The Heterotrimer of the Membrane-peripheral Components of Transhydrogenase and the Alternating-site Mechanism of Proton Translocation*

Transhydrogenase undergoes conformational changes to couple the redox reaction between NAD(H) and NADP(H) to proton translocation across a membrane. The protein comprises three components: dI, which binds NAD(H); dIII, which binds NADP(H); and dII, which spans the membrane. Experiments using isothermal titration calorimetry, analytical ultracentrifugation, and small angle x-ray scattering show that, in the crystalline state, a mixture of recombinant dI and dIII from Rhodospirillum rubrum transhydrogenase readily forms a dI2dIII1 heterotrimer in solution, but we could find no evidence for the formation of a dI2dII1dIII1 tetramer using these techniques. The asymmetry of the complex suggests that there is an alternation of conformations at the nucleotide-binding sites during proton translocation by the complete enzyme. The characteristics of nucleotide interaction with the isolated dI and dIII components and with the dI2dIII1 heterotrimer were investigated. (a) The rate of release of NADP+ from dIII was decreased 5-fold when the component was incorporated into the heterotrimer. (b) The binding affinity of one of the two nucleotide-binding sites for NADH on the dI dimer was decreased about 17-fold in the dI2dIII1 complex; the other binding site was unaffected. These observations lend strong support to the alternating-site mechanism.

Transhydrogenase, found in the cytoplasmic membranes of bacteria, and in the inner membranes of animal mitochondria, couples the redox reaction between NAD(H) and NADP(H) to the translocation of protons.

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

Its function in energy metabolism, biosynthesis, and detoxification has been discussed at length (1, 2). In different organisms (and possibly in different tissues of the same organism) transhydrogenase can either utilize the proton electrochemical gradient (\(\Delta p\)) to drive reduction of NADP+ by NADH, or it can use NADPH oxidation by NAD+ to augment \(\Delta p\) formation. Energy coupling in transhydrogenase is indirect. In the forward direction (Reaction 1), protein conformational changes accompanying proton translocation bring together the nicotinamide rings of the bound nucleotides to allow the redox reaction (3). This “binding-change mechanism” may share common features with energy coupling in some ion-translocating ATPases. More generally, transhydrogenase has a number of properties that make it an excellent model for understanding the principles of operation of conformationally linked pumps in biology.

The polypeptide organization of transhydrogenases varies between species, but the arrangement of the three components, dI, dII, and dIII, is essentially the same in all (Fig. 1). NAD(H) binds to dI, and NADP(H) binds to dII; these two components protrude from the membrane (into the bacterial cytoplasm or mitochondrial matrix). The dII component spans the membrane, probably in 13 or 14 transmembrane helices (reviewed in Ref. 4). There is cross-linking and hydrodynamic evidence that both the bovine (5, 6) and Escherichia coli (7) transhydrogenases have two copies each of dI, dII, and dIII. We shall refer to this structure as a generic dI2dII2dIII2 “hexamer,” although, in terms of polypeptide composition, it corresponds to a homodimer in the bovine enzyme, an \(\alpha_2\beta_2\) tetramer in E. coli, and a (PntAA)2(PntAB)2(PntB)2 hexamer in Rhodospirillum rubrum. Observations on the intact bovine enzyme indicate that interactions between the two dI-dII-dIII polypeptides are important in turnover. First, measurements of rates of transhydrogenation after partial chemical modification indicate reactivity in half of the sites (8, 9); second, only one NADH and one NADPH can bind per dimer (10).

The dI and dIII components of transhydrogenases can be separately expressed and isolated as stable, water-soluble proteins, which bind their cognate nucleotides. Those from R. rubrum have been studied in the most detail (11–14), but the equivalent components from E. coli (12, 15), bovine (16, 17), human (18), and Entamoeba histolytica (19) transhydrogenases have similar properties. High resolution structures of both dI (20) and dIII (21–24) have now been published. Remarkably, a simple mixture of dI and dIII proteins, even from different species, forms a complex that catalyzes transhydrogenation. Measurement of activity during titration of dI with dIII indicates that the two proteins become tightly associated (the apparent \(K_d \approx 20 \text{ nM}\) for the R. rubrum components) (13). The x-ray structure of the complex in crystals grown from an equimolar mixture of R. rubrum dI and dIII (in the presence of NAD+ and NADP+) has been recently solved at 2.5-Å resolution.

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

† To whom correspondence should be addressed. Tel.: 44-121-414-5423; Fax: 44-121-414-5925; E-mail: j.b.jackson@bham.ac.uk.

