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Biochimica et Biophysica Acta 1413 (1999) 81–91

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Evidence for the stabilization of NADPH relative to $NADP^+$ on the dIII components of proton-translocating transhydrogenases from *Homo sapiens* and from *Rhodospirillum rubrum* by measurement of tryptophan fluorescence

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Received 14 June 1999; accepted 22 July 1999

Abstract

A unique Trp residue in the recombinant dIII component of transhydrogenase from human heart mitochondria (*hs*dIII), and an equivalent Trp engineered into the dIII component of *Rhodospirillum rubrum* transhydrogenase (*rr*dIII.D155W), are more fluorescent when $NADP^+$ is bound to the proteins, than when NADPH is bound. We have used this to determine the occupancy of the binding site during transhydrogenation reactions catalysed by mixtures of recombinant dI from the *R. rubrum* enzyme and either *hs*dIII or *rr*dIII.D155W. The standard redox potential of $NADP^+$/NADPH bound to the dIII proteins is some 60–70 mV higher than that in free solution. This results in favoured reduction of $NADP^+$ by NADH at the catalytic site, and supports the view that changes in affinity at the nucleotide-binding site of dIII are central to the mechanism by which transhydrogenase is coupled to proton translocation across the membrane.

Keywords: Transhydrogenase; Proton translocation; Tryptophan fluorescence; Nucleotide binding; Redox protein; *Rhodospirillum rubrum*; *Homo sapiens*

1. Introduction

Transhydrogenase is a proton pump, found in the inner mitochondrial membranes of animal cells, and in the cytoplasmic membranes of bacteria. It uses the energy of the proton electrochemical gradient (Δp) to drive the reduction of $NADP^+$ by NADH, and it probably functions (a) in the supply of NADPH for biosynthesis, and for glutathione reduction [1], and (b) in the regulation of flux through the tricarboxylic acid cycle [2]. The mechanism by which transhydrogenase couples the translocation of protons across the membrane to the transfer of reducing (hydride) equivalents between NAD(H) and

Abbreviations: *rr*dI, the NAD(H)-binding component of *Rhodospirillum rubrum* transhydrogenase; *hs*dIII the NADP(H)-binding component of human transhydrogenase; *rr*dIII.E155W, the NADP(H)-binding component of *R. rubrum* transhydrogenase with a Trp residue substituted for Glu155; $AcPdAD^+$, acetyl pyridine adenine dinucleotide (oxidized form)

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0005-2728/99/$ – see front matter
PII: S0005-2728(99)00084-5

NADP(H) is only poorly understood (for reviews, see [3–5]).

$$NADH + NADP^+ + xH^+_{OUT} \Leftrightarrow NAD^+ + NADPH + xH^+_{IN} \quad (1)$$

Earlier determinations of the value of x were unreliable (reviewed, in detail, [6]). A recent investigation yielded $x = 1$ [7].

The enzyme has a tripartite structure. The dII component spans the membrane, probably via 10–14 hydrophobic helices [8–11]. The dI and dIII components protrude from the membrane (on the matrix side in mitochondria and on the cytoplasmic side in bacteria), and possess the binding sites for NAD^+/NADH and $NADP^+$/NADPH, respectively. The amino acid sequence of dI is similar [12] to that of the soluble protein, alanine dehydrogenase, whose crystal structure has recently been solved [13]. We suggested [14,15] that, like alanine dehydrogenase, dI comprises two domains, each of which has the open, twisted βαβ structure of the Rossmann fold; one of these (domain I.2) binds the NAD^+/NADH (see also [16]), the other (domain I.1) has an unknown function. An emerging NMR structure shows that the dIII protein also adopts the Rossmann fold [17,18]. We [19] and others [20] have stressed that coupling between proton translocation and transhydrogenation is probably achieved through Δp-induced changes in nucleotide-binding affinity. Our view is that dI.NADH serves as a passive hydride donor in the reaction, and that the crucial changes in binding energy occur at the dIII nucleotide-binding site [3,19]. Thus, the mechanism of H^+ pumping by transhydrogenase might have more in common with the cation-transporting ATPases than with the redox proteins of the respiratory and photosynthetic electron transport chains.

During the last 5 years, the dI and dIII components of transhydrogenases from several species have been isolated using recombinant DNA technology, and purified [21–27]. Even in the absence of the membrane-spanning dII component, simple mixtures of dI and dIII yield complexes which catalyse transhydrogenation [23–28]. The hydride transfer step in the dI:dIII complexes, and nucleotide exchange at the dI-binding site, are rapid, but the net rates of forward and reverse transhydrogenation (see Eq. 1) are very slow due to limiting rates of release of NADPH and $NADP^+$, respectively, from dIII in the absence of dII, as first shown [24].

