Stopped-flow Reaction Kinetics of Recombinant Components of Proton-translocating Transhydrogenase with Physiological Nucleotides*

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New information on the high resolution structure of the membrane proton pump, transhydrogenase, now provides a framework for understanding kinetic descriptions of the enzyme. Here, we have studied redox reactions catalyzed by mixtures of the recombinant NAD(H)-binding component (dI) of *Rhodospirillum rubrum* transhydrogenase, and the recombinant NADP(H)-binding component (dIII) of either the *R. rubrum* enzyme or the human enzyme. By recording changes in the fluorescence emission of native and engineered Trp residues, the rates of the redox reaction with physiological nucleotides have been measured under stopped-flow conditions, for the first time. Rate constants for the binding reaction between NAD\(^+\)/NADH and the *R. rubrum* dI-dIII complex are much greater than those between nucleotide and isolated dI. For the redox step between the physiological nucleotides on the *R. rubrum* dI-dIII complex, the rate constant in the forward direction, \(k_f \approx 2900 \text{ s}^{-1}\), and that for the reverse reaction, \(k_r \approx 110 \text{ s}^{-1}\). Comparisons with reactions involving an analogue of NAD(H) indicate that the rate constants at this step are strongly affected by the redox driving force.

Transhydrogenase, which is found in the inner membranes of animal mitochondria and the cytoplasmic membranes of some bacteria, catalyzes the reaction shown below.

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

**REACTION 1**

A single proton is translocated across the membrane, from the p-aqueous phase (the “outside” of intact mitochondria and bacteria) to the n-aqueous phase (the “inside”), for each hydride equivalent transferred from NADH to NADP\(^+\). Under most conditions, this is the *in vivo* direction; the reaction is driven by the proton electrochemical gradient (\(\Delta p\)) resulting from the action of respiratory (or photosynthetic) electron transport. For recent reviews, see Refs. 1–3.

The gross structure of transhydrogenases from different species is strikingly invariant. The enzyme has three components; dI and dIII, which bind NAD(H) and NADP(H), respectively, protrude from the membrane (on the cytoplasmic side in bacteria, and the matrix side in mitochondria), and dII spans the membrane. Crystal structures of the dIII components of human and bovine transhydrogenases were recently described (4–6), and the solution structure of the *Rhodospirillum rubrum* transhydrogenase equivalent was determined by NMR.\(^1\) The basic fold of dIII is similar to the classical, dinucleotide-binding domain of lactate dehydrogenase, but NADP\(^+\) is bound with an unusual, “reversed” orientation. The nicotinamide ring of the bound NADP\(^+\) is exposed on a ridge of dIII. A homology model of dI (8), based on the crystal structure of the sequence-related alanine dehydrogenase (9), suggests that the nicotinamide ring of NADH is located in a deep cleft. It was proposed that, in the complete transhydrogenase, the ridge of dIII inserts into the cleft of dI to bring the nicotinamide rings of the two nucleotides into apposition to effect direct hydride transfer (5, 6). The protruding helix-D/loop-D of dIII is thought to interact with the membrane-spanning, dII and, together with the adjacent, lid-like loop E, which passes over the bound nucleotide, might be responsible for the energy transmission. The proton translocation steps during turnover are probably coupled specifically to changes in the mode of NADP(H) binding (1, 5, 10).

Kinetic studies of transhydrogenase have also developed, following discoveries that fragments of the protein retain their nucleotide binding and some catalytic capacity. Thus, recombinant dI and dIII proteins, which bind their cognate nucleotides, have now been isolated from a number of species (11–16); mixtures of these proteins, even from different species, catalyze transhydrogenation reactions, albeit with properties that are modified relative to those of the complete enzyme. Transient state experiments, in particular, have revealed useful information on the hydride transfer step (17–19). Without exception, experiments with dI-dIII complexes have been carried out with nucleotide analogues having altered absorbance spectra to facilitate measurement of the rate of reaction. In this report we describe transient state experiments with the physiological nucleotides. A procedure is described, in which single-turnover hydride transfer between NAD(H), on dI, and NADP(H), on dIII, is monitored by following changes in protein fluorescence. We use a natural Trp residue in human recombinant dIII, and an engineered Trp in the equivalent *R. rubrum* protein (20). The oxidation/reduction step is faster than expected from studies with substrate analogues, and we can detect a driving force effect that arises from differences in the redox potentials of the natural and analogue substrates.