William D. Venning‡, Daniel J. Rodrigues‡, Chris J. Weston‡, Nick P. J. Cotton‡, Philip G. Quirk‡, Neil Errington‡, Stephanie Fint‡, Scott A. White‡, and J. Baz Jackson‡

From the ©School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom, the ©National Centre for Macromolecular Hydrodynamics, School of Biosciences, University of Nottingham, Sutton Bonington, Leicestershire LE12 5RD, United Kingdom, and the ©European Synchrotron Radiation Facility, 6 Rue Jules Horowitz, 38043 Grenoble, France.

Received for publication, May 16, 2001
Published, JBC Papers in Press, June 8, 2001, DOI 10.1074/jbc.M104429200

The Journal of Biological Chemistry Vol. 276, No. 33, Issue of August 17, pp. 30678–30685, 2001 Published in U.S.A.

This paper is available online at http://www.jbc.org/ by guest on January 1, 2018

http://www.jbc.org/
The isolation of DNA coding for the dI and dIII components of *R. rubrum* transhydrogenase, the mutagenesis procedures, and the expression, purification, and storage of the proteins have been described (11, 13). The dIII proteins were prepared either in their NADP⁺ form or their NADPH form, as described (14). When necessary, proteins were concentrated by centrifugation through Vivapak spin filters. All preparations were checked for purity by SDS-polyacrylamide gel electrophoresis and were assayed for transhydrogenase activity with appropriate protein partners to ensure comparability with published data, again by established methods. Protein concentrations were determined by the micro tannin procedure (26) with bovine serum albumin as a standard; previous results have shown that this assay gives similar results to other methods (13). For isolated dI and dIII, all concentrations are given in terms of the monomers unless otherwise stated.

Isothermal titration calorimetry was carried out using the Microcal MCS as described in the figure legends. Data were analyzed using the instrument software.

Sedimentation velocity experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge. Solutions were loaded into standard 12-mm double-sector cells and run at a rotor speed of 45,000 rpm, at 20 °C. Solute distribution was recorded at 4 min intervals using scanning absorbance optics at a wavelength of 280 nm. The data were analyzed using the time-derivative g(α)(t) method (27) to produce sedimentation coefficient distributions, from which information on heterogeneity, sedimentation coefficient, and diffusion coefficient were obtained. These data were then used in the Svedberg equation (28) to yield molecular weight information.

Small angle x-ray scattering (SAXS) data were collected at the ID2 high brilliance beamline at the European Synchrotron Radiation Facility, Grenoble, France. The scattering curves were measured using a wavelength (λ) of 0.995 Å and with the detector positioned at 2.5 m from the sample (momentum transfer limits of 0.10 < Q < 2.67 nm⁻¹, where Q = 4πsinθ/λ and 2θ is the scattering angle). Following data reduction, the scattering curves were examined for radiation damage. Guinier analysis (29) was performed using Igor Pro (Wavemetrics), and a macro designed by Kenneth Littrel and Diego Pontoni. Observed scattering patterns were compared with the predicted scattering curves produced by CRYOSOL, version 2.1 (30), using the Protein Data Bank file 1HZZ (and truncated forms thereof). Low resolution envelope functions were created using the program SASHA, version 2.04b (31), and were subsequently viewed using ASSA, version 2.0 (32).

The rate of NADP⁺ release from dIII was measured using an enzyme trap (15). The dIII protein (1.0 μM), with or without 1.0 μM dI protein, was incubated in 20 mM Mops, pH 7.2, at 25 °C before addition of 5 units of isocitrate dehydrogenase. The subsequent fluorescence change accompanying the reduction of nucleotide as it dissociated from the dIII was measured using a Spex FluoroMax (340-nm excitation, 450-nm emission, 2.1-nm band pass). The change in NADPH fluorescence as it rebound to dIII was negligible. NADP⁺ release from the E155W mutant of dIII following addition of excess NADPH was measured from the change in Trp fluorescence (15, 33). The dIII protein (with or without dI) was pre-incubated in 20 mM Mops, pH 7.2, containing 20 μM NADP⁺, at 20 °C for 5 min. Nucleotide exchange was then initiated by addition of NADPH (50 μM). The apparent first order rate constant for NADP⁺ release was calculated from the time course of the decrease in protein fluorescence (280-nm excitation, 359-nm emission, 2.1-nm band pass). The pH dependence of NADP⁺ release was determined in similar experiments but using a buffer mixture (20 mM Mes, 20 mM Hepes, 20 mM Mops).

