Catalytic Properties of Hybrid Complexes of the NAD(H)-Binding and NADP(H)-Binding Domains of the Proton-Translocating Transhydrogenases from *Escherichia coli* and *Rhodospirillum rubrum*†

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ABSTRACT: Transhydrogenase couples reversible hydride transfer from NADH to $NADP^+$ to proton translocation across the inner membrane in mitochondria and the cytoplasmic membrane in bacteria. The enzyme is composed of three parts. Domain I (dI) and domain III (dIII) are water soluble and contain the binding sites for NAD(H) and NADP(H), respectively; domain II (dII) spans the membrane. In the present investigation, dI from *Rhodospirillum rubrum* (rrI) and *Escherichia coli* (ecI), and dIII from *R. rubrum* (rrIII) and *E. coli* (ecIII) were overexpressed in *E. coli* and subsequently purified. Also, a preparation of a partially degraded *E. coli* transhydrogenase (ecβ) was examined. Catalytic activities were analyzed in various dI+dIII and dI+ecβ combinations. The abilities of the different dI+dIII combinations to catalyze cyclic transhydrogenation, i.e., the reduction of $AcPyAD^+$ by NADH mediated via tightly bound NADP(H) in dIII, varied in the order: rrI+ecIII ≈ rrI+rrIII > rrI+ecβ ≫ ecI+ecIII; no measurable activities for ecI+rrIII and ecI+ecβ were detected. Thus, rrI has a much greater apparent affinity than ecI for ecIII or rrIII or ecβ. The pH dependences of the cyclic reaction seem to be determined by scalar protonation events on dI, both in rrI+rrIII and ecI+ecIII mixtures as well as in the wild-type *R. rubrum* and possibly in the *E. coli* enzyme. Higher reverse activities for rrI+ecβ than for rrI+ecIII confirmed the regulatory role of dII for the association and dissociation rates of NADP(H).

Under physiological conditions, membrane-bound nicotinamide nucleotide transhydrogenase catalyzes the stereospecific transfer of hydride equivalents from the 4A position of NADH to the 4B position of $NADP^+$. This process is coupled to proton translocation, across the cytoplasmic membrane of bacteria, and across the inner membrane of mitochondria:

$$n\mathrm{H}^+_{\mathrm{out}} + \mathrm{NADH} + \mathrm{NADP}^+ \rightleftharpoons n\mathrm{H}^+_{\mathrm{in}} + \mathrm{NAD}^+ + \mathrm{NADPH} \quad (1)$$

Estimates indicate that $n = 1$ (*1*−*3*). For reviews on transhydrogenase, see Olausson et al. (1995) (*4*) and Jackson (1991) (*5*). The amino acid sequences of the enzyme from some 13 different organisms have been fully or partially determined, and analysis of these data, together with substantial biochemical information, indicates that transhydrogenase has a tripartite structure. Domain I (dI)[1] has approximately 400 residues, and domain III (dIII), approximately 200 residues. These two domains protrude from the membrane, and contain the binding sites for NAD(H) and NADP(H), respectively. The 10−14 α-helices of domain II (about 400 residues) span the membrane and naturally embrace at least part of the proton conducting pathway (Figure 1).

Detailed structural information on transhydrogenase is presently unavailable, although the fold of the NAD(H)-binding site in dI has been predicted (*6*). On the basis of kinetic experiments, there is an emerging view that proton translocation is conformationally coupled to events at the level of NADP(H) binding and release (*7*−*9*).

dI proteins from *E. coli* (*9, 10*) and from *R. rubrum* (*11*), and dIII from *E. coli* (*9*), *R. rubrum* (*12, 13*), and *Bos taurus* (*12, 14*), have recently been overexpressed in water-soluble form, purified, and partially characterized. The dI proteins are isolated as apoprotein dimers, which bind NADH with moderately high affinity and NAD^+ with lower affinity (*15*). The binding of NAD(H) to recombinant dI protein is accompanied by closure of a surface mobile loop onto the adenosine part of the nucleotide (*10*). Single site mutations

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[1] Abbreviations: dI, dII, and dIII, domain I, domain II, and domain III, respectively, of transhydrogenases in general; rrI and rrIII, domain I and domain III constructs, respectively, of *Rhodospirillum rubrum* transhydrogenase; ecI and ecIII, domain I and domain III constructs, respectively, of *Escherichia coli* transhydrogenase; ecβ, preparation of *E. coli* transhydrogenase in which at least the portion of the α-subunit extruding from the membrane has been degraded; $AcPyAD^+$, 3-acetylpyridine adenine dinucleotide (oxidized form).

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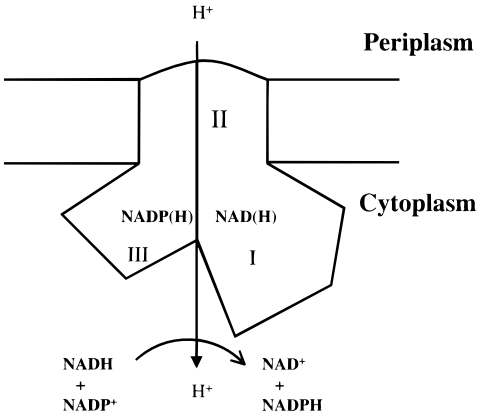

FIGURE 1: Schematic representation of the domains in transhydrogenases.

lead to pronounced inhibition of hydride transfer. The oligomeric state of recombinant dIII is not known with certainty, although the available information suggests that the isolated proteins are monomeric. They contain approximately stoichiometric amounts of NADP(H) (*9*, *13*). Structural differences have been described in dIII from *E. coli* depending on whether the bound nucleotide is oxidized or reduced (*9*).

