnI fragment containing the dIE7 construct was excised from the PCR product, cloned into pET-Duet-1 (Novogen), and designated pTHB31. The DNA of all three plasmids was sequenced to confirm that no errors were introduced by the PCR. The plasmids were then used to transform cells of either *E. coli* C600, in the case of pUMO1 and pGVBS, or *E. coli* BL21(DE3), in the case of pTHB31. The cells were grown, induced, harvested, frozen, thawed and disrupted, as described for the production of wild-type dI. The dIE7 protein was purified using the procedure described for wild-type dI. Only small amounts of dI.Y146A and dI.Y146F were found in the soluble fraction of the induced *E. coli* cells; rather more was associated with cellular inclusion bodies. The inclusion-body fraction was, therefore, used as a starting point for isolation of these two mutant proteins. All operations were carried out at 4° C. After cell breakage, the lysate was centrifuged at 30,000 g for 30 minutes. The resulting pellet was re-suspended in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride and re-centrifuged. The pellet from this operation was re-suspended in 6 M guanidinium hydrochloride, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, incubated for 30 minutes and centrifuged at 100,000 g for 60 minutes. The denatured proteins were refolded by extensive dialysis of the supernatant against 30 mM Na-Hepes, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride and re-centrifuged. The pellet from this operation was re-suspended in 6 M guanidinium hydrochloride, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 15 % (v/v) glycerol. The dialysed material was subjected to chromatography on Butyl Toyopearl (see above). Pooled fractions containing the mutant dI proteins were supplemented with 25 % (v/v) glycerol and stored at -15° C. On the basis of stain intensity with Page Blue 83 on SDS-PAGE, the protein was >95% pure. To confirm that dI completely refolds under the conditions
described, the guanidinium-hydrochloride denaturation and subsequent steps were performed with purified wild-type protein. The appearance of the resulting protein on SDS-PAGE was unchanged relative to that from the conventional preparation. It was fully active in reconstitution assays, and the very short wavelength fluorescence emission spectrum was preserved (23). Protein concentration was determined by the microtannin procedure (27). Unless otherwise stated, dI concentrations are given as monomers. Before use, proteins were thawed on ice and either used directly or concentrated in Vivaspin centrifugal filters with a molecular weight cut-off of 10 kDa for dI and 5 kDa for dIII.

*R. rubrum* was grown under photosynthetic conditions and everted membrane vesicles (chromatophores) were prepared and stored, as described (28;29). To remove the dI polypeptide of transhydrogenase, the membranes were repeatedly washed by centrifugation (29). The bacteriochlorophyll concentration of the vesicles was measured using the *in vivo* extinction coefficient of 140 mM⁻¹ cm⁻¹ at 880 nm (30).

Transhydrogenation rates in *R. rubrum* membranes were measured in the "reverse" direction (compare equation 1) as the reduction of AcPdAD⁺ (an analogue of NAD⁺) by NADPH at 375 nm (ε = 6.1 mM⁻¹ cm⁻¹) on a Shimadzu UV-2401 spectrophotometer. Low concentrations of carbonyl cyanide-p-trifluoromethoxy phenylhydrazone were added to the assay buffer to prevent build-up of an unfavourable proton electrochemical gradient (see figure legends). "Cyclic transhydrogenation" in dI₂dIII₁ complexes was measured as the reduction of AcPdAD⁺ by NADH, utilising the tightly-bound NADP⁺ that results from the standard purification procedure of dIII.

Trp fluorescence was measured using a Spex FluoroMax. Isothermal titration calorimetry was carried out using a MicroCal VP-ITC, and data were analysed using ORIGIN software, as previously described (15). Differential scanning calorimetry was performed with a MicroCal VP-DSC, again using ORIGIN for analysis. CD spectra were recorded on a Jasco J-810 polarimeter purged with dry nitrogen. One dimensional ¹H-NMR spectra were recorded on a Bruker AMX500 equipped with a cryoprobe. Pulse and collect spectra with water pre-saturation were acquired using 11 ppm spectral width and comprised 256 transients with a total acquisition time of 11 minutes. Analytical ultra centrifuge experiments were carried out using an 8-piece An-60 Ti rotor in a Beckman-Optima XL-I using either absorbance or interference optics, as described in the figure legends. A 2-sector cell was used in the sedimentation-velocity experiments, and a 6-sector cell in the sedimentation-equilibrium experiments. Sedimentation-velocity data were analysed using the continuous c(s) distribution method in the SEDFIT program (31). Sedimentation-equilibrium data were fitted using both the Beckman XL-I software, implemented in ORIGIN, and the SEDPHAT program (32).

Unless otherwise stated, amino acid residues are specified for the *Rhodospirillum rubrum* transhydrogenase according to the numbering system described (16).

