The Membrane peripheral Subunits of Transhydrogenase from 
Entamoeba histolytica Are Functional Only When Dimerized*

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Unlike their bacterial and mammalian counterparts, the NADP(H)- and NAD(H)-binding components of proton-translocating transhydrogenase from the protozoan parasite Entamoeba histolytica (denoted ehdIII and ehdI, respectively) are tethered by a polypeptide linker. The recombinant tethered fragment, ehdIIIE-ehdI, was prepared without its membrane-spanning II component. Dimers of ehdIIIE-ehdI catalyzed transhydrogenation, but monomers were inactive. The addition of ehdIII to ehdIIIE-ehdI monomers did not lead to an increase in the rate of transhydrogenation, showing that this inactivity is not the result of an unfavorable topology introduced by the linker. The addition of a bacterial dI to ehdIIIE-ehdI led to an increase in the rate of transhydrogenation, showing that the linker is flexible. A hybrid protein in which ehdIII is tethered to the bacterial dI (denoted ehdIII-rrdI) more readily formed active dimers. Data from small angle x-ray scattering by the hybrid dimers were fitted to models derived from the high-resolution crystal structure of the bacterial dIIddIII complex (Cotton, N. P. J., White, S. A., Peake, S. J., McSweeney, S., and Jackson, J. B. (2001) Structure 9, 165–176). The results show that the ehdIII-rrdI dimer is asymmetric; one dIII associates with dI, as in the bacterial complex, but the other is displaced. The results provide evidence for the alternating site, binding change model for proton translocation by intact transhydrogenase.

Some membrane proteins, through changes in their conformation, can link a scalar chemical reaction to the translocation of solutes or ions. Often in these proteins, oligomeric interactions are central to the mechanism. For example, the FvF0 ATPase operates by a 3-site rotary mechanism (1) and the P-glycoprotein undergoes a 2-site alternation during turnover (2). Although it catalyzes a redox reaction rather than ATP hydrolysis, transhydrogenase is in this category of proteins, and its amenable properties make it an excellent model for studying conformational coupling (reviewed in Refs. 3 and 4). The enzyme links the hydride transfer between NAD(H) and NADP(H) to the translocation of protons across the inner membranes of mammalian mitochondria and the cytoplasmic membranes of bacteria as shown in Reaction 1.

\[
\text{NADH} + \text{NAD}^+ + \text{H}^+_\text{out} \rightleftharpoons \text{NAD}^+ + \text{NADPH} + \text{H}^+_\text{in}
\]

\text{Reaction 1}

The reaction is driven from left to right by the proton electrochemical gradient generated by the respiratory (or sometimes photosynthetic) electron transport chain. Thus, transhydrogenase is important as a source of NADPH for biosynthesis and glutathione reduction (for protection against free-radical damage) and in the regulation of flux through the tricarboxylic acid cycle (5, 6). Genes encoding transhydrogenase have also been found in several protozoan parasites. In Plasmodium falciparum the enzyme is probably located in the mitochondrial membrane, but in Eimeria tenella it is thought to be associated with so-called “refractile bodies” (7) and in Entamoeba histolytica, with “mitosomes” (8), otherwise known as “cryptons” (9). The functions of the E. tenella refractile bodies and the En. histolytica mitosomes and the nature of the partner proteins that are coupled to the transhydrogenase in the local chemiosmotic proton circuits are not known. The partner proteins are probably not enzymes normally associated with oxidative phosphorylation (10).

All proton-translocating transhydrogenases seem to have a similar structural organization. There is a dII component, which spans the membrane, and dI and dIII components, which protrude from the membrane on the matrix side in mitochondria and on the cytoplasmic side in bacteria. The dI component binds NAD+/NADH, and dIII binds NADP+/NADPH. The manner in which the three components relate to the polypeptide composition varies in different species (Fig. 1). In animal mitochondria, all three transhydrogenase components are on the same polypeptide chain; it runs dI-dII-dIII, N terminus to C terminus. In bacteria, gene sequences show that the dII component is always separated into dIIA and dIIB. In Escherichia coli transhydrogenase (and in other bacterial species) an α polypeptide comprises dI plus dIIA, and a β polypeptide comprises dIIB plus dIII. In transhydrogenase from Rhodospirillum rubrum (and some other bacterial species) there are three polypeptides; PntAA comprises dI, PntAB comprises dIIA, and PntB comprises dIIB plus dIII. The predicted order of components in the single polypeptide chain of transhydrogenase from the protozoan parasites (from the gene sequences) is dIIB-dIIA-dI. Thus, the N terminus of the protein corresponds to the N terminus of the bacterial β polypeptide (PntB). An extra segment (38 amino acid residues in both E. tenella and En. histolytica) is predicted between dII and dI and probably serves as a linker.

We recently expressed the dIII-linker-dI fragment of En. histolytica transhydrogenase from cloned DNA in cells of E. coli and purified the protein (henceforth denoted ehdIII-ehdI) in

* This work was supported by the Wellcome Trust and the Biotechnology and Biological Sciences Research Council. 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.