Even in the absence of membrane-spanning domain II, mixtures of recombinant dI and dIII catalyze "forward" and "reverse" transhydrogenation (cf. eq 1) and the so-called "cyclic reaction" which involves the reduction of protein-bound $NADP^+$ by NADH, followed by the oxidation of protein-bound NADPH by $AcPyAD^+$ (*9*, *12*, *13*). Previous experiments revealed some similarities, and some differences, between the behavior of mixtures of rrI+rrIII on the one hand (*13*) and ecI+ecIII on the other (*9*). Both rrI+rrIII and ecI+ecIII catalyzed very low steady-state rates of forward and reverse transhydrogenation. The evidence suggested that the reactions were limited, in the absence of domain II, by slow release from domain III of product NADP(H) (*9*, *13*). However, whereas rrI+rrIII catalyzed a very rapid rate of cyclic transhydrogenation, approximately as fast as is achieved in the complete enzyme (*13*), ecI+ecIII catalyzed only a low rate of this reaction (*9*). In the case of the *R. rubrum* proteins, the hydride transfer step is very rapid, which was strongly supported by stopped-flow experiments demonstrating that reduction of $AcPyAD^+$ by NADPH within the dI·dIII complex proceeds with an apparent first-order rate constant of $\sim 600\ s^{-1}$ (*16*). Hydride transfer catalyzed by this complex proceeds directly from one nicotinamide ring to the other without proceeding through an intermediate hydride acceptor (*16*).

Yamaguchi and Hatefi (*14*) showed that a hybrid mixture of domain I from *R. rubrum* transhydrogenase and domain III from the bovine enzyme was capable of catalyzing transhydrogenation. In this report, other hybrid systems are explored, using domains from *R. rubrum* and *E. coli* transhydrogenases. It is demonstrated that the nucleotide-binding domains of the enzyme are remarkably nonspecific in their ability to form catalytically active complexes. A new preparation (ecβ) of *E. coli* transhydrogenase is also described, in which the α-subunit is selectively degraded. The catalytic activity of this preparation is restored by addition of *R. rubrum* dI protein (rrI), but not by the equivalent *E. coli* protein (ecI). It is discussed whether the dependence of the rates of reverse and of cyclic transhydrogenation on the concentrations of the domains in different types of complexes provides indications of affinity and turnover. In view of the coupling of transhydrogenation to proton translocation, the effects of pH on the reactions catalyzed by dI + dIII and by rrI + ecβ mixtures are investigated.

EXPERIMENTAL PROCEDURES

Expression and Purification of dI and dIII Proteins. Domain I and domain III proteins from *R. rubrum* (rrI and rrIII, respectively) were expressed in *E. coli*, from pCD1 and pCD2, respectively, recovered from cell-free extracts and purified by column chromatography, as described (*11*, *13*, *17*). Domain I protein from *E. coli* (ecI) (previously referred to as EcTHSα1) was expressed and purified essentially according to the method described earlier (*9*). In the latter procedure, immobilized metal chelate affinity chromatography was employed as the sole purification step. Following elution from the $ZnSO_4$-loaded Chelating Superose column (Pharmacia Biotech, Sweden), the purest fractions of ecI, as judged by SDS−PAGE, were pooled and concentrated to approximately 10 mL using a Centriprep-10 concentrator (Amicon Inc., USA). The sample was then extensively dialyzed against 25 mM sodium phosphate buffer, pH 7.0. Glycerol was added to 20%, and the final ecI concentration was 3.2 mg/mL. Aliquots were frozen in liquid nitrogen and stored at −80 °C. The protein was stored on ice during experiments. When indicated, a second independent preparation of ecI was used (*10*). The expression and purification protocol for ecIII (previously abbreviated to EcTHSβ1) has also been described (*9*), although for the present experiments the gel filtration step was performed using a 25 mM Hepes buffer, pH 7.0, containing 100 mM NaCl. After subsequent concentration with a Centriprep-3 concentrator (Amicon Inc.) and addition of glycerol to 20%, the sample was frozen in liquid nitrogen and stored at −80 °C at a concentration of 5.0 mg/mL. The protein was found to be more stable at 4−25 °C than at 0 °C, and was therefore stored in the refrigerator or at room temperature when used for experiments.

The preparation of ecβ was derived from purified wild-type transhydrogenase from *E. coli*, to which a six residue long, N-terminal histidine tag had been added (*9*). Long-term storage (several weeks) of this protein on ice in buffer containing 15 mM sodium phosphate, pH 7.0, 0.05% dodecyl maltoside resulted in specific degradation of the α-subunit. Analysis by SDS−PAGE indicated that the β-subunit remained intact. The stock solution of ecβ was 5.0 mg/mL.

Catalytic Activities. Reverse and cyclic transhydrogenase activities were measured spectrophotometrically following the reduction of $AcPyAD^+$ at 375−455 nm using a Shimadzu UV-3000 dual wavelength spectrophotometer or at 375 nm using a Shimadzu UV-1601 spectrophotometer. The absorption coefficient used was $6.10\ mM^{-1}\ cm^{-1}$ (*18*). Unless otherwise specified, the reaction buffer contained 20 mM Mes, 20 mM Mops, 20 mM Tris, 20 mM Ches, and 50 mM NaCl, pH 7.0. This buffer will be referred to as the dIdIII assay buffer. When different pHs were required, adjustments were made by addition of either HCl or NaOH. For measurements involving ecβ, 0.01% Brij was added to the dIdIII assay buffer. See figure legends for details.