**RESULTS**

**Catalytic activities of dI.Y146F and dI.Y146A.** The dI components of some transhydrogenases, like those from *E. coli* and mammals, are linked to dII through the polypeptide chain. However, the dI of *R. rubrum* transhydrogenase exists as a separate polypeptide. In currently-available gene sequences the *R. rubrum*-like organisation is, in fact, the most common (13). The dI can be removed from inverted membrane vesicles of *R. rubrum* simply by centrifugation washing in the absence of NADP(H) (28;33); activity is restored to the dII/dIII components in the depleted membranes by adding recombinant dI (26). Freshly-purified dI.Y146F was almost as effective as wild-type dI in restoring transhydrogenation activity: the maximal rate of reaction with the mutant was ~80% that with wild-type, and the concentrations of protein required to achieve the half-maximal rates were similar, 5 nM for dI.Y146F and 3.5 nM for wild-type dI (supplemental Fig. S1). In contrast, much higher concentrations of dI.Y146A were needed to produce an increase in the rate of transhydrogenation with dI-depleted membranes; the maximal rate was not reached at >15 µM mutant...
protein (Fig. 2). In a complementary series of experiments, dl-depleted membranes were supplemented with a fixed concentration of wild-type dl (enough to restore activity to ~67%) together with a variable concentration of dl.Y146A (Fig 2). Low concentrations of the mutant dl led to inhibition of the transhydrogenase reaction originating from the wild-type enzyme. Even concentrations of dl.Y146A, which in the absence of wild-type dl had barely any restorative effect on transhydrogenation, caused substantial inhibition.

A complex comprising two polypeptides of dl and one polypeptide of dIII (the dl-dIII1 complex) forms with high affinity in mixtures of the two recombinant proteins. The rates of forward and reverse transhydrogenation catalysed by the dl-dIII1 complex in steady state are extremely low because, in the absence of the membrane-spanning dl, the products NADPH and NADP+, respectively, remain very tightly bound to dIII. However, the dl2dIII1 complex catalyses a high rate of "cyclic transhydrogenation" in which tightly-bound NADPH is re-oxidised by AcPdAD+ (26). The addition of fresh dl.Y146F to a solution of dIII led to the formation of complexes capable of performing cyclic transhydrogenation at ~70% of the rate of wild-type complexes. The dependence on protein concentration was similar to that obtained with wild-type dl: approximately 40 nM of wild-type dl, and 45 nM dl.Y146F gave half-maximal rates of reaction with 20 nM dIII (supplemental Fig. S2). However, in close parallel to the results of the experiments on membranes, much higher concentrations of dl.Y146A were required to give catalytically-active complexes, and the reaction did not reach its maximal rate even when >20 µM mutant protein was added (Fig. 3). Experiments were also performed to investigate whether dl.Y146A can compete with wild-type dl in the formation of complexes with dIII. Thus, a fixed concentration of wild-type dl (enough to give 46% of the maximum rate) was added to dIII together with a variable concentration of dl.Y146A (Fig. 3). The rate of cyclic transhydrogenation was inhibited by dl.Y146A. The inhibitory effect was evident even at concentrations of the mutant which barely supported transhydrogenation in the absence of wild-type dl.

After a few days of storage at either 4º C or -15º C (see Methods), purified dl.Y146F lost its capacity to restore transhydrogenation activity to depleted membranes and to form active complexes with dIII. This decline was reflected in changes in the protein fluorescence spectrum (see below). In marked contrast, wild-type dl and dl.Y146A in identical solution conditions remained stable for >2 days at 4º C, and for >3 months at -15º C.

The oligomeric state of wild-type dl and dl.Y146A. By several criteria, isolated wild-type dl is a dimer in solution at concentrations >1 µM (15). The X-ray structure reveals the interaction surfaces of the R. rubrum dl dimer (34;35) and the E. coli dl dimer (36). The dimer interfaces of the proteins from the two species are very similar. Loaded at a concentration of 12 µM, dl.Y146A from R. rubrum transhydrogenase ran with Mr ≈ 44 kDa on gel-permeation chromatography, whereas wild-type dl ran with Mr ≈ 84 kDa (supplemental Fig. S3). The Mr of the monomers from amino acid sequence is calculated to be ≈ 40.3 kDa.

From the results of sedimentation-velocity experiments on wild-type dl in the analytical ultracentrifuge, the relation between c(s) and S showed a single peak (at S = 3.75) independent of protein concentration in the range 3-12 µM (Fig. 4A). The c(M) distribution of the data yields Mr = 81 ± 7 kDa with a frictional ratio of 1.3. The results clearly show that wild-type dl is a dimer at these concentrations. However, the main peak in the c(s) versus S relation for dl.Y146A shifted from S = 3.0 to S = 3.6 as the protein concentration was increased from 3 to 12 µM, and a minor peak at S = 2.2 became evident at 12 µM protein (Fig. 4A). This behaviour is characteristic of a monomer-dimer equilibrium which is in fast exchange on the time scale of the moving boundary. The results of sedimentation-equilibrium experiments on dl.Y146A were satisfactorily fitted by a monomer-dimer equilibrium with Ka = 20 ± 3 µM (Fig. 4B).

