Fast Hydride Transfer in Proton-translocating Transhydrogenase Revealed in a Rapid Mixing Continuous Flow Device*

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Transhydrogenase couples the redox reaction between NAD(H) and NADP(H) to proton translocation across a membrane. Coupling is achieved through changes in protein conformation. Upon mixing, the isolated nucleotide-binding components of transhydrogenase (dI, which binds NAD(H), and dIII, which binds NADP(H)) form a catalytic dI2dIII complex, the structure of which was recently solved by x-ray crystallography. The fluorescence from an engineered Trp in dIII changes when bound NADP* is reduced. Using a continuous flow device, we have measured the Trp fluorescence change when dI2dIII complexes catalyze reduct of NADP* by NADH on a sub-millisecond scale. At elevated NADH concentrations, the first-order rate constant of the reaction approaches 21,200 s⁻¹, which is larger than that measured for redox reactions of nicotine amide nucleotides in other, soluble enzymes. Rather high concentrations of NADH are required to saturate the reaction. The deuterium isotope effect is small. Comparison with the rate of the reverse reaction (oxidation of NADPH by NAD⁺) reveals that the equilibrium constant for the redox reaction on the complex is >36. This high value might be important in ensuring high turnover rates in the intact enzyme.

The enzyme provides NADPH for biosynthesis and glutathione reduction, and in mitochondria, it also helps to control flux through the tricarboxylic acid cycle. The enzyme provides NADPH for biosynthesis and glutathione reduction, and in mitochondria, it also helps to control flux through the tricarboxylic acid cycle (1, 2). The relative simplicity of transhydrogenase, the emergence of methods for determining its rate of reaction in real time (3–6), and recent high resolution structural information (7–12) make it a good model for understanding the general principles of operation of conformationally coupled ion translocators. The enzyme is composed of three components. dI and dIII, which bind NAD(H) and NADP(H), respectively, protrude into the mitochondrial matrix (or the bacterial cytoplasm), and dII spans the membrane. The intact enzyme is effectively a dimer of two dI-dII-dIII “trimers” (3, 14), though there are species variations in the way the polypeptide chains are joined.

The findings that the transfer of hydride-ion equivalents between the bound nucleotides on transhydrogenase is direct (3, 5) and that there is no exchange of the transferred hydride with water protons (15) together establish that coupling to proton translocation does not occur at the redox step. We proposed an NADP(H) binding change model in which NADP⁺ (or NADPH) from the solvent can only bind to (or leave from) an “open” state of the dII component of the protein and in which the redox reaction can only take place in an “occluded” state. Association and dissociation of protons during translocation, gated by the redox state of the NADP(H), drives the protein between the open and occluded states (16, 7). Recent observations on pronounced structural asymmetries in transhydrogenase suggest that the two dI-dII-dIII trimers of the complete enzyme undergo reciprocating alternations of conformation during turnover (12). Thus, as the dIII in one trimer enters the open state to permit product release and substrate binding, the other enters the occluded state to permit hydride transfer; the two trimers run 180° out of phase. In general, a mechanism of coupling involving changes in NADP(H) binding is also favored by other authors (17–19).

Isolated recombinant dI and dIII readily form a complex. That from Rhodospirillum rubrum transhydrogenase has been studied in most detail (3–6, 12, 20) (see also Refs. 21 and 22). It is a dI2dIII heterotrimer in both the crystalline state and in solution (12, 23). The two characteristic properties, (a) a capacity to catalyze a rapid redox reaction between NAD(H) and NADP(H) (or their analogues) and (b) an extremely slow rate of release of bound NADP⁺ and NADPH, suggest that the isolated complex adopts a conformation similar to that of the occluded state in the intact enzyme. These properties of the dI2dIII complex can be partly rationalized in terms of features seen in the x-ray structure of the protein. The redox reaction between NADP(H) and AcPdAD(H)1 (an NAD(H) analogue) on the dI2dIII complex was studied by stopped-flow spectrophotometry (3–5). A rapid, single turnover burst of hydride transfer precedes the slow steady-state reaction that is limited by product NADP(H) release. A E155W mutant of dIII was subsequently isolated to study these processes with physiological nucleotides from changes in protein fluorescence, but the forward reaction was too fast to measure by stopped-flow (6). In this report we describe measurements of the forward reaction with physiological nucleotides using a newly developed continuous flow device. The reaction is faster than predicted from the stopped-flow experiments, and thus the on-enzyme equilibrium constant is greater than expected.