**EXPERIMENTAL PROCEDURES**

DNA coding for the dI and dIII proteins of *R. rubrum* transhydrogenase (rrdI\(^\dagger\) and rrdIII, respectively), and the human dIII protein (hs-

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\(^2\) The abbreviations used are: rrdI, the dI (or NAD(H)-binding) component of *R. rubrum* transhydrogenase; rrdIII, the dIII (or NADP(H)-

19490 This paper is available online at http://www.jbc.org
Temperature was 20.2 °C.

**RESULTS**

Single-turnover Transhydrogenation Revealed by Changes in Trp Fluorescence—Stopped-flow analysis of transhydrogenation (with substrate combinations AcPdAD\(^+\) + NADPH (Refs. 17 and 18) and AcPdADH + NADP\(^+\) (Ref. 19)) in mixtures of dI and dIII proteins revealed a burst of reaction before onset of the very slow steady-state rate. The burst is limited to a single turnover of dIII by the slow release of product, either NADP\(^+\) or NADPH. In mixtures of rdI + rdIII, the burst is biphasic. The fast phase (phase A), which is predominant at high concentrations of dI (relative to dIII), is thought to correspond to hydride transfer in dI-dIII complexes present at the instant of mixing. The slow phase (phase B), which is predominant at low concentrations of dI (relative to dIII), is thought to result from the multiple turnovers of dI that are required to complete the single turnover of dIII. Phase B is limited by the dissociation of dI-dIII complexes; the re-association of the proteins and the exchange of nucleotides on dI are both relatively fast. Fig. 1 shows that the stopped-flow kinetics of AcPdAD\(^+\) reduction by NADPH with mixtures of rdI and rdIII.E155W (trace b) were indistinguishable, within error, from those obtained with mixtures of rdI and wild-type rdIII (trace a). The reaction was measured from the absorbance change at 375 nm, which corresponds to formation of AcPdADH. The apparent first order rate constants for phase A (558 and 610 s\(^{-1}\)) and for phase B (32 and 28 s\(^{-1}\)) were similar for the wild-type and the mutant dIII proteins. We previously showed that the steady-state kinetics of transhydrogenation are not significantly affected by this amino acid substitution (20) and, furthermore, \(^{1}H,^{15}N\) HSQC NMR spectra of rdIII.E155W (in the NADP\(^+\) and NADPH forms) differed from spectra of wild-type protein (25, 30) only in those resonances that are assigned to residues close to the site of the mutation (data not shown).

Wild-type rdII has a single Trp residue (at position 72), and
wild-type rrdIII has no Trp. The change in protein fluorescence that accompanies the burst of AcPdAD$^{+}$ reduction by NADPH with a mixture of wild-type rrdI and rrdIII is also shown in Fig. 1 (trace c). There was a very small decrease in fluorescence with approximately similar kinetics to those of hydride transfer. It probably resulted from the quenching of dI-Trp$^{72}$ fluorescence by the weakly binding product, AcPdADH (Ref. 11), but the signal/noise ratio was too small for detailed analysis. The protein fluorescence of a mixture of rrdI and rrdIII.E155W under similar conditions substantially increased during the reduction of AcPdAD$^{+}$ by NADPH (trace d). The signal/noise ratio was more than adequate to show that the kinetics of the fluorescence change were very similar in character to those of the 375-nm absorbance change. Both were distinctly biphasic. Within error, the $k_{\text{app}}$ values for phase A (603 ± 71 s$^{-1}$ and 571 ± 58 s$^{-1}$ in fluorescence and absorbance measurements, respectively), and for phase B (30.5 ± 6.8 s$^{-1}$ and 29.3 ± 7.2 s$^{-1}$) were similar. For reasons that will be discussed below, the amplitude of phase A of the fluorescence change was routinely a slightly greater fraction of the total amplitude of the burst, than was the amplitude of phase A of the absorbance change (e.g. 74 ±10% compared with 59 ± 7% for 37.5 μM dI and 37.5 μM dIII). The very slow, steady-state increase in the absorbance at 375 nm due to the reduction of AcPdAD$^{+}$ during multiple turnovers (seen only on long time scales (Ref. 13)) was not evident in fluorescence measurements (data not shown). We propose that the fluorescence increase during the burst of hydride transfer results mainly from the change in occupancy of the dIII protein; bound NADP$^{+}$ leads to greater Trp$^{152}$ fluorescence than bound NADPH (14, 15, 20).