Fluorescence Measurements. The rate of release of $NADP^+$ from ecIII was measured from changes in the level of tryptophan fluorescence. Excitation and emission wavelengths were 280 and 339 nm, respectively, with both slits set to 4.3 nm. The temperature was 25 °C, and the medium contained dIdIII assay buffer adjusted to the desired pH with either HCl or NaOH. The protein concentration was typically 1.22 μM, and the NADPH was added to a final concentration of 10 μM. For additional information including the correction for the inner filter effect, see Fjellström et al. (1997) (*9*).

The determination of $k_{off(NADP^+)}$ was based on fitting the fluorescence quenching traces to biexponential functions, i.e., $I = A_0 + a_1 \exp(-k_1 t) + a_2 \exp(-k_2 t)$, where I is relative fluorescence intensity, t is time, k_1 and k_2 are the rate constants for the two exponential phases, A_0 is the final amplitude, and a_1 and a_2 are the amplitudes of the two component phases. The values of $k_{off(NADP^+)}$ referred to in the text correspond to the rate constant associated with the dominating amplitude (normally about 90%); this rate constant was, in general, 5−10 times greater than the second. The reason for the complexity of the fluorescence kinetics is presently not known.

Isotope Effects. Deuterated substrate [4A-^{2}H]NADH (NADD) and nondeuterated substrate [4A-^{1}H]NADH (NADH) were synthesized essentially as described (*19, 20*). The reaction buffer contained 6 mM Ches−KOH and 50 mM KCl. Deuterated ethyl-d_5 alcohol-*d* (99+ atom % D) was obtained from Sigma Aldrich. To remove the proteins in the final step, the reaction mixtures were centrifuged at 4 °C for approximately 1 h through Centriprep-3 concentrator tubes (Amicon Inc.). The final concentrations of substrates were determined from the absorption at 340 nm.

Determination of Protein Concentration. rrI, rrIII, ecI, and ecβ concentrations were determined by the bicinchoninic acid assay (*21*) (with BSA as standard), and ecIII was measured optically (*9*).

Gel Electrophoresis. SDS−PAGE was performed essentially according to the protocol of Laemmli (1970) (*22*).

RESULTS

Characteristics of the Isolated Domains. *E. coli* domain I (ecI), *R. rubrum* domain I (rrI), *E. coli* domain III (ecIII), and *R. rubrum* domain III (rrIII) were expressed and purified as described under Experimental Procedures. The SDS−PAGE representation in Figure 2 shows the purified products. Nondenaturing size-exclusion chromatography showed that, in accordance with previous reports (*9, 13*), both ecI and rrI were isolated as dimers. The ecIII and rrIII behave anomalously on nondenaturing size-exclusion chromatography (*9, 13, 14*). Both the ecIII and rrIII preparations used in the present study contained approximately 20% bound NADPH and 80% bound $NADP^+$, as judged by optical absorption analysis for ecIII (*9*) and by nucleotide extraction procedures for rrIII (*13, 23*).

Catalysis by Mixtures of Transhydrogenase Domains I and III from R. rubrum and E. coli. Figure 3 shows the dependence on protein concentration of the rates of the reverse and cyclic reactions catalyzed by mixtures of recombinant domains I and III of *E. coli* transhydrogenase (ecI+ecIII). In each case, the rates tended toward a maximum as the concentration of one component was held constant,

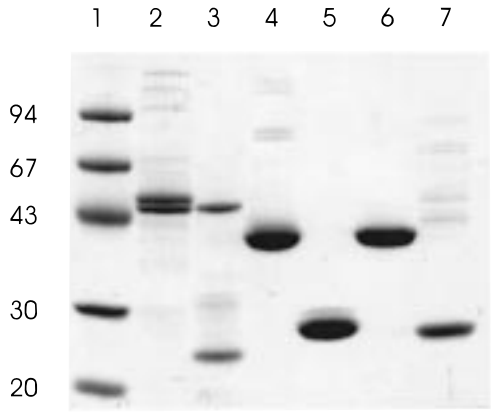

FIGURE 2: SDS−PAGE of *E. coli* transhydrogenase and purified domain I and domain III preparations from *E. coli* and *R. rubrum*. The low molecular weight marker is shown in lane 1, intact *E. coli* transhydrogenase (8 μg) in lane 2, ecβ (12 μg total protein) in lane 3, ecI (8 μg) in lane 4, ecIII (8 μg) in lane 5, rrI (8 μg) in lane 6, and rrIII (8 μg) in lane 7.