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1 The abbreviations used are: ehdIII, the NADP(H)-binding component of transhydrogenase from Entamoeba histolytica; ehdI, the NAD(H)-binding component of transhydrogenase from En. histolytica; ehdIIIE-ehdI, the fragment of En. histolytica transhydrogenase in which dIII and dI are joined by the native polypeptide linker; rrdI, the NAD(H)-binding component of transhydrogenase from Rhodospirillum rubrum; rrdIII, the NADP(H)-binding component of transhydrogenase

Printed in U.S.A.
Fig. 1. The polypeptide composition of transhydrogenases. The single polypeptide of mammalian transhydrogenase, the two polypeptides (α and β) of the E. coli enzyme, the three polypeptides (PntAA, PntAB, and PntB) of the R. rubrum enzyme, and the single polypeptide of *En. histolytica* are shown. The vertical alignment reflects amino acid sequence similarity. Unshaded areas represent the relatively hydrophilic dI and dIII components, and the shaded areas show the membrane-spanning dII. The separation of dII in the bacterial and parasite enzymes is represented by sections a and b.

substantial yields (11). Like complexes formed from mixtures of isolated dI and dIII from the transhydrogenases of other species (12–16), the tethered ehIII-ehII catalyzed so-called “cyclic” transhydrogenation at substantial rates and “reverse” transhydrogenation at much lower rates. This shows that, in other complexes, hydride transfer between nucleotides bound to the dI and dIII components of ehIII-ehII is fast, and the release of NADP(II) from dIII is slow (12).

Cross-linking and hydrodynamic studies show that the mammalian transhydrogenase is a homotrimer (17, 18) and that the *E. coli* enzyme is a comparable α2β2 tetramer (19); both enzymes are essentially “dimers” of the two “trimeric” units of dI, dII, and dIII. The high-resolution crystal structures of isolated dI (20) and the complex of dI and dIII (21) from *R. rubrum* transhydrogenase clearly indicate that the intact enzyme from this species has a similar organization. The structure of the *R. rubrum* complex and its hydrodynamic (22), kinetic (23), and NMR (24) properties reveal another important feature, i.e. only one dIII polypeptide binds to the two polypeptides of the dI dimer. Because hydride transfer across the single dI/dIII interface of the complex is extremely rapid (25), it was proposed that in the complete enzyme the two dI/dIII interfaces must be alternately brought together during turnover. The proposal is supported by earlier studies indicating “half of the sites” reactivity for bovine transhydrogenase (26, 27) and by another investigation in which inactivation of the bovine enzyme by high concentrations of Triton X-100 was attributed to the dissociation of dimers into monomers (18).

In this report, we use the tethered complex of ehIII-ehII and a tethered hybrid complex of *En. histolytica* dIII and *R. rubrum* dI to show the importance of dimeric interactions between dI units in hydride transfer. The unique properties of the parasite transhydrogenase provide further evidence for site alternation during transhydrogenase turnover and begin to reveal the structural basis for this process.

**EXPERIMENTAL PROCEDURES**

**Gene Constructs and Expression—**Tethered ehIII-ehII and isolated rrI and rrIII were expressed from plasmids pCJW3, pCD1, and pCD2, from *R. rubrum*; ehIII-rrI, a hybrid protein in which ehIII is tethered to the bacterial dI (unless otherwise indicated, the abbreviations above are not intended to represent the oligomeric state of the protein); ArFdAD<sup>-</sup>, the oxidized form of acetylpyridine adenine dinucleotide; SAXS, small angle x-ray scattering; MOPS, 4-morpholinepropanesulfonic acid.

DNA coding for isolated ehI (amino acid residues Leu<sup>385</sup>–Lys<sup>393</sup>) (29) was amplified by PCR using oligonucleotide primers made by Alta Bioscience and a construct bearing the complete *En. histolytica* transhydrogenase gene generously provided by Dr. C. G. Clarke of the London School of Hygiene and Tropical Medicine. The DNA sequence of the “sense” primer was chosen to result in Leu-565 being changed to the N-terminal Met residue of the ehI protein. DNA coding for ehIII (amino acids Met<sup>239</sup>–Lys<sup>257</sup>) was similarly amplified. The PCR products for ehI and ehIII were (separately) ligated into pPCR-Script<sup>®</sup>SK (+) using the PCR-Script Amp Kit from Stratagene. After confirming that the DNA fragments were free of polymerase errors, they were cloned into pET21(d) from Novagen to give pcJW1 (for ehI) and pcJW4 (for ehIII). The constructs were transformed into *E. coli* BL21(DE3) (30). Bacterial cells were then grown to mid-log phase at 25 °C in NZCYM medium (see Ref. 11) and induced with 1 mM isopropyl-β-D-thiogalactoside for 14 h. Growth at higher temperatures led to the aggregation of proteins into insoluble inclusion bodies from which active proteins could not be recovered by conventional refolding techniques. Cells bearing the plasmid coding for the dI construct grew very slowly, and relatively small amounts of recombinant protein were obtained. Cells were harvested by centrifugation, washed in a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM dithiothreitol, and stored at –20 °C.

