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A catalytically active complex formed from the recombinant dI protein of *Rhodospirillum rubrum* transhydrogenase, and the recombinant dIII protein of the human enzyme

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Abstract

Transhydrogenase is a proton pump. It has three components: dI and dIII protrude from the membrane and contain the binding sites for NAD(H) and NADP(H), respectively, and dII spans the membrane. We have expressed dIII from *Homo sapiens* transhydrogenase (*hs*dIII) in *Escherichia coli*. The purified protein was associated with stoichiometric amounts of NADP(H) bound to the catalytic site. The $NADP^+$ and NADPH were released only slowly from the protein, supporting the suggestion that nucleotide-binding by dIII is regulated by the membrane-spanning dII. *Hs*dIII formed a catalytically active complex with recombinant dI from *Rhodospirillum rubrum* (*rr*dI), even in the absence of dII. The rates of forward and reverse transhydrogenation catalysed by this complex are probably limited by slow release from dIII of NADPH and $NADP^+$, respectively. The hybrid complex also catalysed high rates of 'cyclic' transhydrogenation, indicating that hydride transfer, and exchange of nucleotides with dI, are rapid. Stopped-flow experiments revealed a rapid, monoexponential, single-turnover burst of reverse transhydrogenation in pre-steady-state. The apparent first-order rate constant of the burst increased with the concentration of *rr*dI. A deuterium isotope effect ($k_H/k_D \approx 2$ at 27°C) was observed when [4B-^{1}H]NADPH was replaced with [4B-^{2}H]NADPH. The characteristics of the burst of transhydrogenation with *rr*dI:*hs*dIII differed from those previously reported for *rr*dI:*rr*dIII (J.D. Venning et al., Eur. J. Biochem. 257 (1998) 202–209), but the differences are readily explained by a greater dissociation constant of the hybrid complex. The steady-state rate of reverse transhydrogenation by the *rr*dI:*hs*dIII complex was almost independent of pH, but there was a single apparent pK_a ($\sim$9.1) associated with the cyclic reaction. The reactions of the dI:dIII complex probably proceed independently of those protonation/deprotonation reactions which, in the complete enzyme, are associated with H^+ translocation.

Keywords: Transhydrogenase; Proton translocation; Membrane protein; Nucleotide-binding site; Human heart; (*Rhodospirillum rubrum*)

1. Introduction

Nicotinamide nucleotide transhydrogenase couples the transfer of hydride ion equivalents between the 4*A*-position of NAD(H) and the 4*B*-position of NADP(H) to the translocation of protons across a membrane:

Abbreviations: $AcPdAD^+$, acetyl pyridine adenine dinucleotide (oxidised form); *rr*dI, the NAD(H)-binding component of transhydrogenase from *Rhodospirillum rubrum*; *hs*dIII, the NADP(H)-binding component of transhydrogenase from *Homo sapiens*, etc.

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$$NADH + NADP^+ + nH^+_{OUT} \Leftrightarrow$$

$$NAD^+ + NADPH + nH^+_{IN}, \quad (1)$$

where *n* is probably 1 [1]. For recent reviews, see [2–4].

The enzyme is found in the inner membranes of animal mitochondria, and in the cytoplasmic membranes of many bacteria. It probably has a role in the production of NADPH for biosynthesis, and for glutathione reduction [5]. In animals, together with the NADP- and NAD-dependent isocitrate dehydrogenases, transhydrogenase might also be involved in the fine regulation of the tricarboxylic acid cycle [6].

About 20 transhydrogenases have been identified in available genome sequences. The organisation of all these enzymes is similar, although some domain shuffling has occurred. The basic structure is tripartite: the enzyme comprises two relatively hydrophilic components (dI and dIII), which protrude from the membrane, and a membrane-spanning component (dII). The dI and dIII components contain the binding sites for NAD(H) and NADP(H), respectively. On the basis of homology modelling with the crystal coordinates of alanine dehydrogenase [7], dI has two domains, each of which has the open, twisted, βαβ structure of the Rossmann fold; domain I.2 binds the NAD(H), but domain dI.1 has no known function [8]. An emerging NMR structure reveals that dIII also has the Rossmann fold [9]. Since hydride transfer between the NAD(H) and NADP(H) is direct [10], the dI and dIII proteins must be arranged to permit close approach of the nicotinamide rings of the two nucleotides during catalysis.

Recombinant forms of dI and dIII from the transhydrogenases of *Rhodospirillum rubrum* [11–13], *Escherichia coli* [14,15], and *Bos taurus* [12] have been described. The isolated, recombinant dI proteins are dimeric. They bind NADH with moderately high affinity, and NAD^+, with somewhat lower affinity. There is probably one nucleotide per monomeric unit, at least in the bacterial enzymes [16]. Recombinant dIII proteins are isolated, apparently as monomers, and are associated with tightly bound $NADP^+$ and NADPH (one NADP(H) per monomer) [13,15]. The tightly bound nucleotides are located at the catalytic site [13].

Mixtures of recombinant dI and dIII proteins (either from the same or from different species) catalyse transhydrogenation, even in the absence of the membrane-spanning dII [12,13,15,17,18]. The most intensively studied dI:dIII complexes, those from *R. rubrum*, catalyse 'forward' and 'reverse' transhydrogenation (Eq. 1) at rates which are ~3 orders of magnitude slower than those observed with the intact enzyme [13]. This was attributed to the very slow release of the product nucleotide (NADPH and $NADP^+$, respectively) from the dIII protein in the absence of dII. The *R. rubrum* dI:dIII complexes also catalyse 'cyclic transhydrogenation' – the alternate reduction of protein-bound $NADP^+$ by NADH, and oxidation of protein-bound NADPH by the NAD^+ analogue, $AcPdAD^+$ [13,17,19]. This reaction takes place as rapidly in the recombinant *R. rubrum* protein complex as it does on the complete enzyme, thus supporting the idea that NADP(H) does indeed remain very tightly bound to dIII in the dI:dIII complex [13]. Furthermore, it indicates that the hydride transfer step of transhydrogenation on the complex proceeds very rapidly, and this was interpreted as evidence that the apparatus for hydride transfer resides entirely within dI and dIII; the membrane-spanning dII does not contribute to the hydride transfer step [13].

More recently it was shown that a mixture of recombinant dI and dIII from *E. coli* transhydrogenase also catalysed low rates of the forward and reverse reactions but, in contrast to the *R. rubrum* system, only low rates of the cyclic reaction [15]. Subsequent experiments [18] established that a mixture of *R. rubrum* dI and *E. coli* dIII, but not a mixture of *E. coli* dI and *R. rubrum* dIII, catalysed rapid rates of the cyclic reaction. Evidently, the recombinant *E. coli* dI is not catalytically effective, either with its homologous partner (in the absence of dII), or in the interspecies complex. On the other hand, *R. rubrum* dI is effective with both *R. rubrum* and *E. coli* dIII, though it appeared to bind more tightly to the former. A mixture of the native dI protein from *R. rubrum* and recombinant bovine dIII can also catalyse transhydrogenation, at faster rates than a mixture of bovine dI and bovine dIII, though fewer details are available [12].