The presence of 200 µM NADH in sedimentation velocity experiments (using interference optics to circumvent problems due to the high absorbance of the nucleotide)
had no significant effect on the relation between \( c(s) \) and \( S \) for wild-type dI in the concentration range 3 - 12 \( \mu M \). However, NADH altered the profiles for dI.Y146A indicating a decreased rate of exchange between monomer and dimer, probably the result of a shift into the dimeric species (37). Fig. 4C shows data with 6 \( \mu M \) protein; results with 3 \( \mu M \) and 12 \( \mu M \) were similar in character.

In differential scanning calorimetry experiments with wild-type dI, the temperature at the maximum of the sharp endothermic transition peak (\( T_m \)) was 61.1°C (supplemental Fig. S4). At higher temperatures the change in heat capacity indicated exothermic protein aggregation. In experiments with dI.Y146A under similar conditions the \( T_m \) was appreciably lower (45.5°C) but, again, higher temperatures led to exothermic protein aggregation. For both wild-type and mutant proteins, the \( T_m \) values were independent of protein concentration between 12 and 24 \( \mu M \), and they were not affected by the presence of 200 \( \mu M \) NADH. The scans were not reversible even from temperatures just above (~1°C) the respective \( T_m \).

**Fluorescence of wild-type dI and its Y146 mutants: NADH binding to dI.** The dI component of *R. rubrum* transhydrogenase has a single Trp residue (at position 72) in the \( \beta \)-sheet of domain dI.1. The fluorescence emission spectrum of Trp72 has a remarkably short-wavelength maximum at 308 nm and distinctive fine structure (23). These features are attributable to the very rigid local environment of the indole side chain of this Trp (38). Both dI.Y146A and freshly prepared dI.Y146F had very similar spectra to wild-type protein (inset of Fig.5). Whereas the emission spectra of dI.Y146A and of wild-type dI remained unchanged after storage either for > 2 days at 4°C or for > 3 months at -15°C, that of dI.Y146F changed roughly in parallel with the loss of transhydrogenation activity described above; the wavelength of maximum emission shifted from 308 nm to ~340 nm, and the emission band broadened and lost its fine structure (not shown).

The binding of NADH to wild-type dI leads to quenching of the fluorescence of Trp72, from which a \( K_d \approx 18 \mu M \) was calculated (23). The addition of NADH to dI.Y146A quenched the Trp72 fluorescence but, for a given concentration of nucleotide, less than in the experiments with wild-type protein (Fig. 5). Because of the tendency of dI.Y146A to dissociate into monomers, the data are not expected to fit to a simple binding model but were analysed as described in the Discussion.

Isothermal titration calorimetry was used to measure the thermodynamic parameters of the binding reaction between NADH and wild-type dI (15). The protein dimer binds two molecules of NADH, each with a similar \( K_d \approx 18 \mu M \), and with \( \Delta H \approx -62 \text{ kJ mol}^{-1} \text{ monomer} \). In the present work, the data for wild-type protein yielded approximately similar values. Fig. 6 is a comparison of the heat changes accompanying titrations of wild-type dI and of dI.Y146A with NADH under similar solution conditions. In the early part of the titration the measured heat changes upon addition of NADH were much smaller for the mutant protein than for the wild-type. Analysis of the mutant data assuming one class of binding site per dI monomer indicated weaker NADH binding (\( K_d = 62 \mu M \)) and a lower binding enthalpy (\( \Delta H = -22 \text{ kJ mol}^{-1} \text{ monomer} \)) than for the wild-type protein. However, because NADH binding to dI.Y146A seems to shift the monomer/dimer equilibrium, and because there may be heat changes associated with this process, it will be suggested in the Discussion that the calorimetry experiments for the mutant cannot be interpreted quantitatively.

**NMR spectra of wild-type dI and dI.Y146A.** The dI component of wild-type transhydrogenase has a "mobile loop" from residues ~220 to ~240. Because of their short segmental correlation time, amino acid residues in the loop give rise to a set of relatively narrow resonances in the \(^1\text{H} \) NMR spectrum of wild-type dI that are superimposed on the much broader resonances derived from the rest of the protein. A few additional sharp resonances arise from residues (eg Ala384) at the C-terminus of the protein, and from presently unidentified residues (39). The \(^1\text{H} \) NMR spectrum of dI.Y146A is very similar to that of the wild-type suggesting that the protein fold is not altered by the mutation. Most of
the relatively narrow resonances previously identified in wild-type spectrum are clearly seen in the Y.146A spectrum with similar chemical shifts. There are some differences between the spectra of the mutant and wild-type dl, notably in the region of the S-methyl groups of Met residues around 2.0 ppm (Fig. 7). Four Met residues producing relatively narrow resonances were identified in this region of the spectrum of wild-type protein. Two, originally described as MetA and MetC at 1.97 and 2.06 ppm were later assigned, respectively, to Met239 and Met226 in the mobile loop, and two (MetB at 2.04 ppm and MetD at 2.08 ppm) are unassigned but are not in the mobile loop (39-41). There are only two peaks in this region of the dl.Y146A NMR spectrum - at 1.97 ppm and 2.06 ppm. On the basis of their chemical shifts, they are assigned to the mobile loop residues, Met239 and Met226, respectively (and see below).