The rate of steady-state “reverse” transhydrogenation (see Reaction 1) in mixtures of dI and dIII was measured as the reduction of AcPdAD⁺ (an NAD⁺ analogue) by NADPH, from the absorbance change at 375 nm, as described previously (13). The dIII protein (final concentration 900 nM), and dI at various concentrations, were incubated at 25 °C in 50 mM Hepes (pH 7.2) containing 15 mM NaF and 20 μM NADP⁺, and 5 units of isocitrate dehydrogenase were added. The rate of NADP⁺ release was determined over 30 min at 375 nm.

### EXPERIMENTAL PROCEDURES

**Fig. 1. Diagram to show the arrangement of the dI, dII, and dIII components in transhydrogenase.** In some species, dI, dII, and dIII are on the same polypeptide chain, and the protein is homodimeric. In other species, breaks in the chain lead to different oligomeric structures but the overall pattern of dI, dII, and dIII probably remains the same.

Alternating Sites of Proton-translocating Transhydrogenase

---

1 The abbreviations used are: SAXS, small angle x-ray scattering; AcPdAD⁺, acetyl pyridine adenine dinucleotide (oxidized form); Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.
the monomer and the dimer (13).

The reaction was initiated by addition of AcPdAD<sup>+</sup> (200 μM final concentration).

Stopped flow experiments were performed on an Applied Photophysics DX-17MV operating in its fluorescence mode with 280-nm excitation light and ~305-nm emission (selected with a WG305 cut-off filter) and with both monochromator slits set to band pass of 9.3 nm. Data were analyzed using the instrument software. For further details, see Refs. 14 and 34.

The kinetic simulations of experimental data were carried out using the program GEPASI, version 3.21 (35).

RESULTS

The Solution Stability of the dI<sub>2</sub>dIII<sub>2</sub>, Heterotrimer of Transhydrogenase—Experiments with the isothermal titration calorimeter showed that the binding of R. rubrum dI to R. rubrum dIII is accompanied by an enthalpy change of +9.48 kJ·mol<sup>-1</sup> of dIII. At the protein concentrations used, the end point of the titration was abrupt, indicating a tight association of the two components (Fig. 2). In three separate experiments, the heat change titrated with a binding stoichiometry of 1.87, 1.76, and 2.05 mol of dI/mol of dIII, consistent with the formation of a dI<sub>2</sub>dIII<sub>2</sub> heterotrimer; the K<sub>d</sub> was < 60 μM. Experiments at higher concentrations of protein (75 μM dI with additions of dIII up to 128 μM) did not reveal any evidence for the formation of dI<sub>2</sub>dIII<sub>2</sub> tetramer (data not shown).

The results of sedimentation velocity experiments in the analytical ultracentrifuge confirm that R. rubrum dI in solution is dimeric (Fig. 3), i.e., in accordance with earlier gel filtration studies of dI from R. rubrum (11), E. coli (12), and bovine (36) transhydrogenases. The calculated mass is 77.5 kDa (compare 21.5 kDa from the amino acid sequence of the monomer). NMR experiments with R. rubrum dIII also indicate that the protein is a monomer (25), although on size-exclusion chromatography it behaves anomalously; the apparent M<sub>r</sub> value is intermediate between those expected for the monomer and the dimer (13). E. coli dIII is monomeric on size-exclusion chromatography (16), but has an unexpectedly large correlation time in NMR experiments, perhaps indicating some tendency to aggregate (37). Fig. 3 finally shows the g(s<sup>*</sup>) profile of an equimolar mixture of R. rubrum dI and dIII from a sedimentation velocity experiment. The results can be fitted...
Thus, the protein’s radius of gyration ($R_g$) is smaller when NADP(H) is bound to the protein than when NADP(H) is bound to the protein. The data are again consistent with the conclusion that dI is a dimer in solution. Scattering profiles are shown for dI (250 μm, dashed line), dIII (440 μm, dotted line), and a mixture of the two components (solid line). The buffer (20 mM HEPES, pH 8.0, 10 mM (NH₄)₂SO₄, 2 mM dithiothreitol, 4 μM NADP⁺) was degassed before use. Guinier plots of the data were linear in the so-called “Guinier region” (29) and thus provided the $R_g$ values predicted by CRY SOL from the x-ray structure of the dimer derived from 2.0-Å crystal structures of dI and dIII and a mixture of the two components (Fig. 4). The Guinier plot of the SAXS data from experiments to show that NADP(H) release is even slower from the dI2dIII complex than from isolated dIII (see Ref. 13 and “Experimental Procedures”). The $k_{off}$ for NADP⁺ release from isolated wild-type protein (0.03 s⁻¹) was similar to that measured in the earlier work (0.025 s⁻¹). However, in the presence of a molar equivalent of dI protein, the apparent $k_{off}$ value decreased to 0.008 s⁻¹. At higher concentrations of dI, the apparent $k_{off}$ decreased even further, but the accuracy of the measurement was compromised by the problem of providing sufficient isocitrate dehydrogenase to reduce the released NADP⁺ before it rebound to the dIII.