The application of stopped-flow spectrophotometry to a study of hydride transfer by the dI:dIII complex of *R. rubrum* transhydrogenase was recently reported [10,20]. Before onset of the slow steady-

state reaction, a rapid, single-turnover burst was revealed. An analysis of the burst kinetics established that the transfer of hydride between NADP(H) and AcPdAD(H) does not involve any redox intermediates [10]. The single turnover burst is biphasic; the faster phase corresponds to hydride transfer within the complex, and the slower component corresponds to dissociation of the two subunits [20].

In this report, we describe the isolation and properties of recombinant dIII from human heart transhydrogenase (*hs*dIII). We find that, in mixtures with *R. rubrum* dI (*rr*dI), it catalyses slow forward and reverse transhydrogenation in the steady state, and a rapid cyclic reaction. Transient state experiments reveal a different behaviour to that observed in the homologous *R. rubrum* dI:dIII complex, but this can be readily explained within the framework of the existing model.

2. Methods

DNA coding for the dIII region of transhydrogenase from *H. sapiens* (*hs*dIII) was isolated on the basis of sequence information given [21], using the polymerase chain reaction with human heart cDNA as template. The 24-base sense primer was constructed from nucleotide residues 2769–2792, modified to include an *Nde*I site. This placed the start codon at Met^{837} (in the numbering system used for the complete enzyme). The 32-base anti-sense primer, constructed from nucleotide residues 3408–3441, was designed to incorporate the natural stop codon, and to include a *Bam*HI site. The PCR product was cloned into pCR-script using the pCR-script Amp SK(+) cloning kit. At this stage the DNA fragment corresponding to the shorter version of *hs*dIII (from Met^{860} to Lys^{1043}) was excised, using the naturally occurring *Nco*I site and the engineered *Bam*HI site, and cloned into pET21d. In order to clone the long form of *hs*dIII (from Met^{837} to Lys^{1043}), the internal, naturally occurring *Nde*I site was first removed using the Quik-change site-directed mutagenesis kit. The DNA coding for the long form of *hs*III was then excised using the engineered *Nde*I and *Bam*HI sites, and cloned into the expression vector pET11c. The two constructs (designated pSJP1 for the shorter form, and pSJP2 for the longer form) were separately transformed into *E. coli* BL21(DE3), and plasmid preparations from selected clones were sequenced using an Applied Biosystems 373A. Expression of the two forms of *hs*dIII was carried out essentially as described for the equivalent *R. rubrum* protein [13]. Similar chromatographic procedures were also employed in purification of *hs*dIII, although there were slight differences in the elution profiles. Purified protein was stored in 20% glycerol at −20°C. SDS-PAGE was carried out as described [22], and the NADP(H) content of the protein was determined as in [13]. Protein concentration was routinely measured by the microtannin assay [23] with bovine serum albumin as standard. Protein concentrations are expressed with respect to the monomeric units.

The dI protein from *R. rubrum* (*rr*dI) was expressed in *E. coli* from pCD1, recovered from cell-free extracts and purified by column chromatography, as described [11].

Absorbance spectra were measured on a Perkin Elmer Lambda 16 double-beam spectrophotometer at 25°C. Fluorescence spectroscopy was performed with a Spex Fluoromax at 25°C in a 1×1 cm cuvette.

Reverse and cyclic activities were measured spectrophotometrically by following the reduction of $AcPdAD^+$ at 375 nm using a Perkin Elmer Lambda 16 spectrophotometer at 30°C, and an absorbance coefficient of 6.10 mM^{-1} cm^{-1} [24]. Forward transhydrogenation rates were measured by following the reduction of thio-$NADP^+$ at 395 nm, using an absorbance coefficient of 11.3 mM^{-1} cm^{-1} [25]. Unless otherwise specified, the reaction buffer was 50 mM Mops-KOH, pH 7.2, 50 mM KCl, 2 mM $MgCl_2$. Prior to the experiments the dI and dIII proteins were dialysed for 20 h against 10 000 volumes of 20 mM Tris-HCl, pH 8.0, 10 mM $(NH_4)_2SO_4$ at 4°C.

Stopped-flow experiments were performed with an Applied Photophysics DX-17MV spectrophotometer in its absorbance mode (2 mm optical path length). The instrument dead time was 1.31 ms [10,20]. The monochromator-slit widths were set to 5 nm. Measurements were made during typically six successive experiments, recorded over a period of < 1 min, and each consuming 50 μl of the protein and substrate solutions. Rate constants and amplitudes were calculated from the raw data using the instrument software, making due correction for the apparatus dead time.

Prior to the experiments the *rr*dI and *hs*dIII proteins were washed repeatedly through centrifugal filters (Vivascience, 10 kDa cut off for dI protein, and 5 kDa for dIII) with 20 mM HEPES, pH 8.0, 10 mM $(NH_4)_2SO_4$. Bound $NADP^+$ on *hs*dIII was then replaced by incubating the protein (75 μM) in the above buffer supplemented with 105 μM NADPH at 4°C for 2 h [10].

[4B-^{2}H]NADPH, for use in the stopped-flow experiments was prepared and purified, as described [26,27]. Control measurements on the deuterium isotope effect were performed with [4B-^{1}H]NADPH prepared and purified in parallel [20].

Vent polymerase was obtained from New England Biolabs. Human heart cDNA, the pCR-script Amp SK(+) cloning kit and the Quik-change site-directed mutagenesis kit were from Stratagene. Restriction enzymes and T4 DNA ligase were from Boehringer Mannheim, and pET21d, pET11c and *E. coli* BL21(DE3) cells were from Novagen. Oligonucleotides were synthesised by Alta Bioscience, University of Birmingham. All nucleotides and enzymes used for biochemical assays were obtained from Sigma.