As a preliminary step in the generation of DNA coding for the tethered hybrid ehIII-rrI, the internal NdeI site was removed from plasmid pcJW3 by site-directed mutagenesis of T to C at position 1360 using the Stratagene QuikChange kit and oligonucleotide primers from Alta Bioscience. This mutation does not change the amino acid sequence of the transcribed and translated gene. A new NdeI site was then introduced at the 5’-end of the DNA coding for the ehI by mutagenesis of 1793G→C, 1796C→A, and 1798A→G. The final construct in this experiment results in the substitution of an Asp residue for a His at the extreme C terminus of the linker. DNA sequencing established that no errors were introduced by the polymerase. The NdeI-BamHI fragment (coding for *En. histolytica* dI) from this construct was replaced by the NdeI-BamHI fragment (coding for *R. rubrum* dI) from pCD1 using standard cloning procedures. The final construct, designated pCJW7, was transformed into *E. coli* BL21(DE3), and cells were grown, induced, harvested, washed, and stored as described above.

**Protein Purification—**Tethered ehIII-ehII and isolated rrI and rrIII were purified by column chromatography as described (11, 12, 25). The purification protocols for ehIII and ehIII-rrI were similar to those for ehIII-ehII except that, in the case of ehIII-rrI, the protein solution was supplemented with 5% (rather than 10%) ammonium sulfate prior to the phenyl-Sepharose HP column, and the reverse gradient was from 5 to 0% saturation. To purify the ehI protein, a substantial modification of our standard procedures was required. The thawed bacterial extract from 3.2 liters of culture was applied to a 5 ml 30-cm column of Q-Sepharose Fast Flow (Amersham Biosciences) pre-equilibrated with 20 mM Tris- HCl, pH 8.0, and 2 mM dithiothreitol at 0 °C. The column was eluted with a gradient of 0 to 0.4 M NaCl in buffer A. Active fractions were pooled and then brought to 40% saturation with ammonium sulfate and incubated overnight at 4 °C. Precipitate was removed by centrifugation, and the supernatant, containing ehI, was applied to a 2.6 × 25-cm column of butyl-Toyopearl (TosoHaas) pre-equilibrated in buffer A supplemented with 40% saturated ammonium sulfate. The column was developed with a reverse gradient of ammonium sulfate (40–0% saturation), and recombinant protein was eluted in a final wash of buffer A. Active fractions were pooled, concentrated to ~3 ml in Vivascience centrifugal filters (10-kDa cutoff), and subjected to a final purification step on a 2.6 × 62-cm column of HiLoad Superdex 200 in buffer A. The protein was stored in 25% glycerol at –80 °C in 2-ml thin-walled vials (Nalgene).

During chromatography, proteins were assayed for transhydrogenation activity (see below) either alone or in the presence of partner nucleotide-binding proteins (dI or dIII) added in excess. Protein concentrations were determined by the microtannin procedure (31); all values are given as polypeptide monomers. The final preparations were routinely examined by SDS-PAGE, all were >95% pure according to staining intensity with Page Blue 33. The amounts of nucleotide associated with the purified recombinant proteins were determined by enzymatic assay after appropriate denaturation as described (12).

**Analytical Procedures—**Prior to all experiments the stored proteins were thawed on ice, concentrated in Vivascience filters (5-kDa cutoff for dIII proteins, 10 kDa for all other proteins), and washed with reaction buffer (see the legends to Figs. 2–6) supplemented with 2 mM dithiothreitol and 4 μM NADP<sup>−</sup>. Reverse transhydrogenase was measured...
as the reduction of the NAD\(^+\) analogue, AcPdAD\(^+\), by NADPH using the absorbance coefficient 6.07 \(\text{mm} \text{cm}^{-1} \text{cm}^{-1}\) at 375 nm (32). Cyclic transhydrogenation is the reduction of AcPdAD\(^+\) by NADH in the presence of NADP\(^+\), or NADPH (33). It corresponds to the reduction of NADP\(^+\) by NADH followed by the oxidation of NADPH by AcPdAD\(^+\); the reaction can occur without NADPH leaving the enzyme. Steady-state rates of transhydrogenation were measured (at 25 °C) in a PerkinElmer Lambda 16 dual wavelength spectrophotometer. Pre-steady-state kinetics were measured in an Applied Photophysics DX-17MV stopped-flow spectrophotometer in its absorbance mode at 25 °C. The optical path length was 2 mm, and the slits were set to give 5-nm half-bandwidth. The mixing dead time was 1.31 ms (34).

Gel exclusion chromatography was carried out using a 2.6 × 62-cm column of Hilo Saurex Superdex 200 calibrated with the following standards: blue dextran (1 mg ml\(^{-1}\)) to give the void volume \((V_0)\), alcohol dehydrogenase (5 mg ml\(^{-1}\)), bovine serum albumin (1 mg ml\(^{-1}\)), carbonic anhydrase (3 mg ml\(^{-1}\)), and cytochrome c (1 mg ml\(^{-1}\)). The sample and standards were separately applied to the column in a solution containing 20 mm Hepes, pH 8.0, 150 mm NaCl, 5% glycerol, 2 mM dithiothreitol, and 4 mM NADP\(^+\). The molecular mass was calculated from a plot of \(M_r \) versus \(V/V_0\), where \(V\) is the volume required to elute the respective protein.