In a second set of experiments, we used a mutant of R. rubrum dIII (dIII.E155W) whose single Trp residue is therefore a reliable indicator of events in wild-type protein. The kinetic and thermodynamic properties of the mutant are unaffected by the introduction of the Trp residue, and measurements of the fluorescence are therefore a reliable indicator of events in wild-type protein. The rate of Trp fluorescence quenching following the addition of excess NADP⁺ to dIII.E155W loaded with NADP⁺ gives the rate constant for NADP⁺ release (16, 33) (see Scheme 1). Results with isolated dIII were very similar to those described previously; $k_{off}$ = 0.022 s⁻¹. Fig. 6, however, shows that, in the presence of excess dI, the apparent $k_{off}$ for NADP⁺ release decreased about 5-fold. Control experiments at appropriate wavelengths showed that, in these experiments, there was no significant contribution to the measured fluorescence changes from the single Trp in the dI protein. It is concluded that NADP⁺ release from the dI2dIII complex is slower than that from isolated dIII. The character of the titration data can then be satisfactorily modeled on the basis that the $dI_2 + dIII \leftrightarrow dI_2dIII$ reaction equilibrates rapidly relative to the rates of NADP⁺ release either from isolated dIII or from the complex (Scheme 2). The fit, using experimentally derived constants, is shown in Fig. 6.

A complementary set of experiments was performed to determine the rate of Trp fluorescence enhancement following the addition of excess NADP⁺ to dIII.E155W loaded with NADPH (see Ref. 33). These results showed that the rate of NADPH release from the dI2dIII complex is also significantly slower than that from isolated dIII (data not shown). Because the rate constants for NADPH release are so low (for example,
The dI₂dIII₁ complex (see above) and the measured rate constants for the association and dissociation of dI₂dIII₁ complex. The data were simulated (dashed line) with Scheme 2 using the program GEPASI. The model used the measured rate constant for dI₂dIII₁ complex dissociation (50 s⁻¹; Ref. 14), a value of 2 × 10⁸ m⁻¹ s⁻¹ for the complex association constant (calculated from the equilibrium constant of 25 ns; Ref. 13), and the limiting measured values of kₐ = 0.0220 s⁻¹ and k₉ = 0.0466 s⁻¹ for NADP⁺ release from isolated dIII and from dI₂dIII₁, respectively.

SCHEME 2. Determination of the apparent k₉ for NADP⁺ from dIII in the presence of dI.

k₉ ≈ 3 × 10⁻⁴ s⁻¹ for isolated dIII), we did not attempt to describe the effect on a quantitative basis.

The lower rate of release of NADP⁺ from dI₂dIII₁ than from isolated dIII provides an explanation for the observation shown in Fig. 7. At a fixed, total concentration of dIII, the steady-state rate of transhydrogenation (AcPdAD⁺ reduction by NADPH) first increased, and then decreased, with the total concentration of dI. At very low dI, transhydrogenation is limited by the second-order interaction between the dI dimer and the dIII monomer that generates the catalytic dI₂dIII₁ complex (Scheme 3, step a). AcPdAD⁺ binding, hydride transfer, and AcPdADH release are all relatively fast (step b) (see Ref. 14). Net dissociation of the dI₂dIII₁ complex (now with bound NADP⁺) is favorable (step c), and thus release of NADP⁺ proceeds mainly from the dissociated dIII (step d). At higher concentrations of dI dimer, the rate limitation of the overall reaction results, not from step a, but from NADP⁺ release. Ultimately, high concentrations of dI suppress dissociation at step c; thus, NADP⁺ release proceeds increasingly from the dI₂dIII₁ complex, step e, i.e. more slowly. The modeling with Scheme 3, using measured rate constants for the association and dissociation of the dI₂dIII₁ complex (see above) and the measured rate constants for NADP⁺ release (from Fig. 6), produces a quite reasonable fit to the experimental data (Fig. 7).