3. Results

3.1. Cloning and expression of the transhydrogenase-dIII protein from H. sapiens

The dIII protein isolated from bovine transhydrogenase [12] was approximately 20 residues shorter at the N-terminus than the dIII protein isolated from the *R. rubrum* enzyme [13]. In the current work, DNA segments coding for both a longer form (Met^{837}-Lys^{1043}), and a shorter form (Met^{903}-Lys^{1043}), of *hs*dIII were isolated, separately subcloned into expression vector and transformed into an *E. coli* host (see Section 2). Following induction of both strains with isopropyl-β-thio-galactoside, analysis by SDS-PAGE revealed that large amounts of recombinant protein were expressed. The proteins were readily purified using procedures developed for *rr*dIII [13]. Fig. 1 shows SDS-polyacrylamide gels of the purified products. As described for dIII proteins isolated from other sources [12,13,15], both the long and the short forms of *hs*dIII migrated with anomalously large M_r values, 28.5 and 27 kDa, compared

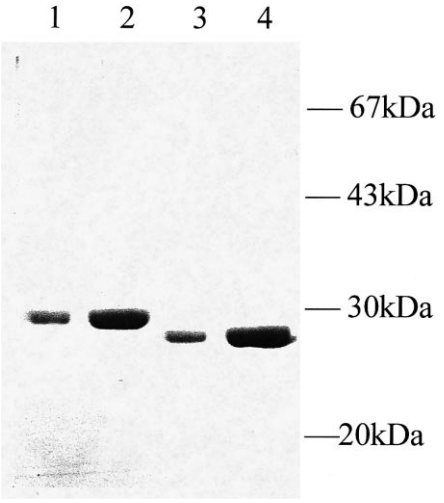


Fig. 1. SDS-PAGE of the two forms of *hs*dIII protein after purification by column chromatography. Lane 1, 0.5 μg *hs*dIII (long form); lane 2, 2.5 μg *hs*dIII (long form); lane 3, 0.5 μg *hs*dIII (short form); lane 4, 2.5 μg *hs*dIII (short form).

with the values predicted from the amino acid sequences of 22.3 and 20 kDa, respectively. All experiments described below were carried out with the short form of *hs*dIII. However, where parallel experiments were performed with the long form of the protein, the data obtained were similar. Thus far, we have been unable to ascribe a function to residues 837–860; they might simply serve as a linker between dII and dIII.

3.2. Bound nucleotides and the fluorescence spectra of hsdIII

As with the isolated dIII proteins from the transhydrogenases of *R. rubrum* [13] and *E. coli* [15], and probably that from *B. taurus* [12], the expressed and purified *hs*dIII protein was associated with tightly-bound $NADP^+$ (0.9 mol per mol protein) and NADPH (0.1 mol per mol protein). The presence of bound nucleotide was reflected in the shifted absorbance spectrum of the protein ($\lambda_{max} = 270$ nm, not shown), though there was insufficient NADPH to detect an absorbance peak at 340 nm.

The rate of dissociation of the bound $NADP^+$ was measured by reducing the nucleotide released from *hs*dIII protein with excess isocitrate and isocitrate dehydrogenase, and recording the fluorescence increase at approximately 450 nm [13]. The reaction was approximately first order ($k_{off} \approx 0.14$ s^{-1}),

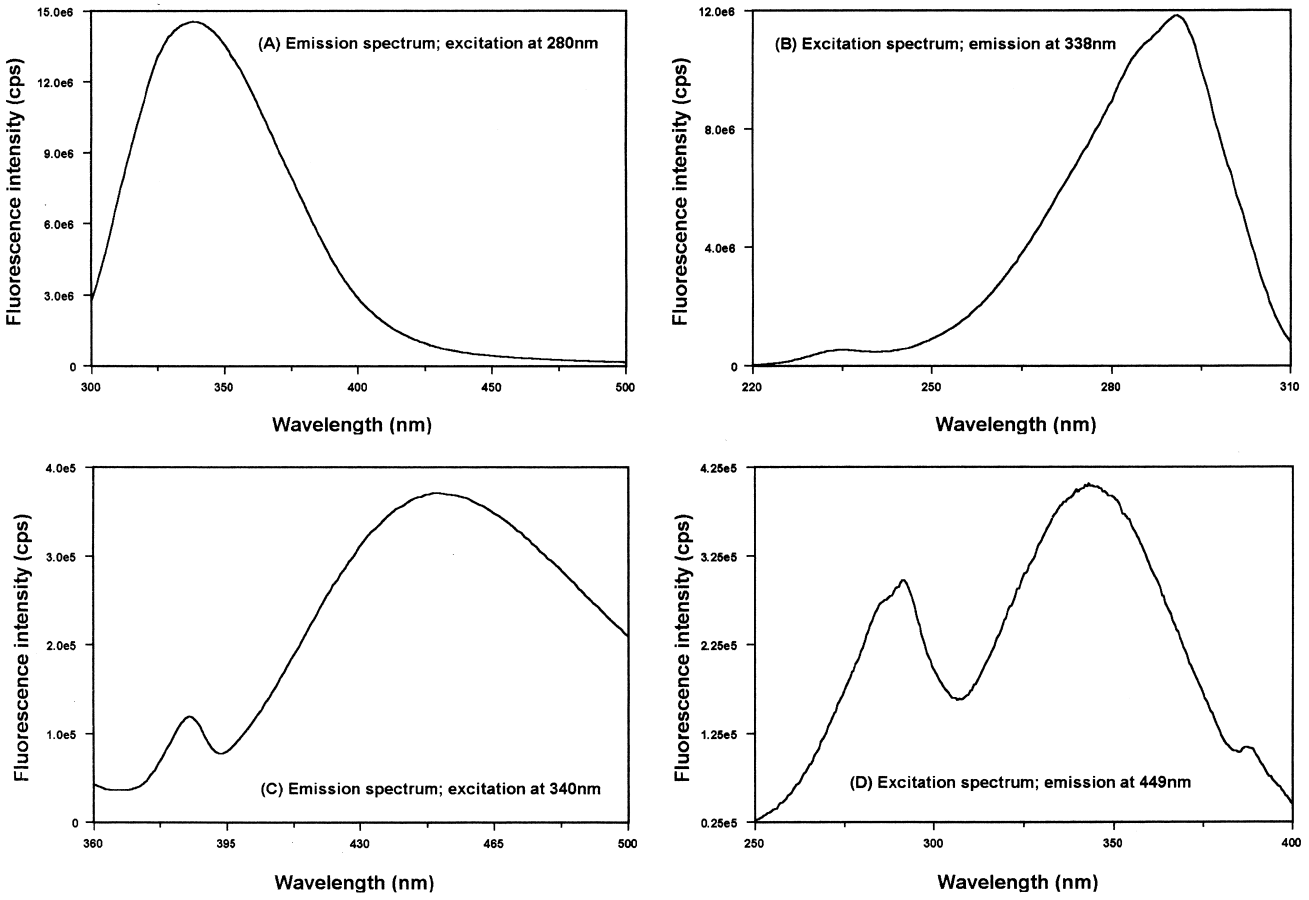


Fig. 2. Fluorescence spectra of purified *hs*dIII protein. The protein concentration was 17 μM. Note that the emission at approximately 385 nm in (C) is the water-Raman band.

though the rate constant was somewhat greater than that observed with *rr*dIII, and should be taken as a lower limit. The rate of dissociation of the bound NADPH was measured by oxidising the nucleotide released from *hs*dIII with excess glutathione and glutathione reductase, and recording the fluorescence decrease [13]. This reaction too was approximately first order, with $k_{off} \approx 0.008\ s^{-1}$.

