Communication

Evidence That the Transfer of Hydride Ion Equivalents between Nucleotides by Proton-translocating Transhydrogenase Is Direct*

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The molecular masses of the purified, recombinant nucleotide-binding domains (domains I and III) of transhydrogenase from Rhodospirillum rubrum were determined by electrospray mass spectrometry. The values obtained, 40,273 and 21,469 Da, for domains I and III, respectively, are similar to those estimated from the amino acid sequences of the proteins. Evidently, there are no prosthetic groups or metal centers that can serve as reducible intermediates in hydride transfer between nucleotides bound to these proteins. The transient-state kinetics of hydride transfer catalyzed by mixtures of recombinant domains I and III were studied by stopped-flow spectrophotometry. The data indicate that oxidation of NADPH, bound to domain III, and reduction of acetylpyridine adenine dinucleotide (an NAD+ analogue), bound to domain I, are simultaneous and very fast. The transient-state reaction proceeds as a biphasic burst of hydride transfer before establishment of a steady state, which is limited by slow release of NADP+.

Hydride transfer between the nucleotides is evidently direct. This conclusion indicates that the nicotinamide rings of the nucleotides are in close apposition during the hydride transfer reaction, and it imposes firm constraints on the mechanism by which transhydrogenation is linked to proton translocation.

Transhydrogenase is found in the inner membranes of animal mitochondria and the cytoplasmic membranes of some bacteria. It couples the transfer of hydride ion equivalents between NAD(H) and NADP(H) to the translocation of protons across the membrane. The net reaction is as follows.

\[ \text{NAD}^+ + \text{H}^+ + \text{NADP}^- \rightleftharpoons \text{NAD}^- + \text{NADPH} + \text{H}_2^+ \]  
(Eq. 1)

For many years, the question as to whether hydride transfer between the nucleotides is direct or indirect has been a matter of controversy. It is central to our understanding of the energy-coupling reactions.

Transhydrogenase comprises three domains. Domains I and III protrude from the membrane (on the matrix side in mitochondria and on the cytoplasmic side in bacteria). Domain II spans the membrane. There are separate sites on the enzyme for NAD(H) and for NADP(H); the former is located on domain I, and the latter on domain III (for reviews, see Refs. 1–3).

The results of some early experiments on transhydrogenases from mitochondria and from Rhodospirillum rubrum were interpreted as evidence for the existence of a stable, reduced-enzyme intermediate (4–6). It was implied that a functional group on the enzyme, presumably either an amino acid residue or an unidentified prosthetic group, can serve alternately as a hydride acceptor and hydride donor. For example,

\[ E + \text{NADH} \rightleftharpoons E(\text{H}) + \text{NAD}^+ \]  
(Eq. 2)

\[ E(\text{H}) + \text{NADP}^- \rightleftharpoons E + \text{NADPH} \]  
(Eq. 3)

where \( E(\text{H}) \) represents the reduced-enzyme intermediate. However, in subsequent work other plausible explanations were found for the earlier data (7, 8). Moreover, the conclusions from steady-state kinetic analysis of transhydrogenase from various sources (7, 9–11) have been interpreted as evidence that the reaction proceeds through the formation of a ternary complex of enzyme and nucleotide substrates. The addition of nucleotides is random, and fast, relative to the rate of a subsequent step in turnover. The reaction does not appear to take place via a substituted enzyme mechanism, and therefore the existence of a reduced enzyme intermediate, which is stable in the absence of nucleotide, is unlikely (viz. reactions exemplified by Equations 2 and 3). However, the steady-state data do not rule out the possibility existence of a reduced enzyme intermediate within the ternary complex, that is a reaction of the following type.

\[ E + \text{NADH} + \text{NADP}^- \rightleftharpoons \text{NADH-E-NADP}^- \rightleftharpoons \text{NAD}^- \cdot E \cdot \text{NADPH} \rightleftharpoons E + \text{NAD}^- + \text{NADPH} \]  
(Eq. 4)