Solution x-ray scattering data were collected following standard procedures on the X33 camera of the European Molecular Biology Laboratory (35) at the Deutsches Elektronen Synchrotron in Hamburg, Germany. Scattering from the ehDI-ehDII protein (271 \(\mu\)M) in a buffer containing 20 mm Hepes, pH 8.0, 10 mm \((\text{NH}_4)\text{SO}_4\), 2 mm dithiothreitol, and 4 mM NADP\(^+\) was measured with a sample to detector distance of 2.5 m. At a wavelength (\(\lambda\)) of 1.5 Å, the data covered the momentum transfer range 0.014 < s < 0.251 Å\(^{-1}\), where \(s = (4\pi\sin\theta)/\lambda\) and \(\theta\) is half the scattering angle. For molecular mass calibration, bovine serum albumin was used as a standard. Data were normalized to the intensity of the incident beam and corrected for the detector response using the program OTOKO (36). The buffer data were subtracted, and the results scaled for concentration using the program PRIMUS.\(^2\) The maximum diameter of the particle, the forward scattering value I(0), the radius of gyration \(R_g\), and the pair-distribution function \(p(r)\) were calculated from the scattering curve using the indirect Fourier transform program GNOM (36) with the data truncated to \(s = 0.1918\) Å\(^{-1}\). Low-resolution dummy atom models were calculated using the program DAMMIN (37) operating in “slow” mode with a spherical initial search space. The models generated by DAMMIN were subjected to an interaxial alignment step with respect to the crystal structure of the \(R.\ rubrum\) di diI monomer (PDB 1HZZ) using the program SUPCOMB (38) followed by a round of manual refinement to permit the inclusion of a second diI polypeptide using the program MASSHA (39). Experimental curves were compared with the scattering curves predicted from the crystal structure of the \(R.\ rubrum\) di diI complex and the truncated/extended forms thereof (see below) using the program CRYSTAL (40).

RESULTS

In a Mixture, Isolated ehDI and ehDII Form Catalytically Competent Complexes, but Hybrid Complexes of ehDI and \(R.\ rubrum\) di Are Considerably More Active—Recombinant forms of isolated diI and diII from \(E.\ coli\) and \(E.\ histolytica\) transhydrogenase were expressed in \(E.\ coli\) and purified (see “Experimental Procedures”). Analysis by SDS-PAGE indicated that the monomer molecular masses of ehDI and ehDII were ~40 and ~28 kDa, respectively (compare 39.7 and 22.0 kDa predicted from amino acid sequence data). Isolated recombinant diI from transhydrogenases of other organisms also behaves anomalously on SDS-PAGE, and the reasons for this are not clear (12, 16). Gel exclusion chromatography under non-denaturing conditions indicated that, at 450 \(\mu\)M, recombinant ehDII is probably monomeric (it eluted in a sharp peak at 29.4 kDa; see “Experimental Procedures”), but the recombinant ehDI protein eluted in a broad peak with molecular mass in the range 46–64 kDa. As found for other transhydrogenase diI components (12, 14, 16), purified ehDII was associated with tightly bound nucleotide (~0.5 mol NADP\(^+\)) and 0.4 mol NADPH mol\(^{-1}\) diI.

Separately, ehDI and ehDII were incapable of transhydrogenation, but together in simple mixtures they catalyzed significant rates of reaction. The dependence of the rate of cyclic transhydrogenation on the concentration of ehDI at fixed ehDII is shown in Fig. 2a. The reaction showed no sign of saturation even at >4 \(\mu\)M ehDI, suggesting that the affinity between the components is quite weak, and the rates were low in comparison with those achieved with tethered ehDI-ehDII (11).

A mixture of isolated \(R.\ rubrum\) diI (rrDI) and \(E.\ histolytica\) diIII gave rise to a very active complex. In the experiments shown in Fig. 2b, the ehDII was saturated by only moderate concentrations of rrDI (apparent \(K_d \sim 500 \text{nM}\)), and the maximum rates were similar to those obtained with mixtures of rrDI and rrDII (12). In contrast, mixtures of ehDI (for example at 975 \(\text{nM}\)) and rrDII (2 \(\mu\)M) gave rise to only very low rates of transhydrogenation (0.2 \(\mu\)mol AcPdADH \(\text{mol}^{-1}\) ehDIII min\(^{-1}\)) (results not shown).

It is concluded that, in its isolated form, ehDIII is a well-behaved protein able to bind a partner diI, resulting in a complex capable of good rates of hydride transfer. On the other hand, during expression and/or isolation, ehDII loses integrity (e.g. relative to that in ehDIII-ehDI). An apparently similar situation was noted with the (non-tethered) nucleotide-binding domains of \(E.\ coli\) transhydrogenase; a mixture of ecDI + ecDII had low activity, and ecDI + rrDII was inactive, whereas mix-
Transhydrogenase from Entamoeba histolytica

Fig. 3. The specific activity of tethered ehdIII-ehdI increases with protein concentration. The reaction buffer was similar to that described in the Fig. 2 legend. The reaction buffer was similar to that described in the Fig. 2 legend. Nucleotide concentrations were 26 μM NADP⁺, 56 μM NADH, and 205 μM AcPdAd⁺ (left-hand axis).

Fig. 4. The addition of ehdIII to monomeric tethered ehdIII-ehdI fails to stimulate transhydrogenation. The reaction buffer was similar to that described in the Fig. 2 legend. Nucleotide concentrations were 26 μM NADP⁺, 56 μM NADH, and 205 μM AcPdAd⁺. The concentration of ehdIII-ehdI was fixed at 127 nM, and the concentration of ehdIII was varied as shown.