The rate constants for release of NADP⁺ and of NADPH from isolated dIII greatly increase at low pH (33). The data suggested that nucleotide release from dIII is preceded by protonation, and the possibility was raised that this might correspond to a step in proton translocation in the intact enzyme. Using the procedure described above (Fig. 6), it was found that the rate constant for NADP⁺ release from the dI₂dIII₁ complex also increased markedly as the pH of the solution was lowered (data not shown). Experiments were performed with 3.0 μM dI plus 1.0 μM dIII, conditions that favor formation of the dI₂dIII₁ complex. Across the pH range (5.3–7.0), the rate constant for NADP⁺ release from dIII decreased by the same factor (~5) upon addition of the 3-fold excess of dI, indicating that protonation of dIII is unaffected by complexation with dI.

The Effect of dI₂dIII₁ Complex Formation on NADH Binding to dI—Experiments using equilibrium dialysis (39) and protein fluorescence quenching (11) showed that NADH binds to isolated R. rubrum dI protein with a K_d ≈ 20 μM. One mol of NADH binds per 1 mol of protomer (i.e. 2/dimer). The results of experiments using isothermal titration calorimetry show that the binding of NADH to isolated R. rubrum dI is accompanied by an enthalpy change of −123.4 kJ·mol⁻¹ of dimer. Consistent with the other methods, the data reveal just a single class of binding site, K_d = 18.3 ± 4.1 μM (Fig. 8, upper panel).

It is difficult to study the binding of NADH to the dI₂dIII₁
complex by either equilibrium dialysis or by protein fluorescence (preliminary results, and the technical reasons for caution, have been discussed elsewhere (Ref. 34)). However, isothermal titration calorimetry provides a way forward. Note that dIII becomes unstable in the absence of bound nucleotide and the protein was therefore pre-loaded with NADPH (see “Experimental Procedures”) before mixing with dI under these conditions, there can be no redox chemistry between the bound nucleotides. Experiments with dI and dIII, added either in a 2:1 ratio (Fig. 8, lower panel) or with dIII in 2-fold excess (data not shown), gave similar results; the data reveal two classes of NADH binding site in the complex. The first has an enthalpy change of $-133.6 \pm 7 \text{kJ mol}^{-1}$ of dI.dIII, a stoichiometry of 0.96 mol of NADH/mol of dI.dIII, and a $K_d = 17.4 \pm 4.7 \mu M$. The enthalpy change and the affinity of this first site are similar, within error, to those of the isolated dI protein. However, the second site has a much lower affinity. It was impossible to titrate the low affinity site to completion because the heat change for NADH binding was small in comparison with the nucleotide’s heat of dilution. However, assuming that the binding capacity of the low affinity site is 1.0 mol of dI.dIII, the data fitted to a $K_d = 311 \pm 91 \mu M$ and an enthalpy change of $-79.4 \text{kJ mol}^{-1}$ of dI.dIII.

In recent stopped-flow experiments, we recorded the kinetics of the fluorescence quenching of the (single) Trp72 in dI during NADH binding (34); the objective was to determine the $k_{on}$ and $k_{off}$ for that nucleotide-binding step. We noted that the amplitude of the dI fluorescence quenching was decreased when dIII.NADPH was present (the wild-type dIII protein, with no Trp residues, was used). In view of the observation that the dI.dIII complex appears to have two classes of NADH-binding site (see above), this experiment was repeated (Fig. 9), with a wider range of concentrations of dIII.NADPH, and with emphasis on an assessment of the relative amplitude of the fluorescence change. As on the previous occasion, the experiment was performed at low temperature, and a low NADH concentration, to slow down the rate of the nucleotide-binding reaction. Consistent with the earlier data, the amplitude of the fluorescence change decreased, but the apparent first order rate constant for that change was unaffected, as the concentration of dIII.NADPH was increased. With the extra data, we now establish that, at high concentrations of dIII.NADPH, the decrease in the fluorescence amplitude became constant at $\sim50\%$ of the level observed in the absence of dIII. Saturation was reached with formation of the heterotrimer, at about 0.5 mol of dIII/mol of dI. Control experiments, using phenylalanine as an inert absorbing solute, showed that the decrease in fluorescence was not a consequence of inner filtering by dIII. This experiment thus provides another indication of the two different NADH-binding sites in the dI.dIII complex. One (with 50% of the total binding capacity) has the same nucleotide-binding kinetics and $K_d$ as the two NADH sites in the isolated dI dimer. The other equates with the low affinity site seen in the microcalorimetry experiments; if its $K_d$ is 311 $\mu M$ (see above), then very little NADH ($<8\%$) will bind to this site at the low nucleotide concentration used in the stopped-flow experiment.