Consistent with this interpretation, the replacement of phase A with phase B, as the concentration of the dI protein was increased (18), was also observed in fluorescence experiments (Fig. 2). The phenomenon was discussed in detail (18, 19). Briefly, it results from an increase in the concentration of dI-dIII complex (and therefore in the fraction of phase A) at increasing dI concentrations, and a corresponding decrease in the concentration of free dIII.NADPH that, for oxidation, requires multiple turnover of dI (the fraction of phase B). In both absorbance and fluorescence experiments, the value of $k_{\text{app}}$ for phase A was independent of the dI concentration, as expected if it reflects intracomplex hydride transfer. The increase in the $k_{\text{app}}$ for phase B with dI concentration, in both sets of experiments, is a consequence of a lower rate of formation of dead end complex (18).

In a separate series of experiments, we monitored the burst of transhydrogenation from AcPdADH to NADP$^{+}$ by following the absorbance decrease at 375 nm. The kinetics were similar in mixtures of rrdI plus rrdIII.E155W (results not shown), and in mixtures of rrdI plus rrdIII (see Ref. 19). With rrdI and rrdIII.E155W (but not with rrdI and rrdIII), a large decrease in protein fluorescence was observed, with similar kinetics to the 375 nm absorbance decrease. In contrast to the experiments with AcPdAD$^{+}$ and NADPH (see above), the amplitude of phase A was a slightly smaller fraction of the total burst in the fluorescence measurements, although, again, there was no significant difference in the apparent rate constants.

Hydride Transfer between Physiological Nucleotides on Complexes of rrdI and rrdIII—The above experiments indicate that it should be possible to monitor transient-state hydride transfer between physiological nucleotides in dI-dIII complexes by recording changes in dIII tryptophan fluorescence. Previously, real-time observations were impossible because of the similar absorbance spectra of NAD(H) and NADP(H). Fig. 3 shows that a biphasic fluorescence decrease was observed upon mixing rrdI plus rrdIII.E155W loaded with NADP$^{+}$ (syringe 1), and NADH (syringe 2) in the stopped-flow spectrophotometer, and a biphasic fluorescence increase was observed upon mixing rrdI
plus rrdIII.E155W loaded with NADPH (syringe 1), and NAD$^+$ (syringe 2). We attribute this, in the “forward” experiment, to reduction of NAD$^+$ on dIII (decrease in Trp$^{155}$ fluorescence), and in the “reverse” experiment, to oxidation of NADPH on dIII (increase in Trp$^{155}$ fluorescence). The release of product NADP(H) is expected to be very slow (13).

The fluorescence burst during transhydrogenation from NADPH to NAD$^+$ was monitored in a set of experiments performed at a fixed concentration of rrdIII.E155W and variable rrdI (Fig. 4). The results were very similar to those obtained for hydride transfer between NADP(H) and AcPdAD(H), as observed in both absorbance measurements (18), and fluorescence measurements (Fig. 2). Notably, the fast phase (phase A) replaced the slow phase (phase B) as the dI concentration was increased, the $k_{\text{app}}$ for phase A was independent of dI concentration, and the $k_{\text{app}}$ for phase B increased with the dI concentration (see above). This clearly establishes the internal consistency of the proposed mechanisms.

Attempts to determine the $k_{\text{app}}$ values of phase A for forward and reverse transhydrogenation at saturating concentrations of NADH and NAD$^+$, respectively, are shown in Fig. 5; both sets of experiments were performed with protein concentrations that ensured dominance by phase A. Phase A of the forward reaction with physiological nucleotides was very fast. The maximum value of $k_{\text{app}}$ that can be measured by the spectrophotometer is approximately 800–900 s$^{-1}$, and this was exceeded at concentrations of NADH in excess of 50–100 $\mu$M. Phase A of the fluorescence change during the reverse reaction was slower and, therefore, easier to resolve. At close-to-saturating concentrations of NAD$^+$, at 20.8 °C, the value of $k_{\text{app}}$ was approximately 600 s$^{-1}$. For reasons that will be explained below, the dependence of the phase A $k_{\text{app}}$ for reverse transhydrogenation on the concentration of NAD$^+$ was also determined at a lower temperature; at 7.8 °C the $k_{\text{app}}$ value at saturating nucleotide was approximately 60% less than that at 20.8 °C (Fig. 5).