Tethered ehdIII-ehdI were very active (with rates in the same range as those obtained with rrdl + rrdIII) (14, 15). The successful expression of mammalian dI has still not been reported. Thus, the exceptional dl is that from R. rubrum transhydrogenase, because, in its isolated state, it maintains the capacity for good reconstitution with its own dIII and those from En. histolytica, E. coli, bovine, and human sources. This could be a consequence of the fact that only in intact R. rubrum transhydrogenase does the dl protein exist as a separate polypeptide (Fig. 1).

Oligomeric Interactions Are Required for Catalytic Activity of Tethered ehdIII-ehdI—At concentrations >100 μM, the mobility of tethered ehdIII-ehdI during gel-exclusion chromatography under non-denaturing conditions at 25 °C indicated a molecular mass of 155 kDa somewhat greater than that expected of a dimer (the molecular mass of the monomer is 65.8 kDa) (11), although at 8 °C the protein repeatedly ran with a molecular mass of 110 kDa (results not shown). Purified ehdIII-ehdII was associated with a tightly bound nucleotide (~0.5 mol NADP⁺ plus NADPH mol⁻¹ protein; the proportions of NADP⁺ and NADPH varied slightly with the preparation, but no significant NAD(II) was detected) (data not shown).

Earlier studies showed that the characteristics of the transhydrogenation reaction catalyzed by ehdIII-ehdI (for example, in relation to nucleotide concentration) were similar to those observed in other dl + dIII systems (11). Fig. 3, however, shows that the specific rates of reverse and cyclic transhydrogenation catalyzed by ehdIII-ehdI were unexpectedly very dependent on protein concentration. The activity tended toward zero at low concentrations and was half-maximal at ~1 μM ehdIII-ehdI for the reverse reaction and ~0.5 μM for cyclic. Note that the cyclic reaction (limited by the rate of hydride transfer and by nucleotide occupation of the dl site) was much faster than reverse (limited by the slow release of NADP⁺ from the dIII site) as described and explained for complexes from the transhydrogenase of other species (12, 14, 41). The results are taken as an indication that ehdIII-ehdI forms catalytically active dimers from inactive monomers with a Kd in the order of 1 μM. Two possibilities are envisaged. (1) For topological reasons, a dIII and a dl tethered together on the same (monomeric) polypeptide chain cannot approach one another to give a catalytically active complex. In dimers, the dIII of one polypeptide interacts with the dl of another to give the active sites for hydride transfer. (2) The interface between monomeric units in a dimer is required to provide the necessary protein conformation (or the necessary movements within the polypeptides) for hydride transfer.

The experiments shown in Fig. 4 were designed to discriminate between these two hypotheses. Because ehdIII can readily form hydride transfer sites with ehdI (both in mixtures of the isolated proteins, and “intra-complex” sites within tethered ehdIII-ehdI; see above), it was reasoned that if the first hypothesis were correct, the addition of isolated ehdIII to a dilute solution of ehdIII-ehdI (where the latter is predominantly monomeric) would lead to the formation of extra hydride-transfer sites. We should then observe a pronounced increase in the specific rate of transhydrogenation up to that observed with dimers of ehdIII-ehdI. For example, the addition of ehdIII to 127 nM ehdIII-ehdI should stimulate the cyclic reaction from ~20 to ~200 min⁻¹ (Fig. 3). In fact, only a very small stimulation was observed (to ~25 min⁻¹ in Fig. 4), and this is taken as firm evidence against the first hypothesis and in favor of the second. The small stimulation that was detected is attributed to an increase in the number of hydride transfer sites due to enhanced association of ehdIII with the low concentration of ehdIII-ehdI dimers in the 127-nM solution (about 10% of the total protein).

The addition of isolated rrdl to a solution of ehdIII-ehdI greatly enhanced its capacity for both reverse (Fig. 5a) and cyclic transhydrogenation (Fig. 5b). The maximal rate of the cyclic reaction in these experiments and the concentration of rrdl needed to reach the half-maximal rate were similar to those observed in the titration of isolated ehdIII with rrdl (Fig. 2b). The results reveal the flexibility of the linker in the En. histolytica complex; the dIII component must be able to move apart from its own dl to interact functionally with the R. rubrum protein and give rise to a highly active rrdl/ehdIII hydride transfer site. Interestingly, for both the cyclic and reverse reactions the degree of stimulation produced by rrdl was more pronounced at low concentrations of ehdIII-ehdI (where the En. histolytica protein is predominantly monomeric) than at high concentrations (where the En. histolytica protein is predominantly dimeric). Thus, the specific rate of transhydrogenation at saturating rrdl was greater at a low ehdIII-ehdI concentration than at high concentration (Fig. 5). This indicates that, when ehdIII-ehdI is in its dimeric form (with moderately active hydride-transfer sites), less of the dIII component is available to form highly active sites with the added rrdl; the dIII is more tightly associated with dl in the ehdIII-ehdI dimer than in the monomer. Although we think that this con-
The concentration of $rr$ and the concentration of concentrations were 21 μM NADPH and 203 μM AcPdAD$^\text{-}$2. The concentration of $eh$III-eh$I$ was fixed at either 501 nM (○) or 2.0 μM (■), and the concentration of rrd$I$ was varied as shown. b, nucleotide concentrations were 20 μM NADP$^+$, 54 μM NADH, and 200 μM AcPdAD$^\text{-}$. The concentration of $eh$III-eh$I$ was fixed at either 103 nM (○) or 1.0 μM (●), and the concentration of rrdIII was varied as shown.

clusion is sound, it is necessary to be cautious in the interpretation of results shown in Fig. 5 because there is a possibility that the proteins can “shuffle” on the time scale of the experiment, i.e., mixed dimers of $eh$III-eh$I$1(rrd$I$)2 might be formed in significant quantities. The degree of shuffling will depend in a complex way upon the relative stability constants of the homogeneous dimers (rrd$I$1 and eh$III$-eh$I$2) and the mixed dimers, and upon the rate constants for dissociation and reassociation.