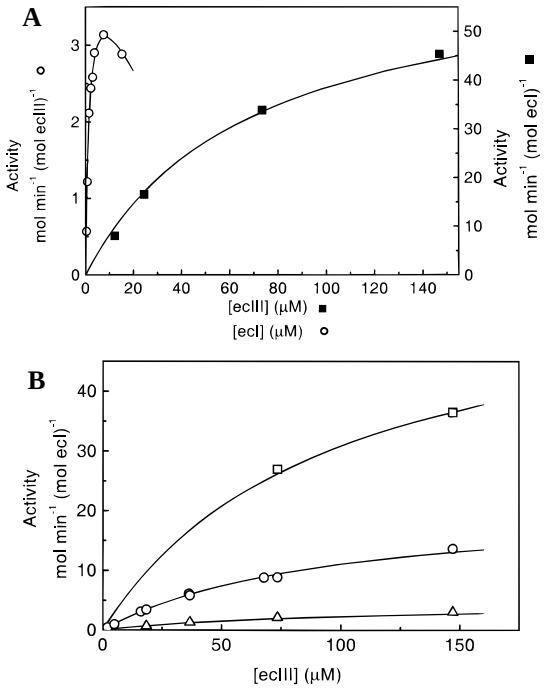

FIGURE 3: Protein−protein titrations of ecI+ecIII mixtures for reverse transhydrogenation (A) and for cyclic activities (B). The experiments were performed in dIdIII assay buffer (Experimental Procedures). pH was (A) 7.0; (B) 6.0 (open squares), 7.0 (open circles), and 7.9 (open triangles). In (A), the concentration of fixed ecI (filled squares) was 0.38 μM, and of fixed ecIII (open circles) 1.96 μM. In (B), the concentration of fixed ecI was 0.79 μM. The substrate concentrations were (A) 2 mM $AcPyAD^+$ and 200 μM NADPH; (B) 400 μM $AcPyAD^+$, 300 μM NADH, and 10 μM $NADP^+$.

and the concentration of the other component was increased. As was found for the *R. rubrum* proteins (*13*), (a) the maximum rate of the reverse reaction (Figure 3A) was much greater when calculated per domain I (with excess domain III), than when calculated per domain III (with excess domain I), and (b) considerably higher concentrations of domain III were required to produce the maximal rate at fixed domain I, than were domain I concentrations at fixed domain III. Generally, however, substantially higher concentrations of the *E. coli* proteins were required to achieve maximal rates of the reverse reaction than was the case with the *R. rubrum*

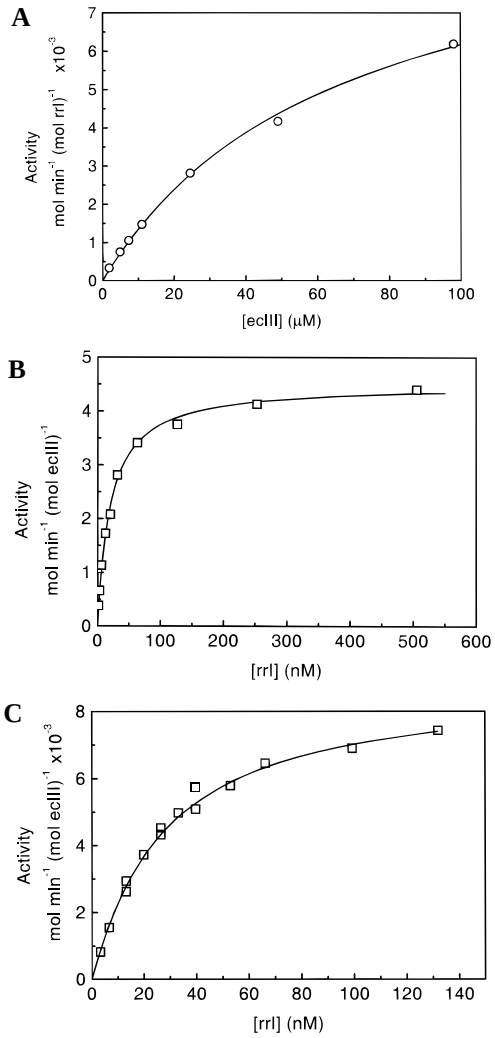

FIGURE 4: Protein−protein titrations of rrI+ecIII mixtures for reverse transhydrogenation (A and B) and for cyclic activities (C). The experiments were performed in dIdIII assay buffer (Experimental Procedures), pH 7.0 (A, B) and pH 6.0 (C). In (A), the concentration of ecIII was variable, and rrI was fixed at 8.8 nM; in (B), the concentration of rrI was variable, and ecIII was fixed at 4.9 μM. The concentrations of AcPyAD$^+$ and NADPH were 500 and 200 μM, respectively, in both the (A) and (B) experiments. For the cyclic reaction (C), the concentration of ecIII was 6.6 nM. Substrate concentrations were 200 μM AcPyAD$^+$, 100 μM NADH, and 10 μM NADP$^+$.

system. Furthermore, in marked contrast to the results of the *R. rubrum* experiments, very high concentrations of the *E. coli* protein were required to give maximal rates of cyclic transhydrogenation (Figure 3B), and those rates were low and of the same order as those of the reverse reaction.

A hybrid mixture of domain I from *R. rubrum* and domain III from *E. coli* (rrI+ecIII) catalyzed very high rates of transhydrogenation as shown in Figure 4. The properties (a) and (b) of the reverse reaction (see above), which are common to both rrI+rrIII and ecI+ecIII, were again evident in the hybrid system, although the ratio between the maximum rate calculated per domain I (with excess domain III) and the maximum rate expressed per domain III (excess domain I) (Figure 4A,B) was even greater than that described for rrI+rrIII (*13*). The protein−protein titration of the cyclic reaction catalyzed by rrI+ecIII (Figure 4C) revealed two features in common with rrI+rrIII (*13*), and different from ecI+ecIII (Figure 3B). Notably, first, much lower concentrations of protein were sufficient to achieve the maximal rate, and, second, the rate (calculated per domain III with excess domain I) was much greater than the equivalent maximal rate of reverse transhydrogenation.