In stopped-flow experiments, the hydride transfer step from NADPH to the NAD^+ analogue, acetylpyridine adenine dinucleotide ($AcPdAD^+$), in dI:dIII complexes, is revealed as a burst prior to establishment of the slow steady-state rate [27,29,30]. There are no redox intermediates in this reaction; hydride transfer proceeds directly and rapidly from one nucleotide to the other, indicating that the nicotinamide rings of the two nucleotides must be brought into apposition during catalysis. A comparison of the characteristics of the 'forward' ($AcPdADH \rightarrow NADP^+$), and 'reverse' ($NADPH \rightarrow AcPdAD^+$), reactions suggested that the equilibrium constant of the hydride transfer step on the *Rhodospirillum rubrum* dI:dIII complex is altered by about 6–16-fold, relative to that in free solution, in favour of NADPH and $AcPdAD^+$ [31]. This has important consequences for the mechanism of transhydrogenation with physiological nucleotides in the forward direction.

In this report, we describe a different approach towards understanding the redox chemistry at the catalytic site. We make use of a recent observation by Fjellstrom et al. [26,28], that fluorescence emission from the single Trp residue of dIII of *Escherichia coli* transhydrogenase is lower when NADPH is bound to the protein than when $NADP^+$ is bound. We find that the equivalent Trp in dIII from human heart (*hs*dIII), and a Trp engineered into *R. rubrum* dIII (*rr*dIII), behave similarly. We also make use of the fact that, in simple mixtures of either *rr*dI+*rr*dIII or *rr*dI+*hs*dIII, nucleotide exchange with dI, and hydride transfer, take place very rapidly, whereas nucleotide release from dIII is extremely slow [27,29,30]. Under these conditions, $AcPdAD^+$/AcPdADH in solution reach equilibrium with bound $NADP^+$/NADPH and, thus, we are able to estimate the standard redox potential of nucleotide in the dIII-binding site.

2. Materials and methods

The dI protein of *R. rubrum* transhydrogenase (*rr*dI), and the dIII protein of the *Homo sapiens* enzyme (*hs*dIII) – the 'long form' (see [27]), were sep-

arately expressed in *E. coli*, strain C600 and strain BL21(DE3), respectively, from the constructs pCD1 [21] and pSJP2 [27], respectively, and purified by column chromatography.

Mutagenesis of *rr*dIII was carried out using the Stratagene Quikchange kit, and mutagenic primers supplied by Alta Bioscience. After mutagenesis, the nucleotide sequences of coding regions were checked for polymerase errors using an Applied Biosystems 373A automatic sequencer according to the manufacturer's instructions. A *Bam*HI site was engineered into the 5′ end of the DNA coding for *rr*dIII in the construct pCD2 [24], and the internal *Nde*I site was removed using the mutagenesis kit. Thus, C^{2757}ATATG in the nucleotide sequence [32,33] was changed to C^{2757}ACATG, which does not alter the amino acid sequence of the translation product. The *Nde*I and *Bam*HI fragment limiting the dIII coding region was excised from an agarose gel, and inserted into the multiple cloning site of pET11c (Novagen) to give pNIC2. This construct was transformed into BL21(DE3), and used for the expression of wild-type *rr*dIII. The DNA coding for mutant protein *rr*dIII.E155W (dIII of *R. rubrum* transhydrogenase, but with Glu155 changed to a Trp residue) was isolated using pNIC2 as the template, designated pJDV1, and transformed into BL21(DE3) for expression. Wild-type *rr*dIII and *rr*dIII.E155W were purified, as described [24]; during column chromatography, the behaviours of the mutant and wild-type proteins were indistinguishable.

Note that in this report, we number amino acid residues according to the sequence of the *isolated* dIII proteins. Thus, Met1 of *rr*dIII is equivalent to Met262 of PntB (the β subunit of the complete *R. rubrum* enzyme), and Met1 of *hs*dIII is equivalent to Met837 of the mature protein of human transhydrogenase (a single polypeptide).

All protein preparations were checked for purity ($>95\%$) by SDS-PAGE, and concentrations were determined using the microtannin assay [34]. The amounts of bound nucleotides in the dIII proteins were measured after acid or alkaline extraction, as appropriate, and as described [24,35]. Where required, NADP(H) on dIII was converted either to the fully oxidized or fully reduced form, as described [18,29].

Steady-state rates of transhydrogenation were measured as the reduction of $AcPdAD^+$ by NADPH, or by NADH in the presence of $NADP^+$, at 375 nm on a Perkin Elmer λ16 spectrophotometer, using an extinction coefficient of 6.1 mM^{-1} cm^{-1} [31,36].

Measurements of tryptophan fluorescence were carried out at 25°C in a magnetically stirred, 1×1 cm cuvette, using a Spex FluoroMax with slitwidths set to 4.25 nm, as previously described [21]. The inner filtering effect of the nucleotides was compensated using the algorithm given [37], or by addition of a non-binding nucleotide (see below). Nucleotide concentrations of stock solutions were determined from their UV absorbance.