The possibility that Cys residues in the polypeptide chain might serve as reducible intermediates in hydride transfer (see, for example, Refs. 1 and 12) has been eliminated by amino acid sequence comparisons, there are no conserved Cys residues in transhydrogenases from different species, and by the fact that complete Cys replacement has only a minimal effect on transhydrogenation activity (13). It is unlikely that other amino acid residues have redox potentials in the appropriate range to serve as intermediates in transhydrogenation between NAD(H) and NADP(H). It has sometimes been stated in the literature that transhydrogenase is devoid of prosthetic groups that might be involved in the hydride transfer pathway, although our survey indicates that studies are incomplete. 1) It was established many years ago that there is no detectable flavin fluorescence from purified preparations of the mitochondrial enzyme (14). 2) Analyses of amino acid sequences of transhydrogenases do not reveal the existence of metal-binding motifs. 3) It has been shown that concentrated solutions of the highly purified recombinant domains I and III (which together are catalytically active, see below) do not have any absorbance that might be attributable to chromophoric groups in the proteins (15, 16). However, we are unaware of studies on transhydrogenase which rule out the presence of weakly, or nonabsorb-
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A mixture of the isolated recombinant forms of domains I and III of *R. rubrum* transhydrogenase catalyzes the so-called “cyclic reaction” at a rate approaching that observed with the complete, membrane-located enzyme (16, 18). Evidently the complex of domains I and III is capable of rapid rates of hydride transfer, even in the absence of membrane-spanning domain II, and thus the apparatus for hydride transfer is located entirely within the two peripheral domains. We report below on the use of electrospray mass spectrometry to determine accurately the molecular masses of these peripheral domains with a view to establishing whether or not they possess covalently bound groups that might participate in the hydride transfer reaction. We also describe an experiment in which we examine the pre-steady-state kinetics of transhydrogenation catalyzed by a mixture of recombinant domains I and III using stopped-flow spectroscopy. In contrast to steady-state kinetic analysis, this procedure can reveal the presence of reaction intermediates. There are no other published descriptions of the pre-steady-state kinetics of reactions catalyzed by transhydrogenase. As hydride donor (binding to domain III), we use NADPH, and as hydride acceptor (binding to domain I), we use the NAD⁺ analogue, AcPdAD⁺. The difference in the absorbance spectra between the reduced forms of the two nucleotides enables us to measure the rate of oxidation of NADPH, and the rate of reduction of AcPdAD⁺, in real time.

**MATERIALS AND METHODS**

Recombinant forms of domain I and domain III of *R. rubrum* transhydrogenase were expressed in *E. coli* C600 from plasmids pCD1 (15) and pCD2 (16), respectively, and purified by column chromatography (15–17). The purity was confirmed by SDS-polyacrylamide gel electrophoresis (7), and the protein concentrations were determined using the microtannin assay (19). As normally prepared, the domain III transhydrogenase were expressed in *R. rubrum* II of *R. rubrum* using the microtannin assay (19). As normally prepared, the domain III transhydrogenase were expressed in *R. rubrum* II of *R. rubrum* using the microtannin assay (19). As normally prepared, the domain III transhydrogenase were expressed in *R. rubrum* II of *R. rubrum* using the microtannin assay (19). As normally prepared, the domain III transhydrogenase were expressed in *R. rubrum* II of *R. rubrum* using the microtannin assay (19).

**TABLE I**

| Domain | Mass spectrometry | Amino acid sequence |
|--------|-------------------|---------------------|
| I | 40,273 | 40,276 |
| III | 21,469 | 21,466 |

**RESULTS AND DISCUSSION**

The Molecular Weights of Domains I and III of *R. rubrum* Transhydrogenase Determined by Electrospray Mass Spectrometry—Table I shows the results of an analysis by electrospray mass spectrometry of the molecular masses of the purified, recombinant domains I and III of *R. rubrum* transhydrogenase. In both cases only a single distinct charge envelope was evident in the raw data. The similarity between the measured molecular masses of domains I and III, and the values calculated from the amino acid sequences (23), eliminates the possibility of a bound prosthetic group or metal center.