The addition of either NAD$^+$ or NADH to wild-type dl causes a broadening of the mobile loop resonances (42). This was interpreted as evidence that the loop closes down on the surface of the protein during nucleotide binding, a view that is supported by crystal structures of isolated dl and the dl.dIII complexes. The addition of NADH to dl.Y146A produced a very similar response. The protein and nucleotide are in a fast to medium exchange on the NMR time scale and titration data do not, therefore, provide accurate $K_d$ values. Nevertheless, similar concentrations of NADH led to similar effects on resonance broadening in the wild-type and mutant proteins (data not shown), thus indicating at least somewhat similar binding affinities. In wild-type dl, the addition of either NAD$^+$ or NADH causes broadening of the resonances due to Met226 and Met229 (amongst others in the mobile loop) but not those of MetB and MetD, which remain prominent in the spectrum (Fig. 7). In dl.Y146A, MetB and MetD are still not evident even after the Met226 and Met239 resonances have been extensively broadened by addition of NADH. This suggests that the MetB and MetD resonances are not detected in the spectrum of dl.Y146A because they are already broadened by the substitution of Tyr146 by Ala.

Monomer-dimer exchange in wild-type dl. A form of wild-type dl (designated dlE7) in which a 7-Glu tag is introduced at the C-terminus of the protein was prepared. With similar profiles to wild-type dl, purified dlE7 was able completely to restore transhydrogenation to dl-depleted R. rubrum membranes (not shown but compare Fig. 2) and to form fully active complexes with dIII (not shown but compare Fig. 3).

Samples of untagged dl and dlE7 were separately subjected to anion-exchange chromatography. As expected from the extra negative charge, higher concentrations of salt were needed to elute the tagged protein. Note that the two forms are readily distinguished on SDS-PAGE ($M_r$ of dl = 40.3 kDa and $M_r$ of dlE7 = 41.2 kDa). In the experiment shown in Fig. 8, samples of dlE7 and untagged dl were mixed and immediately (<5 min) applied to an ion-exchange column at 4°C. Fractions eluted from the column by a salt gradient were analysed by SDS-PAGE. Homodimers of untagged dl were eluted first from the column, followed by a small quantity dlE7 heterodimers, and finally homodimers of dlE7. At the protein concentration used in this experiment the monomeric forms are expected to be present only in low amounts. However, the formation of the heterodimers indicates that the following "scrambling" reactions take place at a significant rate on the time scale of the experiment:

\[
\begin{align*}
dl + dlE7 &\rightarrow dl + dlE7 \\
dl + dlE7 &\rightarrow dl.dlE7
\end{align*}
\]

When the mixture of dl and dlE7 was incubated for 2.5 h at 4°C before applying to the ion-exchange column, the amount of the heterodimer was significantly increased although the scrambling process was still not complete (at complete scrambling, the concentration of dl.dlE7 should be twice that of the dl and dlE7 homodimers). If the second-order rate constant for the association of the two protein monomers, $k_{on} \approx 10^7 \text{ M}^{-1}\text{s}^{-1}$ (generally a good approximation, see (43)), then a first-order rate constant for dissociation of the dimer, $k_{off} \approx 10^3 \text{s}^{-1}$, and hence a $K_d \approx 10^{-10}$ M, gives order-of-magnitude agreement with the observed scrambling kinetics.

**DISCUSSION**
Freshly prepared dI.Y146F of *R. rubrum* transhydrogenase behaves similarly to wild-type dI in the construction of intact transhydrogenase with dI-depleted membranes (supplementary Fig. S1) and in the construction of dI_dIII complexes (supplementary Fig. S2). The H-bond between the -OH of invariant Tyr146 in one monomer of wild-type dI and the -NH of Phe160 in its symmetry-related partner (see Fig. 1) is evidently not essential for catalysis. The decline in activity of dI.Y146F over a period of several days, and the accompanying pronounced red-shift in the fluorescence spectrum of Trp72, indicates that this mutant protein is somewhat labile.

In contrast, dI.Y146A is stable but its catalytic and solution properties differ greatly from those of wild-type dI. Gel-permeation chromatography (supplementary Fig. S3) and analytical ultracentrifuge experiments (Fig. 4) show that unlike wild-type dI, which exists as a tight dimer at protein concentrations >1 µM, dI.Y146A dissociates into monomers with a dissociation constant $K_d \approx 20$ µM. On this basis, the lowered $T_m$ value of the mutant protein observed by differential scanning calorimetry (supplemental Fig. S4) probably reflects its dissociation into less thermostable monomers. A similar lowering of the $T_m$ upon dissociation of dimers into monomers has been observed in other proteins (44).