The addition of isolated eh$I$I also led to an increase in the transhydrogenation activity of $eh$III-eh$I$ but, as expected from the experiments described in the previous section, the effect was much less pronounced than with R. rubrum dI. With 200 nM $eh$III-eh$I$I (predominantly monomeric) the increase was approximately linear with eh$I$ concentrations up to 5 μM (indicating a low affinity interaction), and the rate enhancement of cyclic transhydrogenation was from ~10 min$^{-1}$ with zero eh$I$ to only ~70 min$^{-1}$ with 5 μM eh$I$ (data not shown, and compare Fig. 5). Despite the difference in the affinity of $eh$III-eh$I$ for rrd$I$ and for eh$I$, a similar explanation for the results seems likely.

A Hybrid-tethered eh$III$-rrd$I$ Complex—There is clear evidence that isolated dI of transhydrogenase from several species is dimeric (22, 28, 41, 42). None of the results from a number of different types of hydrodynamic experiments on rrd$I$ gave any suggestion that the protein dissociates significantly into mono-

FIG. 5. The addition of rrd$I$ stimulates reverse (a) and cyclic (b) transhydrogenation catalyzed by $eh$III-eh$I$. The reaction buffer was similar to that described in the Fig. 2 legend. a, nucleotide concentrations were 21 μM NADPH and 203 μM AcPdAD$^\text{-}$2. The concentration of $eh$III-eh$I$ was fixed at either 501 nM (○) or 2.0 μM (■), and the concentration of rrd$I$ was varied as shown. b, nucleotide concentrations were 20 μM NADP$^+$, 54 μM NADH, and 200 μM AcPdAD$^\text{-}$. The concentration of $eh$III-eh$I$ was fixed at either 103 nM (○) or 1.0 μM (●), and the concentration of rrdIII was varied as shown.

mers even in the micromolar concentration range (22). We have therefore constructed a hybrid of $eh$III and rrd$I$ joined in a single polypeptide by a linker equivalent to that found in $eh$III-eh$I$ to explore further the importance of a dimeric organization for hydride transfer. DNA coding for eh$I$ was excised from a plasmid bearing the $eh$III-eh$I$I gene fragment and replaced by a sequence coding for rrd$I$ (see “Experimental Procedures”). The 38-residue linker from $eh$III-eh$I$I was retained with only a single substitution (Asp → His at the C terminus of the linker region). The hybrid protein, designated $eh$III-rrd$I$, expressed to high levels in E. coli and was easy to purify by a modification of earlier protocols for the nucleotide-binding components of transhydrogenase.

As with $eh$III-eh$I$I (see above), the mobility of $eh$III-rrd$I$ during non-denaturing gel-exclusion chromatography indicated a molecular mass (~165 kDa) that was intermediate between that of a dimer and a trimer (the molecular mass of the monomer is 66.4 kDa). However, $eh$III-rrd$I$I proved to be a good subject for structural analysis using SAXS (see below), and the data showed unequivocally that the tethered hybrid is a dimer at protein concentrations in the region of 270 μM. It is likely that flexibility in the linker region of both $eh$III-eh$I$I and $eh$III-rrd$I$ leads to elevated hydrodynamic radii, causing increased retention times of the proteins in the matrix of the gel-exclusion column and therefore an exaggeration of the apparent molecular masses.

Purified $eh$III-rrd$I$ catalyzed high rates of cyclic and low rates of reverse transhydrogenation (Fig. 6a), the familiar pattern for complexes of dI and dIII proteins (12). The cyclic
reaction was faster than that catalyzed by dimeric ehIII-ehI, and the reverse reaction was a little slower (compare Fig. 3). Both the cyclic and reverse rates were comparable with those catalyzed by complexes formed from mixtures of isolated rrdI and either rrdIII (12) or ehI (see above). However, in marked contrast to the results obtained with ehIII-ehI (Fig. 3), the specific activity of ehIII-rrdI (for both reverse and cyclic) was essentially independent of protein concentration down to \(<10\) nM, the lowest concentration at which rates could be reliably measured. We suggest that, because of the tight interaction between the two \(R.\ rubrum\) dI and dIII peptides, active dimers of ehIII-rrdI form with a much higher affinity (\(K_d \ll 10\) nM) than those of ehIII-ehI (\(K_d \approx 1\) \(\mu\)M).