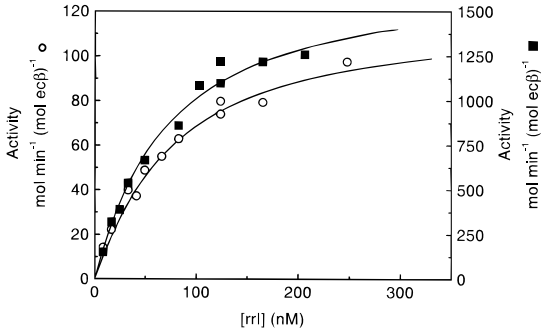

FIGURE 5: Protein−protein titrations of rrI+ecβ mixtures for reverse transhydrogenation and for cyclic activities. The experiments were carried out in dIdIII assay buffer (Experimental Procedures) to which 0.01% Brij 35 detergent had been added. For the reverse reaction (open circles, left *y*-axis), the ecβ concentration was 37 nM, and the AcPyAD$^+$ and NADPH concentrations were both 200 μM. For the cyclic reaction (solid squares, right *y*-axis), the ecβ concentration was 18.6 nM, and the substrate concentrations were 200 μM AcPyAD$^+$, 100 μM NADH, and 200 μM NADPH.

In sharp contrast to these results, mixtures of *E. coli* domain I (up to 23 μM) plus *R. rubrum* domain III (up to 3 μM) did not catalyze detectable transhydrogenation (not shown). This was true of the domain I protein isolated and purified either as described by Diggle et al. (1996) (*10*) or as described by Fjellström et al. (1997) (*9*). Cyclic transhydrogenation by a mixture containing 1.2 μM *E. coli* domain I plus 7.4 μM *E. coli* domain III was unaffected by the presence of 4.7 μM *R. rubrum* domain III (not shown). This suggests that the binding affinity between ecI and rrIII is lower than that between ecI and ecIII.

Reconstitution of R. rubrum Domain I and a Preparation of E. coli Transhydrogenase Lacking the α*-Subunit.* It was found that prolonged incubation of the complete, detergent-dispersed transhydrogenase from *E. coli* at 0 °C led to complete loss of activity of the protein, with respect to both the reverse and the cyclic reactions. Examination of the protein by SDS−PAGE (Figure 2) revealed that the procedure led to selective degradation of the α-subunit, probably through the action of low concentrations of contaminating proteases in the preparation. Indeed, a similar degradation pattern was observed for trypsin-treated *E. coli* transhydrogenase (not shown). Furthermore, the α-deficient preparation (denoted ecβ) presumably did not contain any bound dinucleotides since none was detected in the intact purified native enzyme.

The transhydrogenation activity of ecβ was partly restored by the addition of *R. rubrum* domain I. Low concentrations of rrI were effective in producing maximum rates of the reverse and cyclic reactions as shown in Figure 5. The rate of reverse transhydrogenation (expressed per mole of ecβ), though somewhat lower than that obtained with the complete, detergent-dispersed enzyme, was considerably higher than the maximal rates reached with any of the domain I+III mixtures (see above), suggesting that the rate of product NADP$^+$ release from ecβ was faster than that from domain III (see Discussion). The maximal rate of the cyclic reaction catalyzed by rrI+ecβ was of the same order as that obtained with rrI+rrIII.

Table 1: Deuterium Isotope Effects on the Cyclic Reaction Catalyzed by rrI+ecIII Mixtures[a]

[rrI] (nM)	[ecIII] (nM)	activity [mol min^{-1} (mol of dI/dIII)$^{-1}$] NADH	NADD	ratio V_{NADH}/V_{NADD}
200	6	2555	1005	2.6
6	490	1942	727	2.7
20	6	740	276	2.7

[a] The activity values listed are specific activities for the domain present in lower concentration. The nondeuterated substrate (NADH) and the deuterated substrate (NADD) were synthesized in parallel as described under Experimental Procedures. The assay medium contained 20 mM each of Mes, Mops, Tris, Ches, and 50 mM NaCl; the pH was 7.0. The temperature was 25 °C. The dinucleotide concentrations were: $NADP^+$, 10 μM; $AcPyAD^+$, 200 μM; NADH, 100 μM.

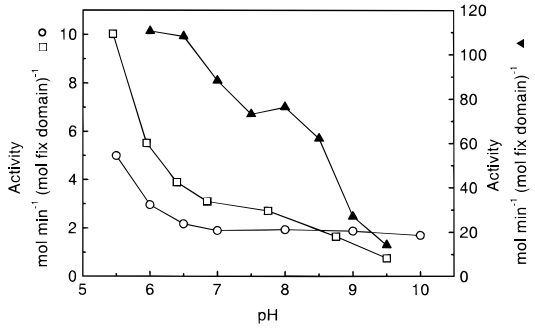

FIGURE 6: pH dependences of reverse transhydrogenation catalyzed by different dI+dIII/ecβ mixtures. Mixtures were ecI+ecIII (open squares), rrI+ecIII (open circles), and rrI+ecβ (filled triangles). Except for the addition of 0.01% Brij 35 detergent in rrI+ecβ mixtures, the dIdIII assay buffer (Experimental Procedures) was used in all experiments. For ecI+ecIII (open squares), the concentrations of ecI and ecIII were 7.69 and 1.96 μM, respectively, and the concentrations of $AcPyAD^+$ and NADPH were 2 mM and 200 μM, respectively. For rrI+ecIII (open circles), the concentrations of rrI and ecIII were 0.16 and 2.45 μM, respectively, and the concentrations of $AcPyAD^+$ and NADPH were 500 and 200 μM, respectively. For rrI+ecβ (filled triangles), the concentrations of rrI and ecβ were 21 and 418 nM, respectively, and the concentrations of $AcPyAD^+$ and NADPH were both 200 μM.

Neither reverse nor cyclic transhydrogenation activity could be detected in mixtures of ecβ (up to 0.5 μM) with ecI (up to 8 μM) (not shown).