The fluorescence emission spectrum of *hs*dIII protein, using 280 nm excitation, had a maximum at approximately 338 nm (Fig. 2A); the complementary excitation spectrum, for the emission band at 338 nm, peaked at 291 nm (Fig. 2B). These features can be attributed to the lone tryptophan residue at position 990 (numbering for complete transhydrogenase). The maximum of the emission band lies at a relatively long wavelength, indicating a quite polar environment for the residue; compare, for example, the short wavelength emission (310 nm) of the lone Trp in dI protein from *R. rubrum* transhydrogenase [11]. The bound NADPH on *hs*dIII (see above) was revealed as a fluorescence emission band centred at 449 nm, from excitation at 340 nm (Fig. 2C). Interestingly, the fluorescence excitation spectrum for the 449 nm emission (Fig. 2D), in addition to a maximum at 343 nm, shows a second peak at 290 nm. This is consistent with tryptophan excitation, and suggests that there is energy transfer between Trp^{990} and the bound nucleotide on dIII.

3.3. Steady state catalysis by a mixture of R. rubrum dI and H. sapiens dIII

Simple mixtures of *rr*dI and *hs*dIII were able to perform forward and reverse transhydrogenation. Fig. 3A,B show steady-state rates of reaction, at fixed *hs*dIII concentration, as a function of the *rr*dI concentration. The data closely resemble those for

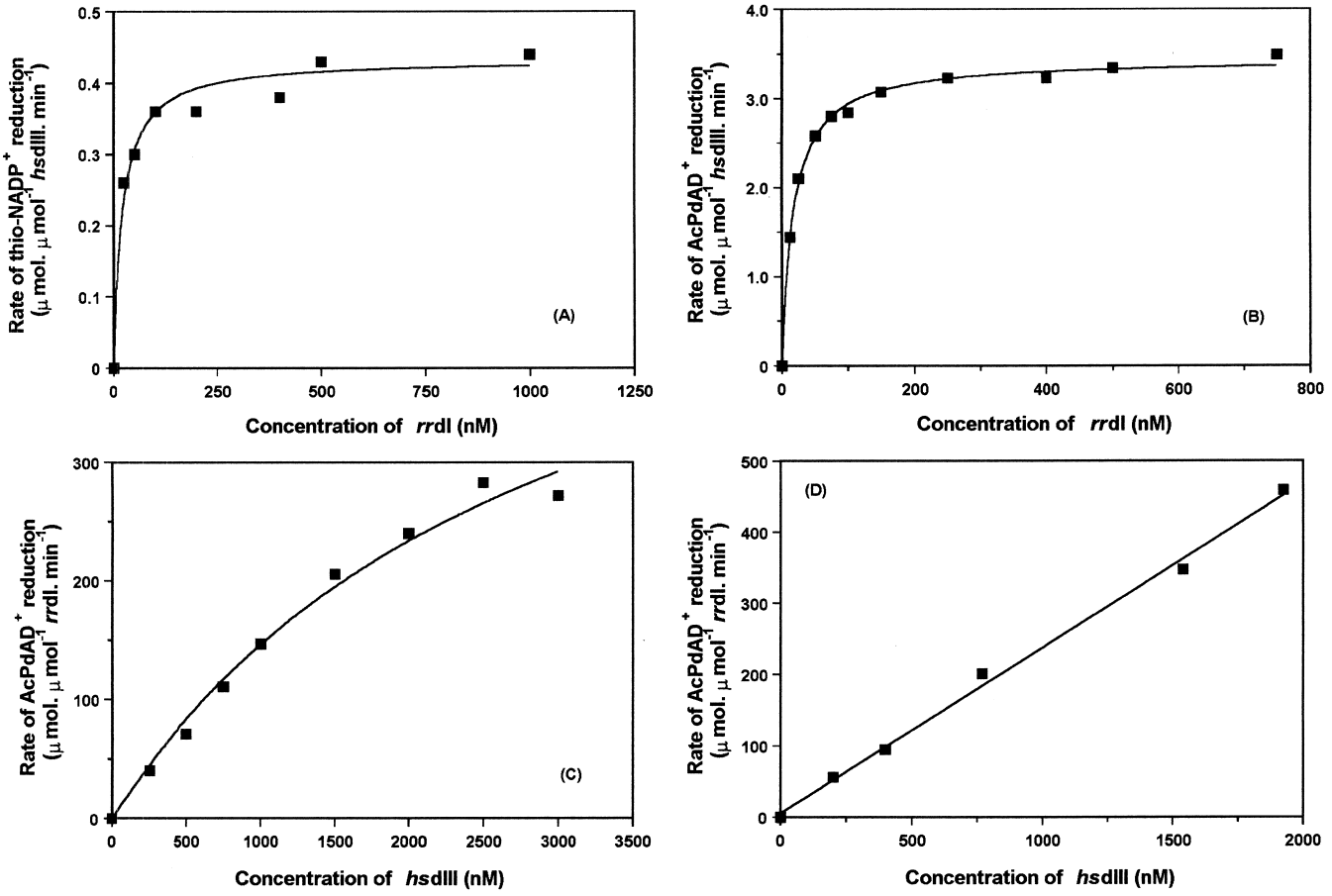


Fig. 3. (A) The rate of the forward reaction as a function of *hs*dIII concentration. The *hs*dIII concentration was 900 nM, the NADH concentration was 200 μM and the thio-NADP$^+$ concentration was 200 μM. (B) The rate of the reverse reaction as a function of *hs*dIII concentration. The *hs*dIII concentration was 830 nM, the NADPH concentration was 200 μM and the AcPdAD$^+$ concentration was 200 μM. (C) The rate of the reverse reaction as a function of *rr*dI concentration. The concentration of *rr*dI was 6.2 nM, the concentration of NADPH was 200 μM and the AcPdAD$^+$ concentration was 200 μM. (D) The rate of the cyclic reaction as a function of *rr*dI concentration. The *rr*dI concentration was 5 nM, the concentration of NADH was 200 μM, the concentration of AcPdAD$^+$ was 200 μM and the concentration of NADPH was 50 μM.

the *rr*dI:*rr*dIII complex [13], and for *rr*dI:*ec*dIII and *ec*dI:*ec*dIII [18]. The rates of reaction catalysed by the recombinant protein complexes, even at high concentrations of dI, are much slower than those measured in complete transhydrogenases, and are probably limited by the release of product NADP$^+$ or NADPH. Thus, the rates of forward (0.007 s^{-1}), and reverse (0.06 s^{-1}), transhydrogenation at elevated dI concentrations (Fig. 3) were in the same order as the apparent first-order rate constants for NADPH release, and for NADP$^+$ release, respectively, from isolated *hs*dIII (see above).