As was observed with ehIII-ehI (Fig. 5), the addition of isolated rrdI to a solution of ehIII-rrdI led to a stimulation of the rate of transhydrogenation, although the effect was not as pronounced (Fig. 6b); for example, with ehIII-ehI predominantly in its dimeric form (i.e. at \(-1\) \(\mu\)M) rrdI increased the rate of cyclic transhydrogenation maximally by \(>18\)-fold, whereas the effect of rrdI on the reaction catalyzed by ehIII-rrdI was maximally about 1.8-fold. The concentration of rrdI giving the half-maximal rate was similar to the values observed in the titration of rrdI against isolated ehIII (Fig. 2b) and of rrdI against ehIII-ehI (Fig. 5b). These results again indicate that the linker is sufficiently flexible to allow the ehIII moietie to move apart from its “own” dI and interact with the supplementary isolated rrdI.

The ability of ehIII-rrdI to catalyze high rates of cyclic transhydrogenation indicate that the hydride transfer step is rapid. This conclusion was supported by stopped-flow experiments. As with simple mixtures of isolated rrdI and rrdIII (23, 34), it was found that the slow steady-state rate of reverse transhydrogenation catalyzed by ehIII-rrdI was preceded by a rapid burst of reaction (results not shown). This probably arises because the binding of AcPdAD\(^+\) and hydride transfer are both fast compared with the rate of product NADP\(^+\) release. As observed with \(R.\ rubrum\) dI-dIII complexes, the amount of AcPdAD\(^+\) reduced during the burst was equivalent to about 50% of the amount of protein (32). The burst comprised two exponential phases of equivalent amplitude, which had apparent first order rate constants of \(-300\) and \(-20\) s\(^{-1}\). These rate constants are similar to those measured for the biphasic burst observed with mixtures of rrdI and rrdIII (\(-550\) and \(-50\) s\(^{-1}\)). There, it was convincingly established that the fast phase corresponds to hydride transfer and the slow phase to dissociation of dIII from the dI dimer prior to rapid reassociation and further oxidation of NADPH on dIII (23). We suggest that the biphasic burst of transhydrogenation of ehIII-rrdI arises for similar reasons, but we cannot of course confirm this by independently varying the concentrations of the two components, the strategy we adopted with rrdI + rrdIII.

**SAXS Structure of the ehIII-rrdI Hybrid Complex**—SAXS data can provide reliable low-resolution information on the size and shape of macromolecules. Preliminary SAXS analyses of recombinant ehIII-ehI were difficult to interpret probably because of the tendency of the molecule to dissociate; the technique is not well suited to solutions that are not monodisperse. Measurements by dynamic light scattering in a range of buffer solutions (not shown) also indicated that solutions of ehIII-ehI were polydisperse. However, ehIII-rrdI did prove to be a good subject for investigation; its SAXS curve is shown in Fig. 7. The molecular mass of the protein calculated from these data, using bovine serum albumin as a reference, was 130 kDa, strongly supporting the notion that ehIII-rrdI is dimeric (see above). Assuming that the two dI components in the ehIII-rrdI dimer interact as in the crystal structure of the isolated dI dimer and dI-dIII complex from \(R.\ rubrum\) transhydrogenase (see “Discussion”), the results of Fig. 7 were used as a basis for discriminating between three plausible models of the hybrid tethered complex. 1) The two dIII components, held by the linkers, extend from the two dI components approximately along the major axis of the dimer. 2) Two of the dI components and one of the dIII components form a structure similar to that of the \(R.\ rubrum\) dI-dIII complex, whereas the second dIII extends from its respective dI. 3) Two of the dI components and one of the dIII components form a structure similar to that of the \(R.\ rubrum\) dI-dIII complex, and the second dIII binds close to the symmetrically equivalent location of the first (rotated 180° about the 2-fold axis running between the two dI polypeptides) (see Fig. 7).

In the first approach toward determining the structural organization of the tethered hybrid complex, a pair-distribution function was generated from the experimental scattering profile using the program, GNOM (36). The function had a smooth profile tending gradually toward zero at a maximum diameter of 150 Å, which is consistent with the second two models but not with the first. Then, low-resolution reconstructions of the particle shape were calculated from the pair-distribution function using the program, DAMMIN (37). The reconstructions from five independent calculations converged as shown in Fig. 8. Only the second model can be fitted satisfactorily into the envelope defined by the dummy atoms of the DAMMIN calculations. Finally, molecular versions of each model were built by manipulating the x-ray structure of the \(R.\ rubrum\) dI-dIII complex (together with a second dIII component) using the program MASSHA (Ref. 39, and see Fig. 7), and these were used to generate theoretical scattering profiles with the program CRYSOL (40). Again, the second model gave the best fit to the experimental data.

**DISCUSSION**

The results described above provide a further illustration that dI and dIII components of transhydrogenase, even in the absence of membrane-spanning dIII, form a complex that is capable of high rates of hydride transfer. Remarkably, hybrid mixtures of dI and dIII from transhydrogenases of widely di-
The crystal structure of the experimental scattering data (see on pair-distribution functions derived from a GNOM analysis of the tions of the overlaid results of five independent DAMMIN calculations.

...homology between dI.1(A) and dI.1(B) are more peripheral and appear to partici-...eh

...genases (43% identity in dI), and therefore similar contact surfaces between monomers in the dI dimer; both hy-

drophobic and H-bond interactions contribute to the dimer interface. As a result of these interactions, the domains desig-
nated dI.2(A) and dI.2(B) (of the A and B polypeptides of dI) pack together to form a central rigid core to the dI dimer; they lie “back-to-back” across a 2-fold axis of symmetry. Domains dI.1(A) and dI.1(B) are more peripheral and appear to partici-

...allel to stabilize the overall dimeric structure.