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‡ The abbreviation used are: Pd, reduced acetyl pyridine adenine dinucleotide, KIE, kinetic isotope effect.

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Figure 1. Single turnover reduction of NADP\(^+\) by NADH on transhydrogenase dI\(_2\)-dIII\(_1\) complexes. Experiments were performed in the continuous flow spectrophotometer, as described under “Materials and Methods.” Trace A, 50 \(\mu\)M NADH; trace B, 1.0 \(\mu\)M NADH.

Materials and Methods

The expression and purification of the isolated wild-type dI (and both the wild-type and the E155W mutant of dIII) from \(R.\ rubrum\) transhydrogenase were carried out as described (3–6). The dIII component was prepared in either its NADP\(^+\) or NADPH form, as required. Protein concentrations were determined using the microtamin assay (24). In both stopped-flow and continuous flow experiments, the dI and dIII proteins were premixed in a 2:1 molar ratio to give the dI\(_2\)-dIII\(_1\) complex and loaded into the appropriate drive syringe. Given the low \(K_d\) and rate of dissociation of the complex, this ensures that the redox reaction proceeds as a monoeponential reaction (3–6). Experiments were performed in 10 mM (NH\(_4\))\(_2\)SO\(_4\) and 20 mM Hepes, pH 8.0, at 20 °C. Sub-millisecond kinetic measurements were performed in a home-built continuous flow instrument based on that described by Shastry \textit{et al.} (25). Our instrument uses their capillary mixer coupled to a 0.25 × 0.25 × 20-mm quartz flow cell (Hellma Cells, Inc.). The dI\(_2\)-dIII\(_1\)-NADP\(^+\) solution and the NADH solution were delivered to the mixer in a 1:10 volumetric ratio using a stepper-motor drive unit developed by Hi-Tech Ltd. (Salisbury, United Kingdom). A 100-watt Hg arc lamp (Osrarm, Germany), a monochromator, and a cylindrical lens were used to excite fluorescence at 291 nm (4-nm bandwidth), illuminating the 20-mm axis of the flow cell along a 250-μm path length. Fluorescence emission (320–400 nm) was selected with a combination of WG320 and UG11 filters (Schott) and was recorded by imaging the flow (total length of image, 16.5 mm) using a digital camera system (Micromax, Roper Scientific) equipped with a SenSys:1401E CCD chip with an array of 1317 × 1035 pixels. Images were converted into intensity profiles along the direction of flow by averaging the rows of pixels across the width of the channel. Relative fluorescence intensities were determined by comparison with images derived from experiments in which NADP\(^+\) (or NADPH) were prepared by similar procedures.

Results

The isolated dIII component of \(R.\ rubrum\) transhydrogenase, as prepared in our laboratory, is associated with tightly bound nucleotide, either NADP\(^+\) or NADPH (3–6, 20). The E155W mutant of the dIII protein has a single tryptophan residue. The mutation does not significantly affect either the NADP(H) binding properties or the catalytic properties of the protein (6, 30). However, the fluorescence emission of the introduced Trp residue is sensitive to the redox state of the bound nucleotide; it is about 25% less intense when the nucleotide is reduced. For the equivalent fluorescence change in \textit{Escherichia coli} dIII, it was proposed that resonance-energy transfer is responsible for the difference, but effects due to conformational changes cannot be eliminated (22). In the experiments shown in Fig. 1, dIII-E155W in its NADP\(^+\) form was premixed with \(R.\ rubrum\) dI (nucleotide-free) at protein concentrations that gave the dI\(_2\)-dIII\(_1\) heterotrimer. The change in Trp-155 fluorescence was recorded after the rapid mixing of this solution with NADH in the continuous flow instrument. In each trace, the fluorescence rapidly decreased with approximately monoexponential kinetics as the bound NADP\(^+\) was reduced. Note that product NADPH is released only extremely slowly from the protein (\(k_{\text{cat}} < 3 \times 10^{-4} \text{s}^{-1}\)) (25), and therefore the redox reaction is limited to only one turnover (4). The dependence of the apparent first-order rate constant of the fluorescence change on the concentration of NADH is shown in Fig. 2. At saturation, this rate constant approached a very high value, \(k_{\text{app}} \approx 21,200 \text{s}^{-1}\), close to the limit of resolution of the instrument.