Kinetic Isotope Effects. To determine if hydride transfer contributed to the rate-determining step, the cyclic reaction rates were compared using nondeuterated NADH and NADH deuterated in the 4A position (NADD). In rrI+ecIII mixtures, a clear isotope effect was demonstrated, and the effects were similar regardless of whether rrI or ecIII was present in excess, as shown in Table 1. Furthermore, when ecIII was mixed with nonsaturating concentrations of rrI, the isotope effect was maintained at the same level. These results indicate that hydride transfer is indeed significantly rate limiting in the cyclic reaction, as was concluded for the intact solubilized *E. coli* enzyme at pH 6.0 (*20*). In contrast, a kinetic isotope effect is only observed during reverse transhydrogenation in the pre-steady-state prior to rate limitation by $NADP^+$ release (*24*). Mixtures of rrI+rrIII and of ecI+ecIII displayed similar isotope effects during the cyclic reaction (not shown).

pH Dependences of Reactions Catalyzed by Homologous and Hybrid Complexes of Transhydrogenase Proteins. Figure 6 summarizes the results of experiments to measure pH dependences of reverse transhydrogenation in various native and hybrid systems. As was described in the case of rrI+rrIII (*25*), for all protein combinations that gave rise to active complexes, notably ecI+ecIII, rrI+ecIII, and rrI+ecβ, the pH dependences of the reverse reaction were much flatter than those described for complete transhydrogenases; the bell-shaped profiles associated with the complete enzymes were essentially lost. Indeed, for all the active dI+dIII mixtures, the pH dependences were remarkably similar. Thus, decreasing the pH from 9.0 to 6.5 typically led to small increases in rate, with a further decrease in pH having a more pronounced effect.

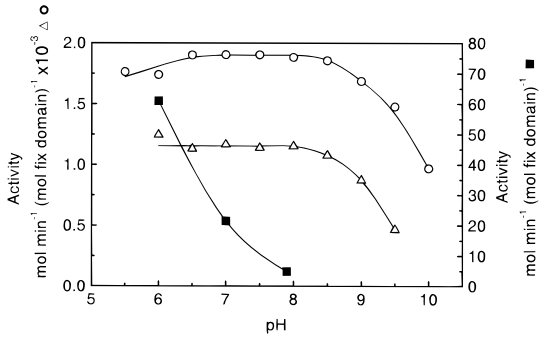

FIGURE 7: pH dependences of cyclic transhydrogenation catalyzed by various dI+dIII mixtures. Mixtures were ecI+ecIII (filled squares), rrI+ecIII (open circles), and rrI+ecβ (open triangles). Buffer conditions were the same as described in Figure 6. The values for ecI+ecIII (filled squares) are estimated maximal rates from Figure 3. For rrI+ecIII (open circles), the concentrations of rrI and ecIII were 3.35 nM and 5.36 μM, respectively; the concentrations of $AcPyAD^+$, NADH, and $NADP^+$ were 200, 100, and 10 μM, respectively. For rrI+ecβ mixtures (open triangles), the concentrations of rrI and ecβ were 20.6 nM and 418 nM, respectively; substrate concentrations were the same as described in Figure 5.

At neutral pH, the rate of reverse transhydrogenation by dI + dIII mixtures is almost certainly limited by slow release of $NADP^+$ from domain III (*9*, *13*). The decrease in the tryptophan fluorescence upon addition of NADPH to $NADP^+$-loaded ecIII reflects the kinetics of $NADP^+$ release (*9*). Using this procedure (*9*), it was found in the present study that the rate of release of $NADP^+$ from ecIII decreased with increasing pH in a way that approximately paralleled the effect of pH on the rate of reverse transhydrogenation catalyzed by ecI+ecIII and by rrI+ecIII (not shown).

For the cyclic reaction a different picture of the pH dependence emerges, as presented in Figure 7. It should be recalled that for *R. rubrum* transhydrogenase, the pH dependence of the dI+dIII-catalyzed reaction was very similar to that found for the complete enzyme (*25*). In both systems, the rate of the reaction was quite constant from pH 6.0 to 8.2, and thereafter declined with an apparent pK_a of approximately 9.1. In the same way, the pH dependence of the ecI+ecIII-catalyzed cyclic reaction (Figure 7) was also very similar to that found for the complete *E. coli* enzyme [see (*7*)]. Note that in the *E. coli* system, the reaction was attenuated with an apparent pK_a of less than 6.5, some 2−3 units lower than that observed in the *R. rubrum* system. Figure 7 also shows that the cyclic reactions catalyzed by rrI+ecIII and rrI+ecβ mixtures both had pH dependences which were similar to that of rrI+rrIII [see (*25*)], but were distinctly different from that of ecI+ecIII (see Figure 7). Thus, the apparent pK_a was in the region of 9.3.

DISCUSSION

The Tripartite Structure of Transhydrogenase; Catalysis by Mixtures of Recombinant Domains I and III. In clear contrast with other proteins associated with the respiratory and the photosynthetic electron-transport chains of mitochondria and bacteria, the gross structure of transhydrogenase has not been subjected to evolutionary change. Although some domain shuffling is observed (*26*), the tripartite arrangement has persisted in all known enzymes. The observations described here and elsewhere (*14*), that hybrid mixtures of the two peripheral subunits/domains of transhydrogenases from *R. rubrum*, *E. coli*, and *B. taurus* are catalytically competent, further indicate that the formation of active complexes is highly tolerant of substantial changes in the primary sequences. This is all the more remarkable in view of the fact that hydride transfer between nucleotides is direct (*16*). The latter observation indicates that the nicotinamide rings must approach one another within 2−3 Å, and that the domain I and III proteins must undergo substantial interdigitation during catalysis (*16, 24*). Either the regions of protein−protein interaction between the two domains must be highly conserved, or they must be flexible enough to accommodate amino acid substitutions.