The Binding of NAD(H) to rrdI—To provide a more complete description of the hydride transfer step in dIII complexes with physiological nucleotides, we have determined the on and off rate constants for NAD(H) and isolated rrdI from changes in Trp$^{72}$ fluorescence (11). The kinetic profile of fluorescence quenching with NADH was too fast to measure accurately in the stopped-flow machine above 15 °C, but at lower temperatures the reaction fitted to a single exponential (not shown). Assuming that binding is a simple, one-step reaction, and provided that the concentration of NADH is much greater (>10-fold) than that of dI, it can be easily shown (e.g. Ref. 31) that $k_{\text{obs}} = k_w[NADH] + k_{\text{off}}$. Thus, values of $k_{\text{on}}$ and $k_{\text{off}}$ are obtained from the gradient and intercept, respectively, of a plot of [NADH] versus $k_{\text{obs}}$. The validity of the approach was supported by the linear dependence of $k_{\text{obs}}$ on NADH concentration (Fig. 6, upper panel). Between 8 and 15 °C, $k_{\text{off}}$ exhibited a
was checked by performing a simulation of each experimental trace using the measured values of $k_{on}$ and $k_{off}$ for NADH (see above) and $K_d \approx 300 \mu M$ for NAD$^+$. Arrhenius plots of the measured values of $k_{off}$ for NAD$^+$ were linear, and gave an activation energy of 54 kJ mol$^{-1}$. Extrapolation to 20 °C yielded $k_{off} \approx 1700$ s$^{-1}$. Using the $K_d$ ($\approx 300 \mu M$) obtained by equilibrium dialysis (34), $k_{off} \approx 6 \times 10^6$ M$^{-1}$ s$^{-1}$.

For reasons that will become clear under “Discussion,” the possibility was considered that the nucleotide-binding parameters of dI might be altered when it is in complex with dIII. An important consideration, in the measurement of these parameters, is that isolated dI protein is dimeric; the species, (dI)$_2$-dIII, forms with high affinity ($K_d < 10^{-6}$ M) and (dI)$_2$-dIII$_2$ forms only with low affinity ($K_d \gg 10^{-6}$ M) (13, 18, 25). Thus, because protein concentrations in the micromolar range are required for ligand binding experiments, the dI population is heterogeneous; we operate within the equilibrium, (dI)$_2$ + dIII $\leftrightarrow$ (dI)$_2$-dIII. Another consideration is that it has not yet been possible to obtain stable preparations of apo-dIII; we must use either dIII.NADP$^+$ or dIII.NADPH, and then design experiments to avoid hydride transfer. Direct measurement of the $K_d$ for NADH in mixtures of rrdI and rrdIII-L.NADPH, using equilibrium dialysis, is difficult because of the elevated background fluorescence from the nucleotide on the dIII protein. However, experiments indicated that the binding constant is similar, within a factor of 3, in isolated dI (34), and in dI-dIII complexes. Because the value is quite high, measurement of $K_d$ from the quenching of Trp$^{72}$ fluorescence suffers from significant error due to the inner-filtering effect of the added nucleotide (11, 30). Nevertheless, results from this kind of experiment strongly supported the conclusion that the $K_d$ of dI for NADH is unaffected by complexation with dIII (data not shown).

Despite these findings, the kinetic experiments shown in Fig. 7 indicate that $k_{on}$ and $k_{off}$ for NADH might be substantially greater with dI-dIII complexes than with isolated dI. These were carried out at a temperature, and with an NADH concentration, that yielded clear, slow, monophasic kinetics of Trp72. Since the steady-state fluorescence spectra of dI and dIII were simply additive (data not shown), it is concluded that a component of Trp$^{72}$ fluorescence quenching takes place very rapidly, i.e. in the apparatus dead time, and is not directly observed. This would indicate that $k_{on}$ and $k_{off}$ for NADH are greater, by at least an order of magnitude, in dI-dIII than in dI. They must increase approximately in parallel, since the $K_d$ is not significantly altered (see above).