3. Results

3.1. Trp fluorescence from hsdIII

The isolated recombinant forms of the dIII components from the transhydrogenases of *R. rubrum* [24], *E. coli* [26], *H. sapiens* [27], and probably that from *Bos taurus* [25], are associated with 1 mole of tightly bound nucleotide ($NADP^+$/NADPH) per mol of protein. Nucleotide extraction experiments indicated that approx. 95% of the nucleotide on the preparations of *H. sapiens* dIII protein (*hs*dIII) used in this report is $NADP^+$, and approx. 5% is NADPH (results not shown). The fluorescence emission from the single Trp residue of the dIII protein of *E. coli* transhydrogenase was lowered when bound $NADP^+$ was replaced by NADPH [26,28]. The *hs*dIII protein has an equivalent Trp residue, Trp154 (position 990 in the complete transhydrogenase sequence [38], and see [27]). Fig. 1A shows that, as in the experiments with *E. coli* dIII, the fluorescence emission of Trp154 was lowered (by approx. 24% without any shift in the wavelength of the emission band), when bound $NADP^+$ was replaced by the reduced nucleotide.

The kinetics of the Trp fluorescence change that follow from the addition of NADPH to *hs*dIII preloaded with $NADP^+$, are shown in Fig. 2A. The initial, rapid fluorescence decrease is the result of 'inner filtering' by the added nucleotide (at both the excitation and emission wavelengths). Following this effect, the Trp fluorescence was quenched with

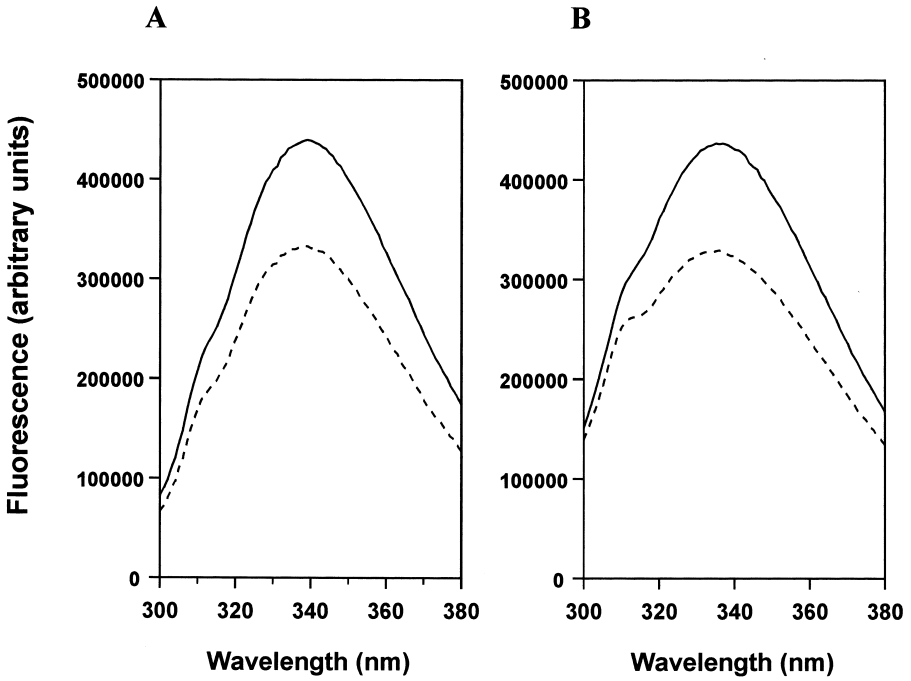

Fig. 1. The fluorescence spectra of *hs*dIII (A), and *rr*dIII.E155W (B), in their $NADP^+$-bound and NADPH-bound forms. Experiments were performed using 1.0 μM *hs*dIII (A) or 1.0 μM *rr*dIII.E155W (B) in 20 mM MOPS, pH 7.2. Excitation was at 280 nm. Upper curves in each panel: the fluorescence emission spectra were recorded with the proteins in their $NADP^+$ forms (see Section 2). Lower curves in each panel: the fluorescence emission spectra with the proteins mainly in their NADPH forms (obtained by adding 20 μM NADPH, see Figs. 2 and 3). In each spectrum, the shoulder at about 310 nm is due to the water Raman band.

approximately first order kinetics, as the added NADPH from solution exchanged with $NADP^+$ on *hs*dIII. The apparent first order rate constant of the fluorescence quenching increased with the initial NADPH concentration, approaching a maximum at approx. 0.2 s^{-1} (not shown). The limiting value at high NADPH concentrations (>20 μM), when nucleotide re-binding becomes relatively fast, should correspond to the k_{off} for $NADP^+$ release from the protein (see also [26,28]). Within error, the value is similar to that calculated from experiments in which $NADP^+$ released from dIII was reduced with isocitrate dehydrogenase (0.014 s^{-1} [24]). The addition of NADH to *hs*dIII under similar conditions to those in Fig. 2A (notably, in the absence of dI) caused only the inner filtering effect (experiment not shown); it did not cause quenching of the Trp fluorescence – NADH does not bind to dIII protein (but see below, and Fig. 2C).