In sedimentation velocity experiments the addition of NADH to dI.Y146A is seen to favour the formation of dimers (Fig. 4C). This suggests that dimers bind NADH more tightly than monomers, as shown by the model in Fig. 9. The sedimentation velocity experiments were performed at protein concentrations close to the value of $K_d$ for dI.Y146A and thus, according to the model, NADH substantially shifts the degree of dimer formation through an effect on the coupled equilibria defined by $K_{d1}$ and $K_{d2}$. Wild-type dI showed no such effect since $K_{d1}$ for that protein is very small - the protein is almost completely dimeric at the concentrations used in the experiments.

The observations on the quenching of Trp72 fluorescence by NADH (Fig. 5) are also explained by the model. These experiments were performed at a protein concentration where, in the absence of NADH, wild-type dI is completely dimeric but where dI.Y146A is predominantly monomeric. The NADH titration on wild-type dI yields a $K_d$ for nucleotide binding $\sim 15$ µM, similar to values determined by isothermal titration calorimetry (see below) and by equilibrium dialysis (45). NADH addition to dI.Y146A caused smaller fluorescence changes, and this is attributed in the model to the significantly lowered binding affinity of the monomeric form for NADH. The adsorption isotherm derived from the model provides a good fit to the experimental data using a $K_d$ value for the monomer-dimer equilibrium similar to that derived above, and $K_d$ values for NADH binding to the dI.Y146A dimer similar to those measured for wild-type dI. The sigmoidal character of the theoretical curve for dI.Y146A (not evident in the scatter of the experimental data) results from coupling between the monomer-dimer and the nucleotide-binding equilibria.

To obtain adequate signal-to-noise ratios, studies using isothermal titration calorimetry are performed at higher protein concentrations (~50 µM) than in the Trp fluorescence assays. Again the wild-type dI will be completely dimeric at these concentrations, and the raw data for this protein in Fig. 6 yields thermodynamic parameters for NADH binding similar to previously published values (15). At 50 µM, about 50% of the dI.Y146A mutant protein is expected to be in the dimeric form. In the model (Fig. 9), the addition of NADH to dI.Y146A will lead to nucleotide binding to the dimer and hence to further association of monomers into dimers. The less negative heat changes observed upon NADH addition in the early part of the dI.Y146A titration compared with those for wild-type dI (Fig. 6) might, therefore, be partly explained by the smaller amount of dimer available for nucleotide binding, and partly by a positive enthalpy of dimer formation in the mutant. However, since the contributions to the measured heat changes from NADH binding and from dimer formation cannot be easily separated, the thermodynamic parameters are not unambiguously determined for the mutant protein.

The NMR experiments of Fig. 7 were performed at protein concentrations where both wild-type dI and dI.Y146A are...
almost completely dimeric. The fact that the dependences of band broadening of the mobile loop resonances on NADH concentration were similar for the two proteins is therefore consistent with the notion (assumed in the analysis of Fig. 5) that dimers of wild-type dI and of dI.Y146A bind the nucleotide with approximately similar affinities.

The finding that the dI.Y146A dimer dissociates more readily into monomers than the wild-type protein, and that the dI.Y146A monomer less readily binds nucleotide, provides a straightforward explanation for the results of the experiments shown in Figs. 2 and 3. At low protein concentrations, where the mutant is predominantly monomeric, it is unable to form either active, complete transhydrogenase with dI-depleted membranes (Fig. 2), or active complexes with isolated dIII (Fig. 3). However, at high concentrations, where dI.Y146A is dimeric, it can form active, complete transhydrogenase with dI-depleted membranes, and active dI₂dIII₁ complexes with isolated dIII. Only the dimeric dI.Y146A can bind NAD(H) and thus form a competent active site. Because the K₄ for dI.Y146A dimer dissociation is rather high, the experiments in Figs. 2 and 3 were not performed with saturating dimer concentrations. Nevertheless, they show that dI.Y146A dimers can give intact enzyme and dI₂dIII₁ complexes which are at least 30% (and possibly much more) as active as those with wild-type dI.

Most of the contact surface between the monomers of the R. rubrum dI dimer is between the two dI.2 domains. This surface is slightly elliptical in shape and, as is often the case in dimer interfaces, fairly flat (46). However, the prominent β-hairpin structures which extend, one from each dI.2, curl around the base of the dI "cleft" to form additional spurs of contact with each dI.1 (see Fig. 1). Tyr146 is located near the middle of helix α6, close to the centre of the elliptical contact surface between the two dI.2 domains. This helix is of particular significance to the alternating-sites hypothesis (16;47) because, as well as making large contributions to the interaction surface between the two dI monomers, with α11 it also links the dI.1 and dI.2 domains of each of the monomers. Furthermore, at its N-terminus is the highly conserved RQD loop which binds the (dihydro)nicotinamide ring of NAD(H), and at its C-terminus is the β-hairpin which is suggested to regulate changes in dI-dIII interaction during enzyme turnover (20), and see below. The energy of the interaction between the two monomers of dI.Y146A is decreased by some 30 kJ mol⁻¹ relative to the wild-type protein. Presumably, the dimer is destabilised as a consequence of water molecules entering the cavity left by the substitution of Tyr146 with Ala and causing disruption at the interface.