That isolated dIII-dI complexes are active only in their dimeric form is probably a reflection of the process of site alternation thought to take place during turnover of the intact enzyme. It was proposed that, coupled to proton translocation through the membrane-spanning dII, conformational changes drive dIII between an open state (in which bound nucleotides can exchange with those in the solvent) and an occluded state (in which hydride transfer with nucleotides on dI can occur) (4).

The important question therefore arises as to whether, also in the tethered complexes, only one dIII component (in its occluded state) binds to the dI dimer; it is located at the side of the cleft between dI.1(B) and dI.2(B). Modeling studies indicate that the binding of a second occluded dIII to the symmetrically equivalent site at the cleft of dI(A) is prohibited because it would lead to side-chain clashing with the first dIII. It was proposed that in the intact enzyme the second dIII (in its open state) is displaced; site alternation would lead to a switching of states at the next half-cycle. It is interesting, therefore, that the current experiments show that the linker in dIII-ehdI allows considerable freedom for relative movement of dIII away from dI, even to the point where supplementary isolated dI (dimer) can bind to ehdIII-ehdI giving additional active hydride-transfer sites (Fig. 5).

The stoichiometry in solution (22) and the crystal structure of the dII-dIII complex of R. rubrum transhydrogenase show that only one dIII component (in its occluded state) binds to the dI dimer; it is located at the side of the cleft between dI.1(B) and dI.2(B). Modeling studies indicate that the binding of a second occluded dIII to the symmetrically equivalent site at the cleft of dI(A) is prohibited because it would lead to side-chain clashing with the first dIII. It was proposed that in the intact enzyme the second dIII (in its open state) is displaced; site alternation would lead to a switching of states at the next half-cycle. It is interesting, therefore, that the current experiments show that the linker in dIII-ehdI allows considerable freedom for relative movement of dIII away from dI, even to the point where supplementary isolated dI (dimer) can bind to ehdIII-ehdI giving additional active hydride-transfer sites (Fig. 5).

The fact that the specific activity of the hybrid ehdIII-rrdI is approximately constant down to the lowest protein concentrations that gave reliable transhydrogenation rates (Fig. 6a) suggests that it forms very stable dimers; its $K_d$ for dimer disso-

ciation must be at least two orders of magnitude lower than that of ehdIII-ehdI (equivalent to $>1.15 \text{ kM}^{-1}$).

...ehdIII-ehdI (equivalent to $>1.15 \text{ kM}^{-1}$). We do not know which amino acid substitutions are responsible for this difference in stability; in principle, differences in only two or three amino acid residues might be sufficient. Of course, in the intact enzyme, interactions between the dII components will also help to stabilize the overall dimeric structure.

FIG. 8. DAMMIN calculations on the SAXS curve of tethered hybrid ehdIII-rrdI. The larger spheres show four different orienta-
tions of the overlaid results of five independent DAMMIN calculations on pair-distribution functions derived from a GNOM analysis of the experimental scattering data (see “Results”). The smaller spheres show the crystal structure of the R. rubrum dI-dIII1 complex; the circle/ellipse representing dIII is appended to give an approximation of model 2 (see “Results” and Fig. 7).
however, in dI-dIII complexes, because there is no dII (and no intact enzyme with first one site reacting and then the other).

Furthermore, several different analyses of the SAXS curve indicate that the second dIII does not bind symmetrically to the dII-bound dIII and a coincident tight association of the other dIII with its dI.

The character of the reaction burst with dIII-rrdI revealed in stopped-flow experiments can also be explained by this model. Thus, the fast phase of the burst corresponds to hydride transfer (NADP → AcPDAD) at catalytic sites in the interface between the closely associated dII and dIII. The slow phase corresponds to: 1) dissociation of that site; 2) association of dI and dIII at the second site; and 3) hydride transfer at the second site. By analogy with experimental results with mixtures of isolated R. rubrum dII and dIII (where the rate constants are very similar), it is suggested that site dissociation is the rate-limiting step. Thus, in a sense the biphasic burst in the tethered complex would correspond to events occurring in the intact enzyme with first one site reacting and then the other. However, in dI-dIII complexes, because there is no dII (and no proton translocation) the dIII component remains in the occluded state (from which product NADP+ cannot escape), and so further turnover is prevented.

In conclusion, we have used a combination of transhydrogenase activity measurements and biophysical analyses of the subunit organization of tethered complexes of dI and dIII to reveal information that was not available from investigations of complexes formed from the isolated components. The future challenge is to extend the methodology to the intact enzyme and to determine precisely how conformational changes accompanying proton translocation through dIII drive the alternating events in the nucleotide-binding subunits.

Acknowledgments—We are grateful to Scott White, Philip Quirk, and Nick Cotton for discussions and to Andy White for help with the construction of the hybrid tethered complex. SAXS data were obtained with the kind help of D. Svergun, M. Malfois, and M. Koch.

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*J. Biol. Chem. 2002, 277:26163-26170. doi: 10.1074/jbc.M203514200 originally published online May 9, 2002*

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