For the experiment shown in Fig. 2B, the *hs*dIII had been pre-loaded with NADPH (see [29]), and washed to remove excess nucleotide. The addition of $NADP^+$ then led initially to an inner filtering effect on the Trp154 fluorescence, followed by a fluorescence increase, presumably as the added nucleotide displaced the bound NADPH. At high concentrations of $NADP^+$, the apparent first order rate constant for the fluorescence increase approached a limiting value of 0.003 s^{-1}, which is consistent with the k_{off} value for NADPH dissociation from *hs*dIII calculated from experiments in which released nucleotide was oxidized by glutathione reductase ($k_{off} \approx 0.008$ s^{-1}) [24]. As expected from its inability to bind, the addition of NAD^+ to *hs*dIII under similar conditions to those in Fig. 2B caused only the inner filtering effect (not shown).

A simple mixture of *hs*dIII and the recombinant dI protein from *R. rubrum* transhydrogenase (*rr*dI) catalyses transhydrogenation [27]. The hydride transfer step in the dI:dIII complex, and nucleotide binding to, and release from, dI are very rapid, but the steady-state rates of the 'forward' and 'reverse' reactions (see Eq. 1) are strongly limited by very slow release from dIII of products, NADPH and $NADP^+$, respectively. Observations on the fluorescence of Trp154 in dIII now present us with the opportunity

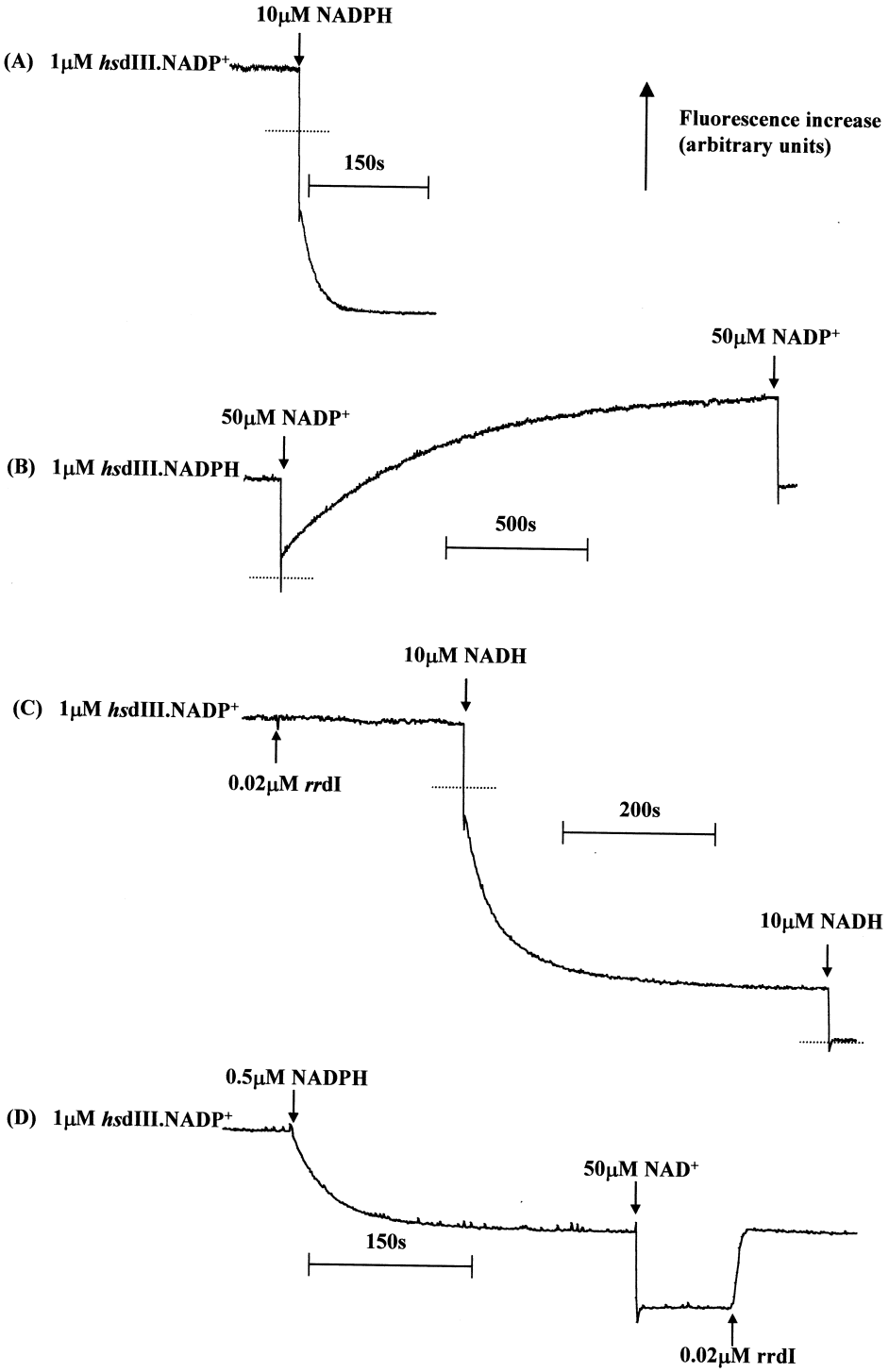

Fig. 2. Changes in the fluorescence of Trp154 in *hs*dIII resulting from changes in nucleotide occupancy. Experimental conditions as in Fig. 1. Fluorescence emission was measured at 338 nm. The dotted lines show the extent of the inner filtering effect of the added nucleotide, calculated as described [37]. Experiments A, C and D were carried out with the protein initially in its $NADP^+$ form (see Section 2). Experiment B was carried out with the protein in its NADPH form [30,31]. The concentrations shown in the figure represent final values. The vertical scale is the same for all parts of the figure, but note differences in the time scales (see horizontal bars).