It is unlikely that the loss of NADH-binding affinity in monomers of dI.Y146A is a trivial consequence of protein unfolding resulting from dissociation of the dimers. Firstly, the inset of Fig. 5 shows that the characteristic short-wavelength fluorescence emission spectrum of wild-type dI is not altered in dI.Y146A, even at a protein concentration (1.0 µM) that greatly favours the monomer. Equivalently, the circular dichroism spectrum of 1.0 µM dI.Y146A was unchanged from that of the wild-type dI dimer (supplementary Fig. S5). Secondly, it was observed that low concentrations of dI.Y146A inhibited transhydrogenation in membranes reconstituted with wild-type dI (Fig. 2), and in dI₂dI₁ complexes (Fig. 3). This indicates that monomers of dI.Y146A can compete with wild-type dI dimers and bind to dI₂dIII₁ complexes in dI-depleted membranes, and to isolated dIII. A possible explanation for the lowered affinity for NADH of the mutant monomer is that substitution of Tyr146 with Ala, independently of causing dimer dissociation, produces an extended conformational change along α6 (some 15 Å) to distort the nicotinamide-binding RQD loop. However, against this suggestion is the observation that dimeric dI.Y146A binds NADH with a similar affinity to dimeric wild-type dI; it would have to be argued that formation of the dimer overcomes, or repairs, the deleterious effect of the mutation. It is more likely that the process of dissociation of dI.Y146 dimers into monomers itself leads to a decrease in NADH-binding affinity. The elliptical contact surface between the two dI.2 domains of the dimer is some distance from the bound nucleotide. However, the
invariant Ala166-Ala167-Gly168 motif in the turn of the β-hairpin of each monomer packs against residues, including Gln132, of the RQD loop of the symmetry-related partner in the dimer. The invariant and essential Gln132 also contacts the NADH nicotinamide (18;29;48). The interaction between the β-hairpin and the RQD loop is lost in monomers of dI, and the consequent disturbance might be expected to affect NADH binding. Such an effect is consistent with an earlier suggestion. X-ray structures of dI and the dIII1 complex showed that the (dihydro)nicotinamide ring of NAD(H) can occupy different positions, "distal" and "proximal" to the NADP(H) bound to dIII. A shift between the two positions is thought to be necessary to gate the redox reaction during coupling to proton translocation across the membrane: hydride transfer is possible only in the proximal position, when the C4 atoms of the nicotinamide rings are ~3.4 Å apart (18;20). The X-ray structures show that the distal-proximal switch correlates with a rotation of dI.1 relative to dI.2, and to movements of the β-hairpin; these motions were thought to be linked, and to be responsible for controlling the structural alternation of the two hydride-transfer sites in the dimer. The indication from the current experiments, that disruption between the β-hairpin and the RQD loop leads to weaker NADH binding, may also be a reflection of the important interactions in this region of the protein.

An understanding of the broadening of the MetB and MetD resonances in the NMR spectrum of dI.Y146A (Fig. 7) may provide an insight into the dynamics of the β-hairpin in the dI dimer. In wild-type dI, the monomer and dimer are in slow exchange on the NMR time scale (from the data of Fig. 8, the mean lifetime (49) is in the order of seconds). The MetB and MetD resonances must arise exclusively from the dimer, which is present at a much higher concentration than the monomer. In dI.Y146A, the monomer and dimer are in intermediate exchange on the NMR time scale (the increase in $K_d$ from the experiments of Fig. 8 indicates a mean lifetime of ~$10^{-3}$ s). The chemical environment of MetB and MetD are different in the monomer and dimer, and the resonances are therefore broadened by the exchange, and become undetectable in the mutant spectrum. Of the 15 Met residues in R. rubrum dI, only Met162, Met163 and Met164 are located close to the dimer interface, making them good candidates for MetB and MetD. This consecutive run of Met residues is in the β-hairpin; their relatively narrow bandwidth thus suggests that the hairpin has significant segmental flexibility in the wild-type dimer. The dynamics of the β-hairpin, which have also aroused the interest of other investigators (36), and the interactions between this feature and both the NAD(H)-binding site on dI and the NADP(H)-binding site on dIII (20), may be critical in the energy coupling reactions of transhydrogenase.

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FOOTNOTES

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The abbreviations used are: AcPdAD\(^+\), acetyl pyridine adenine dinucleotide; NAD(H), both NAD\(^+\) and NADH etc; dI, the NAD(H)-binding component of transhydrogenase; dII, the membrane-spanning component; dIII, the NADP(H)-binding component; dI.Y146A, dI in which Tyr146 is replaced by Ala etc; dI.E\(^{7}\), wild-type dI with a C-terminal tag of 7 Glu residues.