Hydride Transfer between Physiological Nucleotides on Hybrid Complexes of rrdI and hsdIII—Mixtures of rrdI and hsdIII also catalyze a single-turnover burst of hydride transfer between AcPdAD(H) and NADP(H) (16). Because of the relatively weak interactions between the two protein components of the hybrid system, the burst is monophasic. Wild-type hsdIII has a single, natural Trp residue at position 154; it is in an equivalent position to the engineered Trp$^{155}$ in rrdIII (20). Fig. 8 (upper panel) shows that there was a fluorescence increase upon mixing rrdI, and hsdIII loaded with NADPH, with AcP-
...traces are an average of five repeats. Temperature was 20.1 °C.

...fluorescence change (see Fig. 1 and "Experimental Procedures"). The upper panel shows the fluorescence emission from Trp72 of R. rubrum dIII. In this experiment, the dIII protein was pre-loaded with NADP⁺, and NADPH-loaded rrdIII, at a range of concentrations. The second syringe contained 30 μM NADH. The fluorescence change following mixing could be fitted with a single exponential (see "Experimental Procedures"). The amplitude, and the apparent first order rate constant (kapp) of the observed fluorescence change were plotted against the final concentration of NADH. Temperature was 7.8 °C. Each data point is derived from the average of 12–16 repeat experiments.

**Fig. 7. NADH binding to dI-dIII complexes indicated by changes in Trp72 fluorescence.** The first drive syringe contained 2.0 μM rrdI, and NADPH-loaded rrdIII, at a range of concentrations. The second syringe contained 50 μM NADH. The fluorescence change following mixing could be fitted with a single exponential (see “Experimental Procedures”). The amplitude (ΔA), and the apparent first order rate constant (kapp), of the observed fluorescence change were plotted against the final concentration of NADH. Temperature was 20.1 °C.

**Fig. 8. Hydride transfer between physiological nucleotides on hybrid complexes of rrdI and hsdIII measured by protein fluorescence.** Upper panel, the first drive syringe contained 25 μM rrdI and 50 μM hsdIII pre-loaded with NADPH. The second drive syringe contained 2.0 mM AcPdAD⁺. Trace a shows the absorbance change at 375 nm after mixing equal volumes of the two solutions. Trace b shows the fluorescence change (see Fig. 1 and "Experimental Procedures"). The traces are an average of five repeats. Temperature was 20.1 °C. Lower panel, the first drive syringe contained 50 μM rrdI and 50 μM hsdIII. In trace a, the dIII protein was pre-loaded with NADP⁺ and the second syringe contained 50 μM NADH; in trace b, the dIII protein was pre-loaded with NADPH and the second syringe contained 1.2 mM NAD⁺. Both traces represent the fluorescence changes after mixing equal volumes from the syringes, and they are both an average of seven repeats. A fluorescence increase is a downward deflection, and an absorbance increase is an upward deflection. Temperature was 20.7 °C.

dAD⁺ (trace b), which had similar monophasic kinetics to the burst of AcPdADH formation measured as the absorbance increase at 375 nm (trace a). It was previously established that, at a fixed hsdIII concentration, the kapp for the burst of AcPdAD⁺ reduction increases with the concentration of rrdI (16), reflecting the elevated concentration of dI in the mixture. In a series of experiments (data not shown), the kapp values for AcPdAD⁺ reduction and for the protein fluorescence change increased together when the concentration of dI was increased from 6.25 to 37.5 μM (dIII = 25 μM). As in the homologous R. rubrum system (see above), it is proposed that the fluorescence change mainly results from the quenching of Trp154 emission during oxidation of NADPH on dII.

Experiments were then performed on hybrid dI-dIII complexes and physiological nucleotides (Fig. 8, lower panel). There was a monophasic decrease of protein fluorescence following the rapid mixing of rrdI, plus hsdIII loaded with NADP⁺, and NADH (trace a), and a monophasic fluorescence increase upon mixing rrdI, plus hsdIII loaded with NADPH, and NAD⁺ (trace b). Again, it is proposed that the fluorescence change is a reflection of hydride transfer between the physiological substrates. Because the binding between rrdI and hsdIII is quite weak, it is difficult, under stopped-flow conditions, to saturate the latter with the former, and to measure meaningful rate constants for the reaction. However, it is evident from Fig. 8 that hydride transfer between physiological nucleotides, even in the hybrid complexes, is very fast.