Table 1
Catalytic properties of *rr*dIII.E155W

	*rr*dIII.E155W	Wild-type dIII
Apparent first order rate constant for $NADP^+$ release (s^{-1})[a]	0.063	0.030
Cyclic transhydrogenation[b]		
V_{max} (s^{-1})	1540	1610
Concentration of dIII required to give half-maximal rate (nM)	32	26
Reverse transhydrogenation[c]		
V_{max} (s^{-1})	1.31	1.24
Concentration of dI required to give half-maximal rate (nM)	210	170

[a]$NADP^+$ released from the dIII protein (2.0 μM) was trapped with glucose-6-phosphate dehydrogenase (3.3 units ml^{-1}) and glucose 6-phosphate (2.0 mM) in 50 mM KCl, 20 mM MOPS, pH 7.2, and the fluorescence was recorded, as described [24]. The value for the wild-type protein was taken from [24].

[b]Cyclic transhydrogenation is the reduction of $AcPdAD^+$ by NADH in the presence of NADP(H) [19,24]. The reaction was measured in 20 mM MOPS, pH 7.2 with 6.5 nM *rr*dI, 200 μM $AcPdAD^+$, 100 μM NADH and 20 μM $NADP^+$. V_{max} was estimated from double reciprocal plot as the concentration of dIII tended to infinity.

[c]Reverse transhydrogenation was measured as the reduction of $AcPdAD^+$ (100 μM) by NADPH (200 μM) in 20 mM MOPS, pH 7.2. The *rr*dIII concentration (either *rr*dIII.E155W or wild-type) was 845 nM. V_{max} was estimated from double reciprocal plot as the concentration of dI tended to infinity.

to examine changes in the occupation of the nucleotide binding site on this protein that are consequent upon hydride transfer either to or from nucleotide bound to dI.

(1) Fig. 2C shows that the Trp fluorescence from *hs*dIII.$NADP^+$ was quenched following the addition of *rr*dI plus NADH. In experiments in which the order of addition of dI protein and NADH was reversed (not shown), it was established that both components were necessary for the response. Note that the fluorescence emission from Trp72 in dI [21] was negligible under the conditions shown because the concentration of this protein was relatively low. The decrease in Trp fluorescence, shown in Fig. 2C, clearly results from the reduction of the $NADP^+$ on dIII by NADH on dI, and the fact that the product NADPH is slow to dissociate from the dIII protein. Very low *rr*dI concentrations were required to produce this response; in Fig. 2C the dI concentration was one fiftieth that of dIII. This is because the exchange of nucleotides on dI, the dissociation of the complex, and the re-association of dI and dIII, are all fast relative to the rate of nucleotide release from dIII; effectively, dI can visit many dIII molecules during the time taken for dIII to release its NADPH [24]. As expected, the rate of the fluorescence quenching increased markedly when the dI concentration was raised (results not shown).

(2) The experiment shown in Fig. 2D complements that shown in Fig. 2C. To begin, a low concentration of NADPH was added to *hs*dIII partially to displace the bound $NADP^+$; it resulted in the expected quenching of the Trp154 fluorescence (compare Fig. 2A). The subsequent addition of NAD^+ and dI led to a fluorescence increase, as the NADPH on dIII was oxidized by hydride transfer. Again, for the reasons given above, only low concentrations of dI were required to elicit this response. Qualitatively similar results were obtained using a combination of the *hs*dIII and dI proteins *plus* the NAD^+ analogue, $AcPdAD^+$, and this forms the basis for the determination of the standard redox potential of NADP(H) in its binding site (see below).

3.2. Trp fluorescence from rrdIII.E155W

The dIII component of *R. rubrum* transhydrogenase is devoid of Trp residues; its near-ultraviolet fluorescence spectrum is dominated by Tyr emissions [24]. The equivalent of Trp154 in *hs*dIII in the *R. rubrum* protein is a Glu residue (Glu155). In *rr*dIII.E155W we have replaced the Glu residue with a Trp (see Section 2). Steady-state rates of 'reverse' and 'cyclic' transhydrogenation catalysed by this mutant protein in mixtures with wild-type *rr*dI, and its first order rate constants for nucleotide release, were similar to those of wild-type dIII within a factor of two (Table 1). See [24,39] for a detailed