Protein−Protein Titrations. In this report we have investigated the dependence of the rates of reverse and cyclic transhydrogenation on the concentration of recombinant domain I and III proteins from *E. coli* and *R. rubrum*. In addition to yielding information about the interactive properties between various dI+dIII/ecβ combinations (see below), the protein−protein titrations provide the basis for the studies on pH dependences and give indirect information on the role of dII in the intact enzyme.

The dissociation constants of the domain I·III complexes of transhydrogenase (dI·dIII $\rightleftharpoons$ dI + dIII) have not yet been directly determined. The situation might be complicated, not least because domain I is dimeric (*9, 12, 13, 17*) and domain III is possibly monomeric (*9, 12, 13*), and because it is conceivable that the dissociation constants of the complexes are influenced by the occupancy of the nucleotide-binding sites. Evidently, the titrations cannot be regarded as true binding curves, neither for the reverse nor for the cyclic reaction. Nevertheless, useful interpretations may be drawn from measurements of the rates of the reverse and cyclic transhydrogenation reactions during protein−protein titrations.

Cyclic Transhydrogenation Catalyzed by dI+dIII/ecβ Mixtures. Stopped-flow (*16*) and NMR experiments (*27*) indicate that AcPyAD(H) and NAD(H) binding and release to and from isolated domain I are relatively fast. If the same applies to the domain I·III complex, then the rate of the cyclic reaction can, in principle, approach the rate of the slowest hydride transfer step (either NADPH → AcPyAD$^+$ or NADH → NADP$^+$). However, the situation is complicated by the process of substrate inhibition (*20*). If the rate-determining steps remain unaltered, then titrations of the cyclic reaction, in which the concentration of one domain is held constant while the concentration of the other is varied, can be treated as protein-binding curves (*13*). The kinetic isotope effects (Table 1) do suggest that hydride transfer remains at least partly rate limiting independently of the protein concentrations of dI and dIII. Thus, if similar reaction conditions are used, comparisons of the relative apparent binding affinities between various dI+dIII combinations can be made.

Reverse Transhydrogenation Catalyzed by dI+dIII/ecβ Mixtures. The essential characteristics of the dependence of the rate of reverse transhydrogenation on protein concentration in dI+dIII titrations can be understood if (a) the rate of association/dissociation of the complex, (b) the rate of hydride transfer, and (c) the rate of AcPyAD(H) binding to, and release from, domain I are all fast relative to the very slow rate of release of product NADP$^+$ from domain III [$k_{\mathrm{off}(R.\ rubrum)} \approx k_{\mathrm{off}(E.\ coli)} \approx 0.03\ s^{-1}$ (*9, 13*)]. The important feature is that, during the time taken for a domain III protein to release its NADP$^+$, a domain I protein can "visit" many molecules of domain III (some of which will carry NADPH) and catalyze hydride transfer from bound NADPH to bound AcPyAD$^+$. Clearly, for the reverse reaction, the protein−protein titrations for the different complexes do not reflect relative affinities between the domains, but are dependent on the relative rates of various steps in the reaction scheme.

Catalytic Properties of Native and Hybrid Complexes of Domains I and III of Transhydrogenase. The mixture of recombinant ecI+ecIII proteins is a poor transhydrogenase. Although the essential features described above can be recognized in Figure 3, rather high concentrations of protein are required to observe maximal rates of reaction and those rates (notably that of the cyclic reaction) are very low. It is concluded that the dissociation constant of the complex is rather high, and that hydride transfer within the complex takes place only at a low rate.

In contrast, the mixture of rrI+ecIII is a very good catalyst. The protein−protein titrations closely resemble those of rrI+rrIII for the cyclic reaction, indicating a similar hydride transfer rate in both complexes. However, the relatively high reverse rates catalyzed by rrI+ecIII, observed in titrations with fixed rrI and variable ecIII, compared to those for rrI+rrIII (*13*), suggest a shorter lifetime of the former complex.

The mixture of rrI+ecβ provides an intermediate system between the complete enzymes and the reconstituted dI+dIII system, and it allows further analysis of the role of dII. It should be stressed, however, that it is presently unknown if the four predicted transmembrane helices in the α-subunit are intact and still associated with the dII part of the β-subunit in the ecβ preparation. The idea that the low reverse transhydrogenation activities observed both for rrIII and for ecIII with excess rrI are consequences of the lack of dII [this study and (*9, 12, 14*)] is supported by the observation that rrI+ecβ mixtures catalyzed this reaction about 40 times faster than any dI+dIII combination tested so far (*9, 12*−*14*). However, that rate was still about 10−20 times lower than those observed in the intact enzymes. These observations further support the view that the properties of the NADP(H) site are strongly influenced by the transmembrane domain structure in the intact enzyme. The βH91K mutant was previously shown to contain tightly bound NADP(H) (*8*), and it is possible that the predominating conformation of the nucleotide-binding site in this mutant is similar to that in isolated dIII.

On the other hand, the dependence of the rate of the cyclic reaction on the concentration of rrI at fixed ecβ was similar to corresponding dI+dIII titrations. Furthermore, the maximal rates were similar to those observed for the intact *E. coli*

and *R. rubrum* enzymes which suggests that the presence of dII has only a small effect on the hydride transfer rate.