Following experiments to characterize the hydride transfer reaction between NADPH and NAD+ in mixtures of dI and dIII, we showed that the value of the rate constant for product

---

**FIG. 8. Affinities of NADH binding to isolated dI and to the dI.dIII complex.** Measurements were carried out by isothermal titration calorimetry, as described under “Experimental Procedures.” The cell contained either 150 $\mu M$ dI protein (upper panel) or 150 $\mu M$ dI plus 75 $\mu M$ dIII (lower panel) in 10 mM (NH$_4$)$_2$SO$_4$, 20 mM Hepes, pH 8.0. In both experiments the injection syringe contained 3 $\mu M$ NADH in 10 mM (NH$_4$)$_2$SO$_4$, 20 mM Hepes, pH 8.0. The NADH solution was injected in volumes of 1.0 ml ($\times$1), 2.5 ml ($\times$15), and 6.0 ml ($\times$29). Temperature was 18°C. The fitted curve in the upper panel corresponds to two equivalent NADH-binding sites per dI dimer ($K_d = 19.3 \mu M$), and that in the lower panel to two non-equivalent NADH sites per dI.dIII heterotrimer ($K_d = 17.4$ and 311 $\mu M$).

**FIG. 9. The difference in binding capacities for NADH of dI and of the dI.dIII complex, as revealed by the fluorescence change of Trp72.** The fluorescence change of Trp72 in dI accompanying the binding of NADH was monitored in the stopped-flow machine, as described under “Experimental Procedures.” One drive syringe contained dI plus dIII and the other, NADH. The buffer was 10 mM (NH$_4$)$_2$SO$_4$, 20 mM Hepes, pH 8.0, and the mixing ratio was 1:1. Final concentrations were dI (1 $\mu M$), NADH (15 $\mu M$), and dIII (as shown on the abscissa). Temperature was 7°C. The ordinates show the apparent first order rate constant of the observed fluorescence quenching ($<k_{\text{obs}}>\text{)}$ and the amplitude of the fluorescence change ($\times$), as calculated by the instrument software.
NADH release, measured using isolated dI, was inconsistent with the simple kinetic scheme (34). It was suggested that the rate constant for NADH release from the complex of dI and dIII must be much greater than that for isolated dI protein. The finding that one of the NADH-binding sites on the dI-dIII complex has a greatly elevated \( K_r \) consistent with the suggestion. Furthermore, it would appear that this is the site on dI that is functionally involved in hydride transfer.

**DISCUSSION**

The results described above, from several different types of experiment, show that, in solution in the concentration range 10–100 \( \mu M \), the isolated dI component of *R. rubrum* transhydrogenase is a dimer, isolated dIII is a monomer and the complex is a \( dI_2dIII \) heterotrimer. The complex can catalyze a very fast redox reaction between NAD(H) on dI and NADP(H) on dIII (34), but evidently at just one of the two interfaces that can form in the complete, “hexameric” enzyme (\( dI_2dIII_2 \)). It was proposed that, in the complete enzyme, there are conformational changes that *alternately* bring together the two dI/dIII interfaces to permit the redox reaction (23). These conformational changes are coupled to proton translocation across the membrane. They are propagated in the membrane-spanning dI and are associated with changes in NADP*−* and NADPH binding to dIII (22). Thus, dIII may adopt an “open” state, in which bound NADP(H) can readily exchange with nucleotides in the solvent, and an “occluded” state, in which this exchange is blocked. The occluded dIII can interact with dI such that hydride transfer between bound NADP(H) and NAD(H) is facilitated. The open dIII interacts only weakly with dI, and, in this state, redox chemistry between the bound nucleotides is prohibited. The switch is required to ensure the efficiency of energy coupling (i.e. to prevent proton translocation without redox chemistry and *vice versa*). Alternation of the sites is a consequence of out-of-phase co-ordination of the conformational changes: as one dI/dIII site is driven into the dIII-occluded state (e.g. by protonation of dII from the p-phase), the other is driven into the dIII-open state (e.g. deprotonation of dII to the n-phase, see Reaction 1). If the enzyme is undergoing turnover in the “forward” direction (Reaction 1), the first dI/dIII site then catalyzes hydride transfer from NADH to NADP*−*, while the second simultaneously releases product NADPH (and NAD*+) and rebinds substrate NADP*−* (and NADH).