**DISCUSSION**

The experiments described above, with the single species complex, rrdI-rrdIII.E155W, and the hybrid, rrdI-hsdIII, show that a transient-state burst of hydride transfer between physiological nucleotides (both forward and reverse reactions) can be monitored by observing changes in protein fluorescence. Human dIII has its own, single Trp residue, but because of the relatively low affinity between the protein components in the hybrid (16), the single-species complex is the system of choice for studies on the chemistry of the oxidation-reduction step. The mutation, E155W, in rrdIII had no significant effect on the hydride transfer rate (Fig. 1) and, as indicated by HSQC NMR spectra (data not shown; see Ref. 25), only minor effects on the protein structure, and on the conformational change that results from replacement of NADP⁺ with NADPH.

The changes in protein fluorescence that accompany hydride transfer in the dIII complexes mainly arise from the single Trp residues in hsdIII and rrdIII.E155W. It is well documented that the Trp fluorescence of dIII is greater when NADP⁺, rather than NADPH, occupies the nucleotide-binding site (14, 15, 20). There is also a small contribution (~10%) to the fluorescence emission from Trp72 of rrdI during the transhydrogenation burst; it probably results from changes in nucleotide occupation of dI (Fig. 1). This, and perhaps weak, secondary effects of NAD(H) bound to dI on Trp fluorescence in dIII, probably account for the small differences between the relative amplitudes of phase A in absorbance and fluorescence experi-
ments (see Fig. 1). However, there was no significant difference between the \(k_{\text{app}}\) values for phase \(A\) in measurements with AcPdAD(H), and we assume this to be the case also with physiological nucleotides.

There is good evidence (18, 19), which is supported by experiments presented in this report (Figs. 2 and 4), that phase \(A\) of the reaction corresponds to the following kinetic components (Reaction 2).

\[
\text{dI} + \text{dII} \iff \text{dIII}
\]

Crucially, the rates of dissociation of the dI-dIII complex (\(k \approx 50 \text{ s}^{-1}\)) and of NADP\(^+\) (\(k \approx 0.03 \text{ s}^{-1}\)) and NADH (\(k \approx 5 \times 10^{-4} \text{ s}^{-1}\)) from dIII, are slow relative to the rates of hydride transfer; the products NADPH or NADP\(^\text{r}\) remain bound to the complex during the course of phase \(A\). Therefore, when experiments are carried out at either high NADH concentrations (for forward transhydrogenation) or high NADP\(^+\) concentrations (for reverse), then the respective \(k_{\text{app}}\) values are dominated by the hydride transfer step, and by either NAD\(^+\) release or NADH release. The \(k_{\text{app}}\) value for the forward reaction at saturating NADH (\(k_{\text{f(app)}}\)) was too fast to measure, but the extrapolated value was \(2000 \text{ s}^{-1}\) (Fig. 5). The \(k_{\text{app}}\) value for the reverse reaction at saturating NAD\(^+\) (\(k_{\text{r(app)}}\)) was \(600 \text{ s}^{-1}\). It was difficult to determine the values of on and off rate constants for NAD\(^+\)/NADH and the dI-dIII complex (see “Results”). However, even if we take values derived from experiments with isolated dI (Fig. 6), and these are almost certainly lower than those appropriate for the dI-dIII complex (see below), then the rate constants for the hydride transfer step are, \(k_r \approx 2000 \text{ s}^{-1}\) and \(k_r \leq 500 \text{ s}^{-1}\); precise values depend on the relative equilibrium positions of the hydride transfer and of product NAD(H) release steps. The minimal conclusion is that the equilibrium constant of the hydride transfer step with physiological nucleotides on the enzyme (\(K_{\text{eq}} = k_r/k_f\)) is greater than unity. A similar conclusion was reached on the basis of measurements of the equilibrium \(K_d\) values for NAD\(^+\)/NADH on dI and of the redox potential of bound NADP\(^+\)/NADPH on dIII (20, 34), and on the basis of stopped-flow experiments with substrate analogues (19). It is probably an important feature of the complete enzyme. Because there is no isotopic exchange between the hydride equivalents transferred and the solvent (35), and because there seem to be no reaction intermediates on the hydride transfer pathway (17–19), it is unlikely that this step is directly linked to proton translocation. We have presented evidence that proton translocation by transhydrogenase is coupled to changes in the binding of NADP\(^+\) and NADPH, whereas NAD\(^+\)/NADH behave as passive hydride acceptor/donor (reviewed in Ref. 1). A specific suggestion (1, 5, 10, 15) is that, during the forward reaction, H\(^+\)-binding from the p-phase prior to the hydride transfer step drives the enzyme into an occluded state from which bound NADP\(^+\) cannot be released. Following hydride transfer from NADH within the occluded state, H\(^+\) release to the n-phase regenerates an open state from which NADPH can dissociate. The elevated equilibrium constant of the hydride transfer step ensures an intrinsic forward bias between the two power strokes in the reaction, and will have the effect of smoothing the \(\Delta G\) profile of the reaction path (36).