The steady-state rate of reverse transhydrogenation was also measured at a fixed concentration of *rr*dI and a variable concentration of *hs*dIII (Fig. 3C). A comparison of Fig. 3B and C reveals two notable features. (1) Much lower concentrations of *rr*dI (Fig. 3B) than of *hs*dIII (Fig. 3C) were required to give half maximal rates of reaction. (2) The rate of reaction (expressed per mol of *rr*dI) at high concentrations of *hs*dIII (Fig. 3C) was much greater than the rate (expressed per mol of *hs*dIII) in the presence of high concentrations of *rr*dI (Fig. 3B). Both features can be explained on the basis that *rr*dI can (a) exchange its AcPdADH for AcPdAD$^+$, (b) visit many *hs*dIII molecules, and (c) catalyse reaction, in the time taken for NADP$^+$ release. Thus, only a small amount of *rr*dI is required to achieve maximum turn-

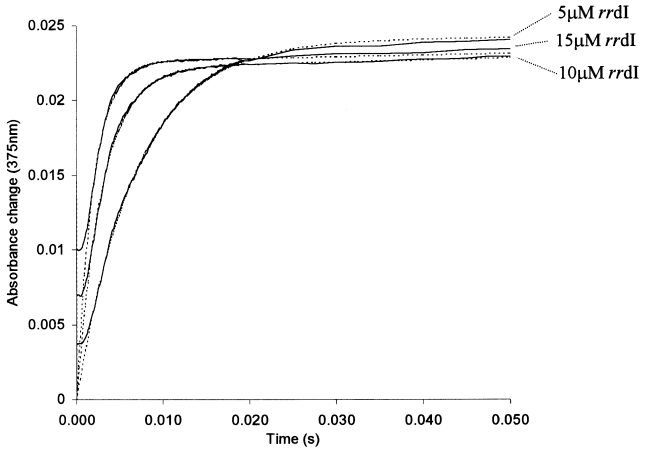


Fig. 4. The pre-steady-state kinetics of hydride transfer from NADPH to AcPdAD$^+$ catalysed by the *rr*dI:*hs*dIII complex. *Rr*dI (10, 20 or 30 μM) *plus hs*dIII (50 μM) and NADPH (70 μM) in 20 mM HEPES (pH 8.0), 10 mM $(NH_4)_2SO_4$ buffer were loaded into one drive syringe of the stopped-flow spectrophotometer. AcPdAD$^+$ (2 mM) in the same buffer was loaded into the other syringe. The traces shown are an average of six recordings of the absorbance change at 375 nm upon 1:1 mixing of the two solutions. The dashed lines show the computer-generated curve taken from data recorded approximately 3 ms after triggering, and extrapolated back to the true zero (see Section 2) using a single exponent.

over of *hs*dIII. A similar conclusion was drawn from the results of experiments with mixtures of *rr*dI *plus rr*dIII [13], and *rr*dI *plus ec*dIII [18].

Having established the catalytic competence of *rr*dI:*hs*dIII complexes, it was shown that the tightly-bound nucleotide on the dIII component (see above) is located at the catalytic site. Thus, upon addition of *rr*dI (2 μM) *plus* AcPdAD$^+$ to a solution of *hs*dIII (16 μM), the fluorescence emission band at 449 nm from the bound NADPH (Fig. 2C) was replaced by a band centred at 469 nm, due to the fluorescence of AcPdADH (data not shown).

Mixtures of *rr*dI *plus hs*dIII also catalysed the 'cyclic' reaction, the reduction of AcPdAD$^+$ by NADH in the presence of (bound) NADP$^+$/NADPH (Fig. 3D). The rates of reaction were rapid, whether expressed per dI protein at variable concentrations of dIII (Fig. 3D), or per dIII protein at variable dI (not shown). However, in marked contrast to experiments with the homologous *rr*dI *plus rr*dIII system [13], neither titration gave any indication of saturation. Experiments with *rr*dI *plus ec*dIII gave intermediate results [18]. It was suggested that, despite complexities in the cyclic reaction [28], protein-protein titrations of the cyclic reaction are an indication of the relative binding affinities between dI and dIII proteins [13,18]. On this basis, the value of the dissociation constant for the *rr*dIII:*hs*dIII complex is greater than that for *rr*dIII:*ec*dIII, which is greater than that for *rr*dIII:*rr*dIII. This is explored further in the following stopped-flow experiments.

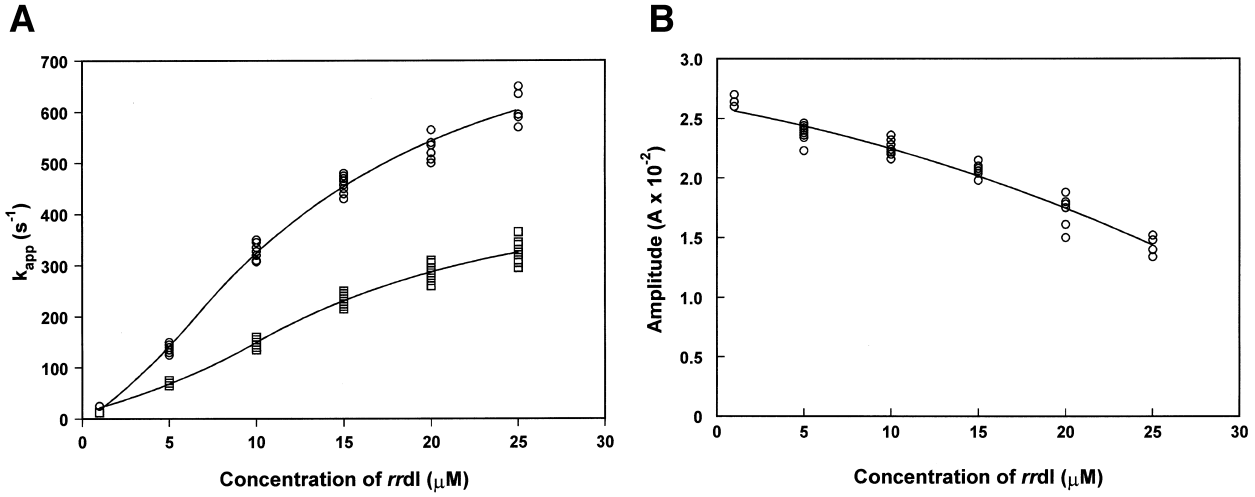


Fig. 5. The dependence of the apparent first order rate constant (A) and amplitude (B) of the pre-steady-state burst on *rr*dI concentration. The experiments were performed as described in Fig. 4. ○, with [4B-^{1}H]NADPH; □, with [4B-^{2}H]NADPH (see Section 2). Each symbol gives the amplitude or apparent first order rate constant from a single recording, as calculated by the instrument software.