Apparent Affinities between Various Domains. With the limitations specified above, the protein concentrations required to achieve half-maximal rates of the cyclic reaction may be interpreted as a semiquantitative measure of the relative abilities of the various domains to form active dI·dIII complexes. These apparent affinities can be listed as follows (from higher to lower apparent affinities): rrI+rrIII ≈ rrI+ecIII ≥ rrI+ecβ ≫ ecI+ecIII. Note that neither ecI+rrIII nor ecI+ecβ displayed any significant activities. That ecI has low and rrI has high apparent affinities for dIII/ecβ proteins might be a consequence of the different subunit arrangements in the *E. coli* and *R. rubrum* enzymes (*4*).

pH Dependences of Reactions Catalyzed by dI + dIII/ecβ Mixtures. The pH dependences of the component reactions of a proton pump take on a special significance in the context of the translocation mechanism; they can help us to identify the steps involved in proton binding and proton release. Detailed studies on the effect of pH on both the intact *R. rubrum* (*25*) and *E. coli* enzymes (*7*, *25*), the *E. coli* βH91E/K mutant (Hu et al., unpublished data), and the rrI + rrIII system (*25*) have been performed. It was suggested that the translocation of protons through the intact enzyme might be responsible for the bell-shaped profile of the forward and reverse reactions. In both *E. coli* and *R. rubrum* transhydrogenases, the rate of the cyclic reaction was attenuated with increasing pH, but the apparent pK_a values of the two proteins differed by 2−3 pH units. They were about 6.5 in *E. coli* (*25*) and about 9.1 in *R. rubrum* (*25*). We now have the opportunity to make comparisons of pH dependences in hybrid complexes of the nucleotide-binding domains from different species. The objective is to identify protolytic reactions that are conserved among all species, and which might, therefore, be central to the mechanism of transhydrogenation, and those which are species-specific.

(i) Cyclic Reaction. From an analysis of the pH dependence of the cyclic reaction catalyzed by ecI+ecIII mixtures, it was noted that the degree of interaction between the two domains seemed to be unaltered in the pH range 6.0−7.9, whereas the maximal rates decreased with increasing pH (Figure 3B). Even though the rates were between 2 and 3 orders of magnitude slower in the ecI·ecIII complex relative to those in the intact enzyme, the pH dependences were remarkably similar. Comparable observations were made previously for the I·III complex and complete enzyme from *R. rubrum* (*25*). It is possible, therefore, that the same functional group(s) on the dI·dIII complex and on the complete enzymes control(s) the cyclic reaction rate. Furthermore, the pH dependence of the cyclic reaction catalyzed by rrI+ecIII mixtures was very similar to that for rrI+rrIII mixtures (Figure 7), and the magnitudes of the rates were similar. Therefore, ecIII does not seem to possess protonatable groups that affect the hydride transfer rates. It is suggested that, in fact, dI carries the acid/base group(s) which determine(s) the pH profiles for the cyclic reaction, and that these groups have different pK_a values in ecI and rrI.

For both the *R. rubrum* and *E. coli* systems, the pH dependence of the rate of the cyclic reaction catalyzed by the dI·dIII complex resembled that of the complete enzyme, suggesting that domain II does not contribute to those pH dependences [see above and (*25*)]. This is supported by the finding that the pH dependence of the cyclic reaction catalyzed by rrI+ecβ (where at least part of domain II is present) displayed a similar pH profile to that of rrI+ecIII (Figure 7). Since the apparent pK_a for the *R. rubrum* transhydrogenase is approximately 9.1, it is considered to be unlikely that the protonation events determining this pH behavior involve vectorial protons; those protons could not be released at a significant rate under physiological conditions. Therefore, the alternative suggestion, that the groups react with scalar protons, is favored. However, the possibility cannot be entirely excluded that the groups responsible for the pH dependence in the intact *E. coli* enzyme are associated with vectorially derived protons.

(ii) Reverse Reaction. The pH dependences of the reverse reaction catalyzed by the dI+dIII mixtures studied in this work (ecI+ecIII and rrI+ecIII) and elsewhere (rrI+rrIII) (*25*) were all very similar. In marked contrast to the pH dependences of the complete *E. coli* and *R. rubrum* transhydrogenases, bell-shaped pH profiles were not observed in any of the dI+dIII or dI+ecβ mixtures. Since the ecβ preparation contains at least the dII portion present in the β-subunit of *E. coli* transhydrogenase, it was interesting to note that the pH profile for rrI+ecβ was not the same as for dI+dIII mixtures; still, however, a bell-shaped curve was not evident. These results support the suggestion that the pH profile in the complete enzyme is affected by dII.

Furthermore, the rrI + ecβ mixture exhibited a $K_{m(NADPH)}$ for the reverse reaction of 15 μM (measured at pH 7.0 with excess rrI, data not shown), the same value as that found in the wild-type *E. coli* enzyme, indicating similar affinities for NADPH. This is in stark contrast to the affinity in the nanomolar range observed for ecIII (*9*) and supports the proposition of an interaction between the NADP(H)-binding site and dII, which is of crucial importance in regulating the affinity of dIII for its dinucleotide substrate.

In conclusion, rrI readily combines with ecIII and ecβ to form complexes that catalyze high rates of transhydrogenation, comparable to those observed in the intact *R. rubrum* and *E. coli* enzymes, whereas combinations involving ecI are not good catalysts. The cyclic reaction is significantly limited by the transfer of hydride equivalents. Protonation events in dI seem to determine the pH dependences of the cyclic reaction both in the dI·dIII complexes and in the wild-type counterparts. Domain II has a profound influence on the binding affinity of dIII for NADP(H), the release rates of the substrate, and the pH dependences for the reverse reaction, whereas it has a limited effect on transfer rates of hydride equivalents.

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