We can now go some way toward estimating the microscopic rate constants and the equilibrium constant for the redox reaction involving the physiological substrates on transhydrogenase. Scheme 1 describes the forward reaction (above), and Scheme 2 describes the corresponding reverse reaction; that is the oxidation of dI\(_2\)-dIII\(_1\)-NADP\(^+\) by NAD\(^+\), as studied earlier in the stopped-flow instrument (6). Because the \(k_{\text{app}}\) for the reaction in Scheme 1 (\(k_{\text{app}} = 21,200 \text{s}^{-1}\)) is much greater than that for Scheme 2 (\(k_{\text{app}} = 590 \text{s}^{-1}\)), the former goes almost to completion and \(k_{\text{app}} \approx k_f\) (the first-order rate constant of the redox step in the forward direction). However, the reaction in Scheme 2 is reversible, and \(k_{\text{app}}\) has contributions from both \(k_f\) and \(k_r\); in the limit \(k_{\text{app}} = k_f + k_r\) (for example, see Ref.
Therefore, $k_f < 590 \text{ s}^{-1}$, and the equilibrium constant of the redox reaction on the protein is $K_{eq} = k_f/k_r > 36$. In fact, earlier experiments show that the reaction of Scheme 2 is readily reversible (6), and thus $k_r$ is quite considerably less than 590 s$^{-1}$ and $K_{eq}$ is quite considerably greater than 36.

Isolated dI2 binds two NADH/mole of dimer, both with a $K_d \sim 20 \mu\text{M}$ (32). However, the dI2-dIII1 complex (in its NADPH form) binds one NADH with a $K_d \sim 20 \mu\text{M}$ and one with a $K_d \sim 300 \mu\text{M}$ (23). It was concluded that the dI protomer whose interdomain cleft is associated with dIII-NADPH (as indicated by the crystal structure (12)) has a decreased affinity for NADH, whereas the dI protomer with the unoccupied cleft has an unchanged affinity for NADH. In Fig. 2, the concentration of NADH giving the half maximal value of $k_{app}$ was $-500 \mu\text{M}$, similar to the $K_d$ of the low affinity site of the dI2-dIII1 complex. This suggests that the nucleotide-binding reactions are fast enough to reach equilibrium and that the high $K_d$ for NADH is the functionally relevant parameter in the steps leading to hydride transfer in the dI2-dIII1 complex. Another more convoluted explanation is that NADH binding is slow relative to the redox reaction and that the high $K_d$ measured for NADH on dI in the dI2-dIII1 complex (23) results from the fact that, in those experiments, NADPH (rather than NADP$^+$) was bound on dIII (the increased $K_d$ might represent a device for minimizing the formation of catalytically dead-end complexes). That is, the effective $K_d$ during catalysis is 20 $\mu\text{M}$.

There is only a small primary deuterium isotope effect (KIE $\sim 2$) on the rate constant for reduction of AcPdAD$^+$ by NADPH catalyzed by dI2-dIII1 complexes at 25 °C (4), but the temperature dependence displays non-classical behavior (as defined in Ref. 33), and this might indicate a contribution from quantum mechanical tunneling at the hydride-transfer step.

DISCUSSION

A reliable lower limit of the equilibrium constant for the physiological nucleotides at the redox step of transhydrogenase ($K_{eq} > 36$) was determined from the data described above. In aqueous solution, the standard redox potential of NAD(H) is
similar to that of NADP(H), and thus the elevated equilibrium constant on the enzyme must result from favored stabilization of the bound nucleotide products relative to the substrates. There might be effects at both the NADP(H) site (5, 6, 30) and the NAD(H) site (see below). The available crystal structures of the nucleotide binding components do not yet provide an explanation for these differences in affinity, but polarization from invariant charged residues (Arg-127 and Asp-135 in dI and Arg-90 in dIII) and hydrogen bonding by invariant Gln-132 at the hydride-transfer site and the caging-like effect of conserved aromatic residues (Tyr-235 of dI and Tyr-55 and Tyr-171 of dIII) are likely to be important.