Isolated dIII is predominantly in the occluded state; it can only release its bound nucleotide extremely slowly into the solvent (see below), but it can bind to dI dimer giving the heterotrimer, which can catalyze a rapid single turnover of hydride transfer between NAD(H) and NADP(H). In the context of the model, the difference in binding affinity of dI2 and of dI2dIII1, for dIII is an indication of the energy required to convert dIII into its open state. There is no direct measure of the \( K_r \) for dissociation of the heterotrimer into dI2+dIII. Analysis of the microcalorimetry (Fig. 2) and ultracentrifugation data (Fig. 3), and indirect observations by stopped-flow spectrophotometry (14, 34), suggest that binding is quite tight (\( K_r < 1 \ \mu M \)) and this is consistent with a \( K_r \sim 25 \ \text{nM} \) calculated from measurements of catalytic activity (13). NMR experiments reveal an interaction between dI2dIII and a second molecule of dIII (25). Significantly, a comparison with the crystal structure shows that some of the amino acid residues involved in this interaction are equivalent to those involved in the interaction of the first dIII with dII within the heterotrimer. From the degree of dIII–resonance broadening at low concentrations of dI2, the \( K_r \) for dissociation of a dIII from dI2dIII1 was estimated to be in the order of 10 \( \mu M \). However, in the microcalorimetry and ultracentrifugation experiments described in this report, we failed to detect the formation of dI2dIII2 tetramer at protein concentrations in the range 10–100 \( \mu M \), suggesting that the \( K_r \) determined by NMR is an underestimate; the value is more likely to be \( > 100 \ \mu M \). Thus, it may be calculated that the \( \Delta G \) needed to switch from the occluded state of dIII to the open state (a free energy change, which, in the complete, “hexameric” enzyme, must be supplied by \( \Delta p \) and delivered through conformational changes in dIII) is \( > 21 \text{kJ mol}^{-1} \). Since one proton is translocated per hydride transferred between nucleotides (40), a \( \Delta G \) of \( -21 \text{kJ mol}^{-1} \) is available to transhydrogenase when \( \Delta p = 0.22 \text{V} \).

We previously observed that the rate of NADP*+ release from isolated dIII is similar to the rate of reverse transhydrogenation catalyzed by the complex of dIII with dI (13). The implication was that reverse transhydrogenation is limited by the rate of product NADP*+ release. However, the observation requires a slight refinement; because NADP*+ release from the complex is substantially slower than from isolated dIII (Fig. 6), it is true only when reverse transhydrogenation is measured within a narrow range of protein concentrations (Scheme 3). Since the rates of NADP*+ release from both isolated dIII and from the dI2dIII1 complex are slow relative to rates of formation and dissociation of the protein complex (and relative to hydride transfer and AcPdAD(H) binding and release), the nucleotide-binding sites on dIII, e.g. at 900 \( \text{nm} \) (Fig. 7), will become fully occupied with NADP*+ even when the dI concentration is only –200 \( \text{nm} \) (i.e. [dI2] = –100 \( \text{nm} \)); effectively, one dI2 protein can productively visit many dIII molecules in the time it takes for the latter to release their nucleotide. At those low protein concentrations, most of the dIII.NADP+, unattached to dI and the nucleotide is released from the protein with a rate constant of 0.022 s\(^{-1}\). As the dI concentration is increased, then more of the dIII.NADP*+ will be in the dI2dIII1 complex, from which the nucleotide is released with a rate constant of 0.004 s\(^{-1}\). Experiments (see above and Ref. 13) indicate that there will be a similar relationship for NADPH release and forward transhydrogenation, but this is difficult to establish on a quantitative basis because the rates are so low.