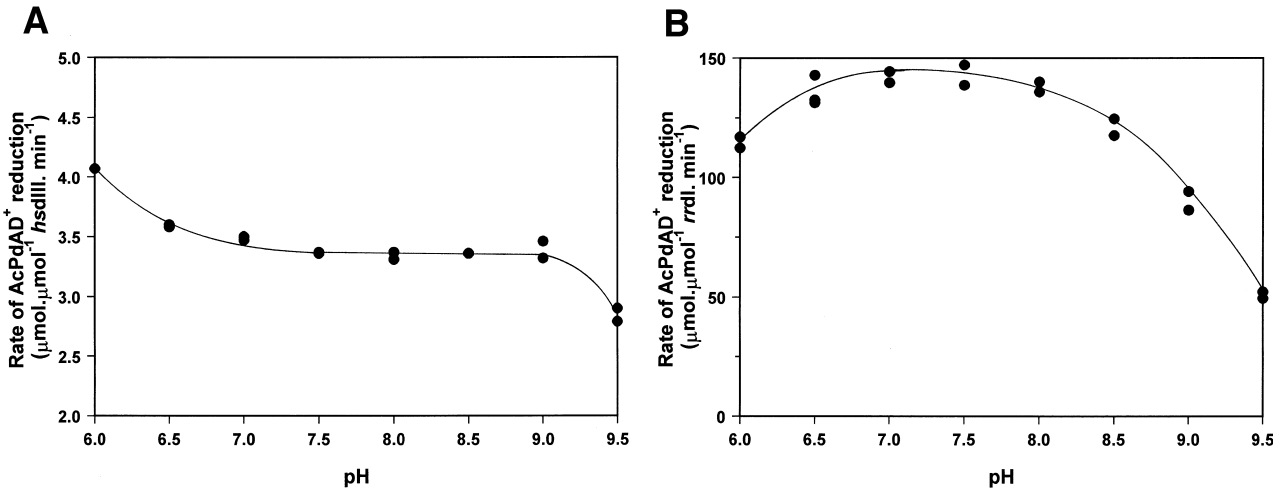


Fig. 6. (A) The pH dependence of the steady-state rate of reverse transhydrogenation catalysed by the *rr*dI:*hs*dIII complex. Experiments were performed with a mixture of *rr*dI (200 nM) *plus hs*dIII (830 nM), NADPH (200 μM) and AcPdAD$^+$ (200 μM) in a buffer containing 10 mM Mops, 10 mM Mes, 10 mM HEPES, 10 mM Tricine, 10 mM Ches, 50 mM KCl, 2 mM $MgCl_2$ (with pH adjusted using NaOH). (B) The pH dependence of the steady-state rate of the cyclic reaction catalysed by the *rr*dI:*hs*dIII complex. Conditions as in (A), except that the concentration of *rr*dI was 5 nM, the concentration of *hs*dIII was 750 nM, and the nucleotide concentrations were NADH, 200 μM, AcPdAD$^+$, 200 μM and NADPH, 50μM.

3.4. *Pre-steady-state kinetics of the reverse transhydrogenation reaction catalysed by rrdI:hsdIII*

In the experiment shown in Fig. 4, the first syringe of the stopped-flow spectrophotometer was filled with a solution of *rr*dI (at one of three different concentrations) *plus hs*dIII loaded with NADPH (see Section 2), and the other syringe with a solution of AcPdAD$^+$. Upon mixing the contents of the two syringes, a rapid burst of AcPdAD$^+$ reduction was observed. The amplitude of the of the burst was equivalent to the reduction of approximately 22 μM AcPdAD$^+$. Since the concentration of each protein in the mixing chamber was 25 μM, it is clear that the burst corresponds to a single turnover of dIII, before the reaction became limited in the steady-state by very slow release of NADP$^+$ ($k_{off} = 0.14$ s^{-1}, see above). Experiments with *rr*dI *plus rr*dIII have given similar results [10,20], but with the following important differences:

1. The burst of AcPdAD$^+$ reduction by *rr*dI *plus rr*dIII was distinctly biphasic [10,20]. In the case of *rr*dI *plus hs*dIII, the burst kinetics were monophasic (Fig. 4A).
2. When the concentration of *rr*dI was increased at fixed *rr*dIII, the slow phase was progressively replaced by the fast phase, but the total amplitude of the burst remained constant [20]. However, the burst observed with the mixture of *rr*dI *plus hs*dIII

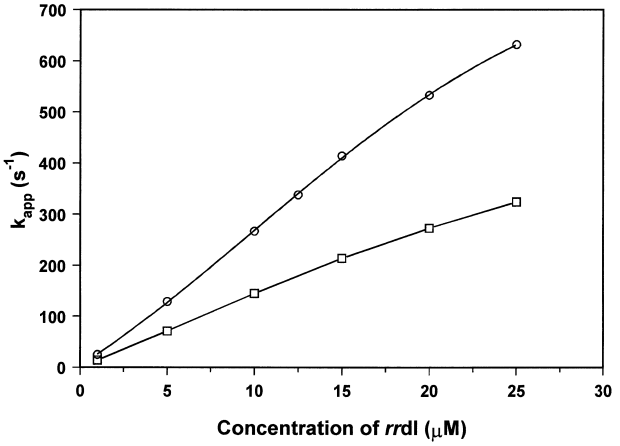


Fig. 7. Simulation of the pre-steady-state kinetics of hydride transfer from NADPH to AcPdAD$^+$ catalysed by the *rr*dI:*hs*dIII complex. Compare with the experimental data in Fig. 5A. The pre-steady-state burst kinetics were generated on the basis of the scheme shown in the text, by numerical integration, using the Euler procedure with a time increment of 0.1 μs. The concentration of *hs*dIII was 25 μM, and that of *rr*dI was as indicated; $k_1 = 10^9$ M^{-1} s^{-1}, $k_{-1} = 2500$, $k_2 = 900$ s^{-1} (for ○) or $k_2 = 450$ s^{-1} (for □). All simulated curves generated under these conditions were approximately first order; the first order rate constants were calculated from logarithmic plots.

was monophasic independently of the concentration of *rr*dI; the apparent first order rate constant increased with the *rr*dI concentration, approaching a value in excess of $\sim 700\ s^{-1}$, the upper limit of accuracy of the instrument (Fig. 5A). The amplitude of the burst observed with *rr*dI *plus hs*dIII declined at elevated concentrations of dI (Fig. 5B) but this is probably a consequence of large errors in extrapolation to zero time that are incurred at high rates of reaction (see Fig. 4).

3. In experiments with *rr*dI *plus rr*dIII, the use of [4B-^{2}H]NADPH in place of [4B-^{1}H]NADPH revealed a primary deuterium isotope effect on the fast phase of the burst ($k_H/k_D \approx 2$–4, depending on temperature), but not on the slow phase [20]. Fig. 5A reveals a deuterium isotope effect ($k_H/k_D \approx 2$ at 27°C) for the monophasic *rr*dI *plus hs*dIII system; the isotope effect was approximately independent of the concentration of the dI protein across the region examined.