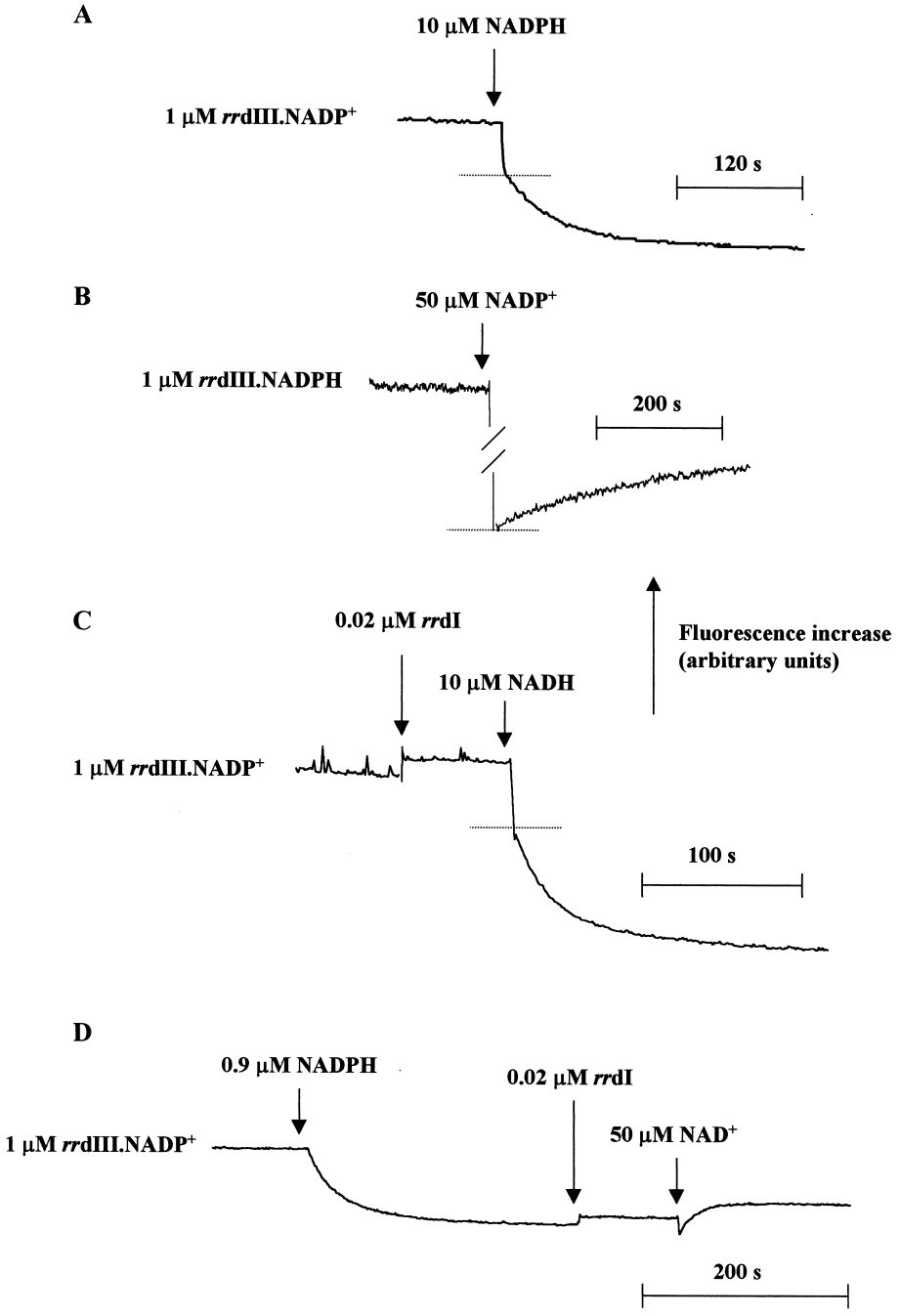

Fig. 3. Changes in Trp fluorescence of *rr*dIII.E155W resulting from changes in nucleotide occupancy. Conditions as in Fig. 1. The dotted lines show the extent of the inner filtering effect of the added nucleotide, calculated as described [37]. Experiments A, C and D were carried out with the protein initially in its $NADP^+$ form (see Section 2). Experiment B was carried out with the protein in its NADPH form [30,31]. The concentrations shown in the figure represent final values. The vertical scale is the same for experiments A, C, and D. In experiment B, the vertical scale is amplified by 4, and a section, equivalent to 75% of the fluorescence change upon addition of the $NADP^+$, has been removed. Note differences in the time scales (see horizontal bars).

discussion of these processes. These results indicate that the amino acid substitution does not have significant effects on the catalytic properties of the protein.