The experimental data used to calculate values of \(k_{\text{on}}\) and \(k_{\text{off}}\) for NAD\(^+\)/NADH and isolated dI were reproducible, and the procedures should be reliable. The values obtained are in the range found commonly for soluble dehydrogenases (31), and are consistent with \(K_d\) values determined by equilibrium procedures (11, 33, 34). We assumed that these values would be similar to the on and off rate constants for NAD\(^+\)/NADH and the dI-dIII complex. However, they are too small to be compatible with a minimal kinetic scheme (such as that shown in Reaction 2), in which a rapid-equilibrium hydride-transfer step is followed by NAD\(^+\) release (in forward transhydrogenation) or NADH release (in reverse transhydrogenation). The same conclusion is reached from experiments performed at 20 °C (using extrapolated values of \(k_{\text{on}}\) and \(k_{\text{off}}\)) and at 7 °C (using measured values). It emerges that \(k_{\text{on}}\) and \(k_{\text{off}}\) for NAD\(^+\)/NADH and the dI-dIII complex must be at least 5 times greater than values measured for the isolated dI protein to provide a reasonable fit to the measured \(k_{\text{f(app)}}\) and \(k_{\text{r(app)}}\). Although it proved difficult to measure the binding constants for NAD\(^+\)/NADH to dI-dIII (see “Results”), there was good evidence (Fig. 7) that \(k_{\text{on}}\) and \(k_{\text{off}}\) at least for NADH, are indeed more than an order of magnitude larger in the complex. We envisage that in the dI-dIII complex (and in the complete enzyme), where the ridge of dIII inserts into the putative cleft of dI, there is a channel between the two protein components for the passage of nucleotide. In isolated dI, the cleft closes up slightly to restrict the nucleotide access to its binding site. This idea receives some support from the finding that the second order rate constants for nucleotide binding to isolated dI are considerably lower than that set by diffusional limitation (see Ref. 37). A difference in the NAD\(^+\)/NADH binding properties of dI in its isolated form and in the complete, membrane-bound protein was also inferred from a steady-state kinetic analysis (7). We previously showed that dissociation of the dI-dIII complex is unnecessary for NAD\(^+\)/NADH exchange between the dI binding site and the solvent (18).

If we assume that the release of the product nucleotide (NAD\(^+\) or NADH) from the dI-dIII complex is fast enough to reach equilibrium during phase \(A\), and if we take the \(K_{\text{eq}}\) for the hydride transfer step from earlier experiments (19, 20), then for the measured \(k_{\text{f(app)}}\) and \(k_{\text{r(app)}}\) (see above), \(k_r\) and \(k_f\) for the physiological substrates converge on values of 2900 and 110 s\(^{-1}\), respectively. A similar exercise using experimental data for transhydrogenation between AcPdAD(H) and NADP\(^r\) (18), yields 40 s\(^{-1}\) for \(k_r\) and 500 s\(^{-1}\) for \(k_f\). It is clear that the rate constant for the reaction NADH → NAD\(^+\) is greater than that for AcPdADH → NAD\(^+\), but that for NADPH → NAD\(^+\) is less than that for NADPH → AcPdAD\(^r\). The \(E^0\) for AcPdAD\(^r\)/AcPdADA in aqueous solution (−0.248 V) is larger than that for NAD\(^+\)/NADH (−0.32 V) (38). It is suggested, therefore, that the differences in rates between the two sets of reactions are primarily the result of a different driving force; differences in transition state geometry, such as the distance of transfer of the hydride equivalent (whether H\(^+\), \(2e^- + H^+\)) or \([e^- + H^+]\) are probably less important.

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