FIGURE LEGENDS

Figure 1. The location of Tyr146 in the dI dimer interface.
(a) The X-ray structure of the dI\(_2\)dIII\(_1\) complex (PDB code, 2OO5) is viewed from the bacterial cytoplasm downwards, as if towards the membrane. The dI(A) polypeptide is colored yellow, the dI(B) polypeptide is green and the dIII polypeptide is pale red; all are shown in a "ribbon" format. The α6 helix, β-hairpin and RQD loop of each dI are highlighted in more intense color. The NADP\(^+\) and the two molecules of H\(_2\)NADH (tetrahydro-NADH), a close analogue of NADH, are shown in space-filling format in atom colors (20). The two Tyr146 residues are also in atom colours in space-filling format. Only the NADP\(^+\) and features associated with the (B) polypeptide are labelled, including the two domains, dI.1(B) and dI.2(B). (b) Stereo representation of the detail around Y146(A) viewed along the direction of the arrow in panel (a).

Figure 2. Restoration of transhydrogenase activity in dI-depleted R. rubrum membranes with dI.Y146A.
•, ♦ Assay mixtures contained 50 mM Na-MOPS, pH 7.2, 50 mM KCl, 2 mM MgCl\(_2\), dI-depleted R. rubrum membranes (10.5 µM bacteriochlorophyll), 200 µM NADPH, 3 µM carbonyl cyanide-p-trifluoromethoxy phenylhydrazone, and either wild-type dI (•) or dI.Y146A (♦) at the concentration shown. The reaction was initiated with 200 µM AcPdAD\(^+\). An equivalent set of the data for wild-type dI is shown in Supplemental Fig. S1 on an expanded scale. ◆ Assays in similar conditions except that 40 nM wild-type dI was present throughout, and the concentration of dI.Y146A was varied as shown on the bottom axis. T = 25°C.

Figure 3. Transhydrogenase activity in complexes of dIII and either wild-type dI or dI.Y146A.
•, ♦ Assay mixtures contained 50 mM Na-MOPS, pH 7.2, 50 mM KCl, 10 mM (NH\(_4\))\(_2\)SO\(_4\), 2 mM MgCl\(_2\), 200 µM NADH, 20 nM dIII (bearing tightly-bound NADP\(^+\)) and either wild-type dI (•) or dI.Y146A (♦) at the concentration shown. The reaction was initiated with 200 µM AcPdAD\(^+\). An equivalent set of the data for wild-type dI is shown in Supplemental Fig. S2 on an expanded scale. ◆ Assays in similar conditions except that 45 nM wild-type dI was present throughout and the concentration of dI.Y146A was varied as shown on the bottom axis. T = 25°C.

Figure 4. Analytical ultracentrifuge analysis of wild-type dI and dI.Y146A.
In all experiments, proteins were first dialysed and spin concentrated (see Experimental Procedures) in 30 mM Hepes, pH 8.0, 10 mM (NH\(_4\))\(_2\)SO\(_4\). Panel A, sedimentation velocity experiment. Closed symbols, wild-type dI: □, 3 µM; ▲, 6 µM; ●, 12 µM protein. Open symbols, dI.Y146A: △, 3 µM; △, 6 µM, ○, 12 µM protein. The centrifuge was operated at 40,000 rpm and 4 °C using absorbance optics. Panel B, sedimentation equilibrium experiment. The centrifuge was operated at the three speeds shown, at 4 °C,
using interference optics. Three protein concentrations, 3 µM, 6 µM and 12 µM, were used at each of the three speeds; only the data for 6 µM protein is shown in the figure. The number of interference fringes was measured and the data was fitted to a self-association, monomer-dimer model using the software described in Experimental Procedures. The weighted residuals for each fitting are also shown. Panel C, sedimentation velocity experiment carried out as in panel A with 6 µM protein but using interference optics. Closed symbols, wild-type dI: ■, no added NADH; ●, with 200 µM NADH. Open symbols, dI.Y146A; △, no added NADH; ◊, with 200 µM NADH.

Figure 5. Quenching of the fluorescence of Trp72 in wild-type dI and dI.Y146A. Main figure: wild-type dI (●) and dI.Y146A (▲) were suspended to a concentration of 1.0 µM in 30 mM Na-HEPES, pH 8.0, 10 mM (NH₄)₂SO₄, at 25°C. Trp72 fluorescence was excited at 280 nm and emission was measured at 308 nm. Slit widths were set to give a band pass of 4.25 nm. NADH was added to give the indicated final concentration, and the fluorescence quenching was determined. The quenching was corrected for the inner-filtering effect of the nucleotide in parallel experiments by adding NADPH, which does not bind to dI (23). Results from three different protein preparations (of both wild-type dI and dI.Y146A) are combined. The wild-type data were analysed assuming that the protein is entirely dimeric, that NADH binds independently to each monomer with $K_d = 15$ µM, and that the fluorescence quenching is proportional to the fractional occupation of the binding sites. The data for dI.Y146A were analysed using the model shown in Fig. 9, assuming that NADH does not bind to monomeric protein, that $K_{d1} = K_{d2} = K_{d3} = 15$ µM, and using the same relation between the fractional occupation of the sites and fluorescence quenching to that found with wild-type dI. The binding equations were solved using the program, MATHEMATICA. The inset shows the fluorescence emission (in arbitrary units) between 290 and 370 nm of wild-type dI (●), dI.Y146A (▲) and freshly prepared dI.Y146F (◊), with excitation at 280 nm.