The coupling process of transhydrogenase is determined by the catalytic specificities (whether the redox reaction is allowed or not) and the ligand binding specificities (for nucleotides, $H_{\text{nuc}}$, $H_{\text{out}}$) of the protein in the open and occluded states. For details, see Ref. 7, and for a discussion of general principles, see Ref. 36. On the other hand, the elevated equilibrium constant of the redox reaction (a reflection of how the nucleotide binding energies are expressed) will have a kinetic effect. In the framework of the model, the elevated equilibrium constant will increase the turnover rate of the enzyme in the forward direction. Thus, the redox reaction takes place in the occluded state. Conversion from the open state to the occluded state precedes and re-conversion to the open state follows the redox reaction. These conversions are driven by appropriately switched protonation/deprotonation reactions associated with proton translocation and will be limited by the proton electrochemical potentials of the bacterial cytoplasm and periplasm. Through an elevation of the equilibrium constant of the redox reaction, the concentration of the occluded intermediate bearing NADH/NADPH will be lowered, and the concentration of that bearing NAD+/NAD+ will be raised, increasing the rates of interconversion of the open and occluded states.

The apparent $K_a$ for NADH during hydride transfer in D$_2$O-dIII complexes is rather high ($\sim 500 \mu M$, Fig. 2) relative to the expected concentration of NADH in the bacterial cell and compared with the $K_a$ for NADH in the intact enzyme in membrane preparations ($\sim 10 \mu M$). This might indicate that during turnover of the complete enzyme, NADH binds predominantly to the dI associated with open dIII ($K_a$ for NADH, $\sim 30 \mu M$). As this dIII (with bound NAD$^+$) is converted to the occluded state, its partner dI-NADH site is shifted to the high $K_a$ form ($\sim 300 \mu M$), but NADH does not significantly dissociate because the hydride transfer step is very fast and proceeds almost to completion (see above). The different conformations of NAD$^+$ seen in each of the four polypeptides of the asymmetric unit of the crystal structure of isolated dI (11) probably reflect these events in the complete enzyme. NAD(H) binding changes are not directly coupled to proton translocation (and in isolated D$_2$O-dIII complexes, NADH binding has to proceed through the "wrong" protein conformation), but they are advantageous (a) to keep the nicotinamide rings apart to prevent hydride transfer in the open state (11, 12) and (b) to permit the relative stabilization of NAD$^+$ during hydride transfer in the occluded state.

The $k_2$ for the reduction of NADP$^+$ by NADH on D$_2$O-dIII complexes of *R. rubrum* transhydrogenase is very large ($\sim 21,200 \text{ s}^{-1}$); its measurement required the use of a novel mixing device and continuous flow observation (25). It is much greater than rate constants reported for redox reactions catalyzed by other enzymes utilizing nicotinamide nucleotide coenzymes, including the soluble dehydrogenases and flavoproteins; typically, these are rather less than 1000 s$^{-1}$. It is not clear what factors are responsible for the very high rate. A $K_a$ of 36 does not represent a very large driving force ($\Delta G = -8.9$ kJ mol$^{-1}$), although judging from comparisons of rate constants measured for NAD(H)$\leftrightarrow$NADP(H) with those for AcPDAD(H)$\leftrightarrow$NADPH, the driving force does have a large effect on the reaction rate (6). At this stage a tunneling mechanism cannot be ruled out but as yet, there is no compelling evidence to indicate non-classical behavior.

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FIG. 3. The kinetic isotope effect on single turnover oxidation of NADPH by NAD$^+$ catalyzed by transhydrogenase D$_2$O-dIII complexes. Experiments were performed in the stopped-flow spectrophotometer, as described under "Materials and Methods." Trace A, dIII-NADPH and 1.0 mM NAD$^+$; trace B, dIII-NADP$^+$H and 1.0 mM NADH.

![Graph showing kinetic isotope effect](image-url)
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