The fluorescence emission spectrum of *rr*dIII.E155W (Fig. 1B) closely resembles that of *hs*dIII and, as with the latter, was less intense ($\approx$30%) when NADPH was bound to the protein, than when $NADP^+$ was bound. Fig. 3 shows a set of experiments with *rr*dIII.E155W that is similar to the set shown in Fig. 2 with *hs*dIII; the results are closely comparable. Fluorescence changes accompanying the displacement of $NADP^+$ from *rr*dIII.E155W. $NADP^+$ by NADPH (Fig. 3A), and the displacement of NADPH from *rr*dIII.E155W.NADPH by $NADP^+$ (Fig. 3B), were slower than in the equivalent human protein (Fig. 2A and B, respectively), but this mirrors the slower rates of nucleotide release from *rr*dIII [24] than from *hs*dIII [27], as measured by other procedures. In particular, the fluorescence increase upon addition of $NADP^+$ to NADPH-loaded *rr*dIII.E155W (Fig. 3B) was extremely slow, and was incomplete with nucleotide concentrations up to about 50 μM (compare $k_{off} \approx 6 \times 10^{-4}$ s^{-1} [24]); above this concentration, the inner filtering effect of the added nucleotide became too large for proper measurement. The value of k_{off} for $NADP^+$, calculated from the Trp fluorescence decrease upon addition of NADPH to $NADP^+$-loaded *rr*dIII.E155W (Fig. 3A), was 0.063 s^{-1}, comparing favourably with the value of 0.058 s^{-1} estimated by trapping released $NADP^+$ with glucose-6-phosphate dehydrogenase (Table 1). Fig. 3C and D show the effects on the Trp155 fluorescence of oxidizing/reducing NADP(H) bound to *rr*dIII.E155W by hydride transfer to, or from, NAD(H) on *rr*dI protein. The results are qualitatively similar to those shown in Fig. 2C,D for the hybrid system.

3.3. The redox potential of bound NADP(H)

The ability to determine the occupancy of *hs*dIII and *rr*dIII.E155W by $NADP^+$/NADPH from the Trp fluorescence also allows us to estimate the standard redox potentials of these nucleotides in their binding sites. Each data point in Fig. 4 was determined as follows. To a mixture of *rr*dI and either *hs*dIII or *rr*dIII.E155W (in the $NADP^+$-bound

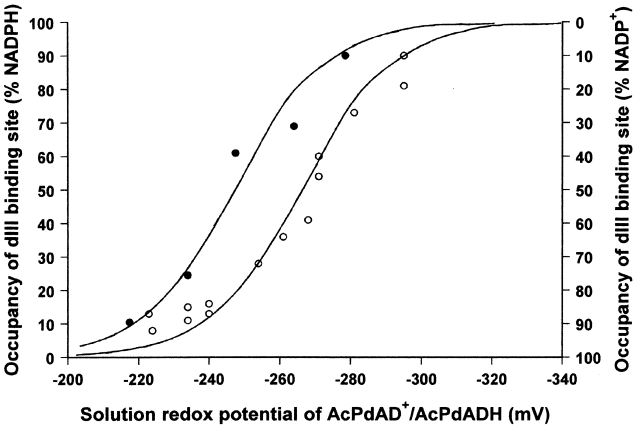

Fig. 4. The standard redox potential of $NADP^+$/NADPH on *hs*dIII and on *rr*dIII.E155W4. The experimental conditions were similar to those in Figs. 2 and 3, but using either 100 nM *rr*dI and 1.0 μM *hs*dIII (○), or 100 nM *rr*dI and 1.0 μM *rr*dIII.E155W (●). Mixtures of $AcPdAD^+$ and AcPdADH (total 20 μM) were added to set the redox state of the $NADP^+$/NADPH on dIII (see text). The fluorescence was scaled on the basis that the emission prior to the addition of nucleotides was equivalent to an occupancy of 95% $NADP^+$, 5% NADPH, and that the emission reached after equilibration with 40 μM NADPH corresponds to an occupancy of 100% of the reduced nucleotide. The solution E_h of the $AcPdAD^+$/AcPdADH was calculated using a value for $E_{m7.2} = -0.254$ V [42].

forms) was added a mixture of $AcPdAD^+$ and AcPdADH. Because (a) the binding and release of $AcPdAD^+$ and AcPdADH to and from dI and (b) the hydride transfer reaction are both very rapid relative to the release of $NADP^+$ and NADPH from the dIII protein, it is assumed that the reaction,

$$AcPdAD^+_{sol} + dIII.NADP^+ \overset{(dI\ protein)}{\Leftrightarrow} AcPdADH_{sol} + dIII.NADPH \quad (2)$$

quickly reaches equilibrium ($AcPdAD^+_{sol}$ and $AcPdADH_{sol}$ represent the solution forms of the nucleotide). The E_h of $NADP^+$/NADPH in the catalytic site is then equal to that of the $AcPdAD^+$/AcPdADH in solution, and the latter can be calculated from the added quantities, after making a small correction for the change in concentrations on the approach to equilibrium. The metastable level of the fluorescence of either Trp154 (for *hs*dIII) or Trp155 (for *rr*dIII.E155W) within a few seconds of mixing was used to determine the fractional occupa-

tion by $NADP^+$ and NADPH of the dIII protein. The relationships between the solution E_h of the $AcPdAD^+$/AcPdADH and the ratio of dIII-bound $NADP^+$/NADPH, shown in Fig. 4, both fit Nernst curves ($n=2$) with an $E_{7.2}$ value for *hs*dIII of −0.266 V, and for *rr*dIII.E155W of −0.253 V.