Figure 6. Heat changes accompanying the binding of NADH to wild-type dI and to dI.Y146A. Heat changes were measured in a solution containing 30 mM Na-HEPES, pH 8.0, 10 mM (NH₄)₂SO₄, and 50.3 µM wild-type dI (top trace) or dI.Y146A (bottom trace). T = 20°C. Each downward spike corresponds to an enthalpy loss resulting from the injection of NADH. The injections were made in the sequence, 1 × 1.0 µl of 1.5 mM NADH, 33 × 3.0 µl and 23 × 6.0 µl. For the wild-type data, the ORIGIN software returned a $K_d = 19$ µM, $\Delta H = -41$ kJ mol⁻¹ monomer and a stoichiometry of 0.8 NADH-binding sites per monomer. The interpretation of the data for dI.Y146A is discussed in the text.

Figure 7. Effects of NADH on the S-CH₃ region of ¹H-NMR spectra of wild-type dI and dI.Y146A. The dI proteins were prepared by dialysing stored, purified material, firstly, against 10 mM Tris-HCl, pH 7.6, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol, then against 10 mM [²H] Tris-HCl, pH 7.6, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol, 0.25 % (w/v) NaN₃ in ²H₂O. Left, wild-type dI (200 µM); right dI.Y146A (200 µM). Bottom spectra, no NADH; top spectra, plus 90 µM NADH. T = 30°C. Met226 and Met229 were assigned (40;41); provisional assignments of MetB and MetD are discussed in the text.

Figure 8. Scrambling of monomers within the dI dimer. Untagged dI and dIE₇ (each 12 µM) were mixed in 20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol. The mixed sample (5 ml) was immediately applied to a 5 ml Q-Sepharose High Performance column and eluted with a gradient of 0-2 M NaCl in the same buffer at 1.0 ml min⁻¹. T = 4°C. Fractions (0.5 ml) were collected and subjected to SDS-PAGE. The bottom row of figures shows the fraction number, and the top row shows the concentration of NaCl in the gradient at that point. Samples of tagged and untagged dI, and a mixture of tagged and untagged protein that had not been subjected to ion-exchange separation are also shown. Control experiments revealed that, when pure dI and pure dIE₇ were separately applied to the column, they eluted at around 340 mM and 600 mM NaCl, respectively.

Figure 9. Model of NADH binding to dI.Y146A.
Here, dI represents dI.Y146A. $K_d$, $K_{d2}$ and $K_{d3}$ are dissociation constants for the reactions indicated. The dI.Y146A monomers bind NADH with a very high $K_d$, and the two dimer species with bound NADH dissociate into monomers with a very low $K_d$. 
Figure 2

Rate of AcPdAD\(^+\) reduction
(mol mol\(^{-1}\)Bacteriochlorophyll min\(^{-1}\))

Concentration of dI (\(\mu\)M)

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Concentration of dI (\(\mu\)M)
Rate of AcPdAD$^+$ reduction

(mol mol$^{-1}$ dIII min$^{-1}$)

Concentration of dI ($\mu$M)

Figure 3
Figure 4A
Figure 4C

Sedimentation velocity (S)

c(s)
Figure 5

Fluorescence quenching (arbitrary units) vs. Concentration of NADH (µM)

- The graph shows a positive correlation between fluorescence quenching and concentration of NADH.
- The data points are scattered, with a trend line indicating a gradual increase in quenching with NADH concentration.

Inset: A closer view of the data points, highlighting the curvature and spread of the values.
Figure 7

Wild-type dI

Met226 MetB Met239

MetD

Chemical shift (ppm)

2.10 2.00 1.90

dI.Y146A

Met226

Met239

plus NADH

no addition
Figure 8

NaCl concentration at which fraction was eluted from the ion-exchange column.

| Fraction number: | 22 | 25 | 35 | 40 | 41 | 54 | 55 | 57 |
|------------------|----|----|----|----|----|----|----|----|
| 320 mM | 360 mM | 460 mM | 500 mM | 520 mM | 640 mM | 660 mM | 680 mM |

original dI
mixture of dI and dIE₇
original dIE₇

dIE₇ ➔
dI ➔
Figure 9

\[ \text{dI} + \text{dI} \xleftrightarrow{K_{d1}} \text{dI} \xleftrightarrow{K_{d2}} \text{dI.NADH} \xleftrightarrow{K_{d3}} \text{dI.NADH} \]
Substitution of tyrosine-146 in the dI component of proton-translocating transhydrogenase leads to reversible dissociation of the active dimer into inactive monomers

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