The finding that NADP(H) release is faster from isolated dIII than from the dI2dIII1 complex might be relevant to our understanding of the process by which these nucleotides are released from the complete enzyme; this is a critical step, thought to be intimately associated with proton translocation. There are two possibilities. (a) The pathway for nucleotide diffusion away from its site is sterically restricted in the complex because of the position of dI. (b) Although the equilibrium still greatly favors the occluded state, there is a shift toward the open form when dIII dissociates from the complex. The crystal structures reveal that there might be some restriction to NADP(H) diffusion from the dIII site resulting from the position of the “RQD” and “mobile” loops on dI (see Ref. 3). Note also that the side-chain amide of Gln\(^{132}\), from the RQD loop of dI, makes an H-bond across the interface with the 2′-OH of the nicotinamide ribose of NADP(H). Loop E (the “lid”) appears to be particularly important in maintaining the high affinity of isolated dIII for NADP(H) (23); its location over the nucleotide, with which it makes numerous H-bonds, appears to be an essential feature of the occluded state, and it is presumably retracted in the formation of the open state of the protein. There are no direct contacts between loop E and dI to be seen in the crystal structure of dI2dIII1 and therefore no clear explanation as to how dI might directly shift the conformation of dIII toward the occluded state. However, an interaction between loop E and the dI mobile loop cannot be ruled out because of the weak electron density of the latter. Moreover, the N terminus of the important helix D/loop D of dIII makes several van der Waals and H-bond contacts (some involving invariant amino acid residues) with loop E. The central region
of helix D/loop D, through highly conserved or invariant amino acid residues, interacts with the RQD loop of dI, and its C terminus is close to the expected position of the membrane-spanning dII. This feature is, therefore, well placed to coordinate conformational changes in loop E and dI and, in the complete enzyme, to couple these to proton translocation.

The organization of the dI.dIIII complex in solution points to an explanation as to why there is a change in $K_d$ of just one of the two NADH-binding sites of the dI dimer upon addition of dIII (Fig. 8). We propose that the dI polypeptide whose cleft is occupied by dIII.NADPH has the greatly decreased NADH-binding affinity, whereas the dI polypeptide whose cleft is unoccupied by dIII has the NADH-binding affinity equivalent to that in isolated dI. This is reminiscent of the finding that, in the crystal structure, the NAD$^+$ electron density in the $B$ polypeptide (which has dIII.NADP$^+$ bound in its cleft) is extremely weak but that in the A polypeptide (whose cleft is not associated with dIII) is well defined (3). The analysis of the crystal structures of both isolated dI (20) and of the dI.dIIII complex (3) indicates that the nicotinamide mononucleotide moiety of the NAD$^+$ can undergo large conformational and positional changes brought about by rotations of the pyrophosphate, ribose-phosphate, and glycosidic bonds. It was suggested that these are functionally involved in positioning the C4 atom of the nicotinamide ring of NAD(H) relative to that of NADP(H) to enable hydride transfer in the occluded state (rings proximal) and to block hydride transfer in the open state (rings distal), i.e. to achieve the necessary gating of the redox and the proton translocation steps. The differences in the binding constants of the two sites of the dI.dIIII complex are presumably a macroscopic reflection of these events. They indicate that conformational changes at the nucleotide-binding sites of the two dI components in the complete enzyme must alternate synchronously with events in dIII during turnover. In the proposed organization of the complete transhydrogenase (3), the dI components make little or no contact with the membrane-spanning dII. Thus, the conformational alterations of dI and its nucleotide must follow those in the open $\rightarrow$ occluded interconversion of dIII, possibly as a consequence of changes in association of the two protein components. Note that the binding-affinity studies, reported here, and the crystallography studies have been performed with "dead-end" complexes (both nucleotides oxidized or both reduced) to prevent redox chemistry. How this situation affects events at the hydride transfer site is not yet clear. Kinetic experiments are currently under way to try to identify steps in the binding of catalytic combinations of nucleotides to the dI.dIIII complex.

In contrast with the two equivalent NADH binding sites on the isolated, recombinant R. rubrum dI dimer, the bovine-transhydrogenase dI dimer was reported to bind only one NADH (10). As discussed previously (39), the difference is surprising in view of the amino acid sequence similarity between the two proteins. In view of the observations described above, the possibility may be considered that the bovine protein spontaneously adopts the asymmetric structure that is only seen in the R. rubrum protein after its association with dIII. This might result if the trypsin treatment used to separate the dI from the detergent-dispersed complete bovine enzyme (39) cleaves unevenly (as was reported; Ref. 36); the low affinity NADH-binding site of the dI dimer would probably not be detected by the centrifugation procedure that was employed. Nevertheless, that report established that the complete bovine enzyme binds only one NADH, and only one NADPH, per dI.dIIII.dIII, "hexamer," which is important evidence supporting the notion of functional asymmetry in transhydrogenase.

Acknowledgments—We thank D. Pontoni, V. Urban, and T. Narayanan for discussion.

REFERENCES

The Heterotrimer of the Membrane-peripheral Components of Transhydrogenase and the Alternating-site Mechanism of Proton Translocation


doi: 10.1074/jbc.M104429200 originally published online June 8, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104429200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 5 of which can be accessed free at http://www.jbc.org/content/276/33/30678.full.html#ref-list-1