**AcPdAD⁺ + E-NADPH → AcPdADH + E-NADP⁺ (Eq. 5)**

A good fit to the burst was obtained by the sum of two exponentials. For AcPdAD⁺ reduction, the rate constants of the two phases were 490 and 8.6 s⁻¹, and for NADPH oxidation, 511 s⁻¹ and 8.5 s⁻¹. These values are similar within the experimental error.

While it is clear that the product NADP⁺ remains on domain III during the burst (see above), we are not yet certain of the time scale of dissociation of the product AcPdADH. If the nucleotides do remain bound to the protein, then their absorbance coefficients might be somewhat distorted from those in free solution (for example, see Ref. 24). Nevertheless, using solution absorbance coefficients for the reduced nucleotides (see “Materials and Methods”) the amplitudes of the fast and slow phases corresponded to approximately 60% of the concentration of domain I and domain III in the reaction mixture (25 μM), indicating that the burst corresponds to a single turnover event. Although there will be some perturbation of the redox potential of the nucleotides in the protein binding sites (25), the reaction is expected to go substantially to completion (the *E₅₀* values of NADPH) and of AcPdAD(H) in solution are −320 and −248 mV, respectively (26).
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FIG. 1. Simultaneous oxidation of NADPH and reduction of AcPdAD\(^+\) by a mixture of the recombinant domains I and III of transhydrogenase from \( R. \ rubrum \). Syringe one contained purified recombinant domain I (50 \( m \mu \) M), NADPH-loaded domain III (50 \( m \mu \) M), and carryover NADPH from the preincubation (final concentration: 70 \( m \mu \) M, see text) in 10 \( m \) M \((NH_4)_2SO_4, 20 \( m \) M Hepes, pH 8.0 (NaOH). Syringe two contained 2 \( m \) M AcPdAD\(^+\), prepared by diluting a 20 \( m \) M stock solution of nucleotide in water with 10 \( m \) M \((NH_4)_2SO_4, 20 \( m \) M Hepes, pH 8.0 (NaOH). The reaction was initiated by mixing 50 \( \mu \) M from each syringe in the stopped-flow spectrophotometer. Each trace shown in the figure is an average of 10 recordings. A, AcPdAD\(^+\) reduction at 375 nm. B, NADPH oxidation at 320 nm, \( T = 8^\circ C \).

Fig. 2 shows the wavelength dependence of the fast and slow phases of the burst. Both components have a similar spectrum to that obtained by subtracting the molar absorbance spectra of NADPH and AcPdADH, though the possibility that the spectra of the reduced nucleotides on the enzyme are distorted by a few nanometers (see Ref. 24) cannot be excluded. No other intermediates can be identified in the spectrum.

Consequences for the Mechanism of Action of Transhydrogenase—It is clear from this work that hydride transfer between nucleotides bound to domains I and III of transhydrogenase proceeds directly and not by way of a reduced enzyme intermediate. This observation indicates that, during catalysis, the peripheral, nucleotide-binding domains must bring the C-4 atoms of the nicotinamide rings of NAD(H) and NADP(H) into juxtaposition. It rules out a number of models in which proton translocation was assumed to arise from chemical interactions between nucleotides and a putative hydride-accepting intermediate (reviewed (1)), and it complements our recent evidence that hydride transfer in preformed domain I-III complexes is extremely rapid, and NADP\(^+\) release is very slow, lend further weight to the hypothesis.

Factors giving rise to the biphasic kinetics of the pre-steady-state burst remain to be investigated. Our preliminary interpretation is that the fast phase \( (k \approx 500 \text{ s}^{-1}) \) corresponds to hydride transfer in preformed domain I-III complexes appropriately loaded with AcPdAD\(^+\) and NADPH, it therefore reflects the true intramolecular rate constant for the reaction, whereas the slow phase \( (k \approx 8 \text{ s}^{-1} \text{ at } 8^\circ C) \) results from protein rearrangements and/or AcPdADH release. The steady-state cyclic reaction catalyzed by the \( R. \ rubrum \) domain I-III complex (the alternate reduction/oxidation of protein-bound NADP\(^+/\)NADPH by NADH/AcPdAD\(^+\)), with a \( k_{cat} \) in the region of 100–200 \text{ s}^{-1} \text{ at } 30^\circ C \), is expected to include contributions from both components.

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