3.5. The pH dependences of reactions catalysed by the rrdI:hsdIII complex

Studies of the pH dependences of the reaction components of transhydrogenase, in the complete enzyme, and in the reconstituted dI:dIII complexes lacking the proton-conducting dII, might provide useful information on the sites of protonation and deprotonation involved in the process of ion translocation. Typically, forward and reverse transhydrogenation by the complete enzymes have distinctive bell-shaped pH dependences with optima at around pH 7.0 [18,19,29,30]. In previously studied dI:dIII complexes this characteristic is lost (at least for the more easily studied reverse reaction) [18,29]. Reverse transhydrogenation by *rr*dI:*hs*dIII was even less dependent on the solution pH than the complexes described earlier (Fig. 6A).

In the complete *R. rubrum* enzyme, the rate of cyclic transhydrogenation was attenuated at high pH, with an apparent pK_a of approximately 9.1 [29]. In the complete *E. coli* enzyme a similar dependence was seen, but with a considerably lower pK_a at approximately 6.5 [19]. The pH dependences of the cyclic reaction catalysed by the *rr*dI:*rr*dIII and the *rr*dI:*ec*dIII complexes were both similar to that of the complete *R. rubrum* enzyme [18]. The pH dependence of the cyclic reaction catalysed by the *ec*dI:*ec*dIII complex resembled that of the intact *E. coli* enzyme [18]. Fig. 6B shows that the pH dependence of the cyclic reaction in the *rr*I:*hs*III complex is similar to that observed for the *rr*I:*rr*III and *rr*I:*ec*III complexes. The pattern beginning to emerge is that the pH dependence of the cyclic reaction is dominated by the dI component, with *rr*dI, the p$K_a \approx 9$, with *ec*dI, the p$K_a \approx 6.5$.

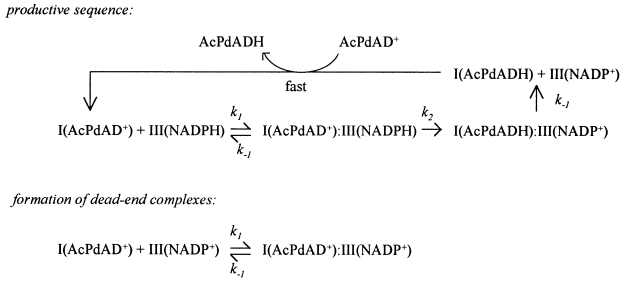
4. Discussion

In this report, we extend earlier investigations which show that the dI and dIII proteins from transhydrogenases of different species can combine to give catalytically active complexes [12,13,15,17,18]. It has either been directly demonstrated, or inferred, that the dIII proteins isolated to date have tightly bound $NADP^+$/NADPH [13,15,17]. The nucleotides are located at the active site on the protein (this work and [13]). The fact that release of $NADP^+$ and of NADPH from dIII is very slow in the absence of dII, explains the low rates of reverse and forward transhydrogenation by dI:dIII complexes. However, the high rates of the cyclic reaction indicate that the hydride transfer step, and the binding/release of nucleotides from dI, operate at rates commensurate with those in the complete enzyme. These accumulated observations, on both bacterial and mammalian transhydrogenases, indicate that interactions with dII lead to accelerated release of $NADP^+$ and NADPH from dIII, as first suggested [13], and see [2]. Furthermore, they provide support for the view that changes in the interaction between dII and dIII, brought about by proton conduction through dII, are coupled to changes in the binding energy of $NADP^+$ and NADPH [19,2]. In contrast, the available evidence suggests that events taking place during the hydride transfer step, and during nucleotide binding/release to/from the dI protein, are not coupled to proton translocation [16,20,31].

We have previously examined, in detail, the pH dependences of the component reactions (forward, reverse, cyclic) of transhydrogenases from various sources (*R. rubrum*, *E. coli*, *B. taurus*), in the complete enzymes, and in dI:dIII complexes

[18,19,29,30]. The most plausible explanation for the shift from a typically bell-shaped pH dependence for reverse (and probably forward) transhydrogenation in the intact enzyme (detergent-dispersed and membrane-bound) to a flat response in dI:dIII complexes is that it represents the loss of protonation/deprotonation associated with H^+ translocation through dII [18,29]. The flat response in *rr*dI:*hs*dIII, reported above, adds further support for this view; all dI:dIII complexes examined to date behave in this manner. In contrast, the single, high pK_a ($\sim$9.0) for the cyclic reaction in *rr*dI:*hs*dIII (Fig. 6B) is very similar to that in *rr*dI:*rr*dIII [29], *rr*dI:*ec*dIII [18] and the complete *R. rubrum* enzyme [29], but is distinctly different from that in *ec*dI:*ec*dIII [18] and the complete *E. coli* enzyme [19], which have a lower pK_a ($\sim$6.5). This suggests that the pH dependence of the cyclic reaction is determined predominantly by the properties of dI; as suggested [18], it probably represents the binding/release of scalar protons, not involved in the energy-coupling process.

Also in this report we have used stopped-flow spectroscopy to investigate the nature of the catalytic and protein-protein interactions in the hybrid complex of *rr*dI and *hs*dIII. As previously described for mixtures of *rr*dI *plus rr*dIII [10,20], the reduction of $AcPdAD^+$ by NADPH catalysed by *rr*dI *plus hs*dIII has burst kinetics in the pre-steady state, and this results from a single turnover of hydride transfer before $NADP^+$ release becomes rate-limiting. However, there are pronounced differences in the character of the burst between the homologous, and the hybrid complexes, which are summarised in Section 3. Even so, the reaction scheme that was proposed earlier to explain the data with *rr*dI *plus rr*dIIII, with only minimal modification, readily accounts for observations with *rr*dI *plus hs*dIII.



Note that this scheme has only three independent rate constants. Earlier experiments justify the assumption, implicit in this scheme, that, at the nucleotide concentrations used in Fig. 5, AcPdADH displaces $AcPdAD^+$ from dI at rates that are rapid relative to the rates of other reactions [20]. The experimental data of Fig. 5A are modelled in Fig. 7, and the following points are noted:

1. The major difference with *rr*dI *plus rr*dIII is that, in *rr*dI *plus hs*dIII, the rate constant for the dissociation of the protein complex (k_{-1}) has to be larger by a factor of 50. This means that the dissociation constant ($K_D = k_{-1}/k_1$) is much greater for the hybrid than for the homologous complex, which, of course, is entirely plausible, and is qualitatively in agreement with the fact that considerably higher *rr*dI and *hs*dIII protein concentrations are required to 'saturate' the cyclic reaction in steady-state (Fig. 3D, compare [13,18,28]). It is the large increase in the value of k_{-1} that changes the kinetic behaviour from biphasic in the *rr*dI *plus rr*dIII system, to monophasic in the *rr*dI *plus hs*dIII system.
2. We assume that, as with *rr*dI *plus rr*dIII, a substantial fraction of the protein can be trapped in the dead-end complexes, in which both nucleotides are oxidised. The effect on the pre-steady-state kinetics is more pronounced at low dI concentrations (results not shown).
3. The value of k_1 used in Fig. 7 is a factor of 10 larger than that used for *rr*dI *plus rr*dIII [20]; however, the latter is rather insensitive to changes in the value of k_1 – a 10-fold increase in the value used previously did not significantly affect the simulation (results not shown). The value $k_1 \approx 10^9$ M^{-1} s^{-1} (Fig. 7) is close to the diffusion-controlled limit for a second order reaction.
4. The value of k_2 in Fig. 7 was slightly higher than that used for *rr*dI *plus hs*dIII, suggesting that within the complex, the hydride transfer rate is actually higher in the hybrid than in the homologous system.
5. The effect of substituting [4B-^{1}H]NADPH with [4B-^{2}H]NADPH (Fig. 5A) was simulated convincingly by a single change on k_2 (Fig. 7). Thus, as expected, a primary isotope effect is exerted only at the hydride transfer step.

The accuracy with which the scheme outlined

above and [20] accommodates the two quite different sets of kinetic data (for *rr*dI *plus rr*dIII [20], and for *rr*dI *plus hs*dIII, this work) suggests to us that it is essentially correct. The fact that catalytic complexes can be generated by mixing separately isolated and purified dI and dIII proteins of transhydrogenases from only distantly related species indicates that contact between the reactive surfaces might be partly non-specific, with an absolute requirement for very few pairwise interactions. Thus, the presence of hydrophobic or ionic patches in the contact regions might be sufficient to maintain the structure of the complex long enough for hydride transfer to take place. Nevertheless, because hydride transfer between nucleotides is direct, and therefore the C4 atoms of the nicotinamide rings must be brought into apposition [10], there must be critical distance, and orientation, constraints on the reaction. In the homologous *rr*dI *plus rr*dIII system the greatly reduced rate of dissociation of the complex suggests additional, probably more specific, interactions between the two components.

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References

[1] T. Bizouarn, L.A. Sazanov, S. Aubourg, J.B. Jackson, Biochim. Biophys. Acta 1273 (1996) 4–12.
[2] J.B. Jackson, P.G. Quirk, N.P.J. Cotton, J.D. Venning, S. Gupta, T. Bizouarn, S.J. Peake, C.M. Thomas, Biochim. Biophys. Acta 1365 (1998) 79–86.
[3] J. Rydstrom, X. Hu, O. Fjellstrom, J. Meuller, J. Zhang, K. Johansson, T. Bizouarn, Biochim. Biophys. Acta 1365 (1998) 10–16.
[4] P.D. Bragg, Biochim. Biophys. Acta 1365 (1998) 98–104.
[5] J. Rydstrom, J.B. Hoek, Biochem. J. 254 (1988) 1–10.
[6] L.A. Sazanov, J.B. Jackson, FEBS Lett. 344 (1994) 109–116.
[7] P.J. Baker, Y. Sawa, H. Shibata, S.E. Sedelnikova, D.W. Rice, Nature Struct. Biol. 5 (1998) 561–567.
[8] P.J. Baker, P.A. Buckley, D.W. Rice, P.G. Quirk, J.B. Jackson (1999) submitted.
[9] M. Jeeves, K.J. Smith, P.G. Quirk, N.P.J. Cotton, J.B. Jackson (1998) J. Biomol. NMR, in press.
[10] J.D. Venning, R.L. Grimley, T. Bizouarn, N.P.J. Cotton, J.B. Jackson, J. Biol. Chem. 272 (1997) 27535–27538.
[11] C. Diggle, M. Hutton, G.R. Jones, C.M. Thomas, J.B. Jackson, Eur. J. Biochem. 228 (1995) 719–726.
[12] M. Yamaguchi, Y. Hatefi, J. Biol. Chem. 270 (1995) 28165–28168.
[13] C. Diggle, T. Bizouarn, N.P.J. Cotton, J.B. Jackson, Eur. J. Biochem. 241 (1996) 162–170.
[14] C. Diggle, N.P.J. Cotton, R.L. Grimley, P.G. Quirk, C.M. Thomas, J.B. Jackson, Eur. J. Biochem. 232 (1995) 315–326.
[15] O. Fjellstrom, C. Johansson, J. Rydstrom, Biochemistry 36 (1997) 11331–11341.
[16] T. Bizouarn, C. Diggle, J.B. Jackson, Eur. J. Biochem. 239 (1996) 737–741.
[17] M. Yamaguchi, Y. Hatefi, Biochim. Biophys. Acta 1318 (1997) 225–234.
[18] O. Fjellstrom, T. Bizouarn, J. Zhang, J. Rydstrom, J.D. Venning, J.B. Jackson, Biochemistry 38 (1999) 415–422.
[19] M.N. Hutton, J.M. Day, J.B. Jackson, Eur. J. Biochem. 219 (1994) 1041–1051.
[20] J.D. Venning, T. Bizouarn, N.P.J. Cotton, P.G. Quirk, J.B. Jackson, Eur. J. Biochem. 257 (1998) 202–209.
[21] E. Arkblad, C. Betsholtz, J. Rydstrom, Biochim. Biophys. Acta 1273 (1996) 203–205.
[22] T.M. Lever, T. Palmer, I.J. Cunningham, N.P.J. Cotton, J.B. Jackson, Eur. J. Biochem. 197 (1991) 247–255.
[23] S. Mejbaum-Katzenellenbogen, W.J. Drobryszycka, Clin. Chim. Acta 4 (1959) 515–522.
[24] T. Palmer, J.B. Jackson, Biochim. Biophys. Acta 1099 (1992) 157–162.
[25] R.R. Fisher, N.O. Kaplan, Biochemistry 12 (1973) 1182–1188.
[26] T. Bizouarn, R.L. Grimley, N.P.J. Cotton, S. Stilwell, M. Hutton, J.B. Jackson, Biochim. Biophys. Acta 1229 (1995) 49–58.
[27] J.W. Little, Anal. Biochem. 48 (1972) 217–224.
[28] T. Bizouarn, R.L. Grimley, C. Diggle, C.M. Thomas, J.B. Jackson, Biochim. Biophys. Acta 1320 (1997) 265–274.
[29] T. Bizouarn, S.N. Stilwell, J.D. Venning, N.P.J. Cotton, J.B. Jackson, Biochim. Biophys. Acta 1322 (1997) 19–32.
[30] L.A. Sazanov, J.B. Jackson, Biochim. Biophys. Acta 1231 (1995) 304–312.
[31] T. Bizouarn, C. Diggle, P.G. Quirk, R.L. Grimley, N.P.J. Cotton, C.M. Thomas, J.B. Jackson, J. Biol. Chem. 271 (1996) 10103–10108.