In principle, these titrations can be performed with NAD^+/NADH instead of $AcPdAD^+$/AcPdADH. Indeed, trial experiments gave results that were in qualitative agreement with the data of Fig. 4. However, because the $E_{m7.2}$ of NAD^+/NADH is approx. 60–70 mV more negative than that of $AcPdAD^+$/AcPdADH (and of the bound $NADP^+$/NADPH), a quantitative analysis with the physiological nucleotides was technically more difficult. Thus, even very low ratios of NADH/NAD^+ were sufficient extensively to reduce the dIII.$NADP^+$, and consequently the error on the measured ratio was considerably greater.

4. Discussion

Our results support the conclusion that a non-essential Trp residue in the dIII protein of transhydrogenase (Trp154 in *hs*dIII and Trp155 in *rr*dIII.E155W, this work, and βTrp415 in *E. coli* dIII [26,28]) is more fluorescent when $NADP^+$ is bound to the protein than when NADPH is bound. Furthermore, we show that the fluorescence change of the Trp can be used to monitor the occupancy of the nucleotide-binding site in dIII during hydride transfer to and from NAD(H) bound to dI protein. The Trp residue is located in, what we have provisionally called, loop D, of the dIII NMR structure [18], and is close enough to the bound nucleotide (in the region of 17 Å from the nicotinamide ring, M. Jeeves, personal communication) to transfer resonance energy by a Förster mechanism.

Recently, we showed that, during the pre-steady-state burst of transhydrogenation from AcPdADH to $NADP^+$ by dI:dIII complexes, the reaction proceeded beyond the position expected for the hydride transfer equilibrium in free solution [31]. This was evidently the result of a bias to the equilibrium constant *on the enzyme* in the 'forward' direction. We estimated the on-enzyme equilibrium constant from the extent of the burst at different nucleotide concentrations, and we attempted to fit k_{app} values for the burst of 'forward' and 'reverse' transhydrogenation to k_f and k_r for the hydride transfer step. In the present experiments a different approach is employed. We have determined the fractional occupancy of the NADP(H)-binding site by monitoring Trp fluorescence under conditions in which exchange of $AcPdAD^+$ and AcPdADH on dI with that in solution, hydride transfer *and* dissociation/re-association of dI:dIII are all very fast relative to release of $NADP^+$ and NADPH from dIII. Under these conditions the reaction described by Eq. 2 will rapidly reach equilibrium, i.e. the redox potential of the bound $NADP^+$/NADPH will take on the same value as that of the $AcPdAD^+$/AcPdADH in solution. Because the dI concentration is kept low, the concentration of bound $AcPdAD^+$ and AcPdADH will be negligible at all but extreme ratios of oxidized to reduced nucleotide, and can be ignored. Thus, because the standard redox potential of $AcPdAD^+$/AcPdADH in solution is fortuitously similar to that of the bound $NADP^+$/NADPH, the most reliable E_h values, where $AcPdAD^+/AcPdADH \approx 1$, are those close to the E_m of the bound nucleotide on dIII (Fig. 4). The fact that the data fit to a Nernst curve support the view that the reaction is operating at equilibrium.

The standard redox potential of the NADP(H) in the binding site of dIII is shifted by +0.073 V (*rr*dIII) or by +0.060 (*hs*dIII) and this must result from stabilization on the protein of the reduced relative to the oxidized nucleotide by a factor of approx. 270 in *rr*dIII and approx. 100 in *hs*dIII. Now, isolated *rr*dI binds NADH about ten times more tightly than NAD^+ (K_d values, determined by equilibrium dialysis, are approx. 30 μM and approx. 300 μM, respectively [40]). Thus, assuming that the nucleotide binding constants are not affected by interactions between dI and dIII, the equilibrium constant for hydride transfer on dI:dIII complexes,

$$\text{dI.NADH} : \text{dIII}\text{NADP}^+ \Leftrightarrow \text{dI.NAD}^+ : \text{dIII}\text{NADPH} \quad (3)$$

is ≈27 for *rr*dI+*rr*dIII (which compares with a value in the range of 6–16 in our earlier stopped-flow determination [31]), and ≈10 for *rr*dI+*hs*dIII. In view of the errors, particularly in the estimation of K_{eq} from the pre-steady-state burst, the agreement is rea-

sonable. The key finding is that the equilibrium constant is significantly greater than the value of approx. 1.0 in free solution.

These results can be considered in the context of the 'binding-change mechanism' of proton pumping by transhydrogenase [3,19,31], so described because of its analogy with that proposed for the F_1F_0-ATPase [43]. In this mechanism NADH binds to dI of the intact enzyme and serves as a passive donor of hydride equivalents; the binding affinities for NADH and NAD^+ are thought not to change during the catalytic cycle [40,41]. $NADP^+$ binds with moderate affinity to the 'open' conformation of the dIII component of the intact enzyme. Hydride transfer does not take place in this state, but an interaction with Δp (e.g. through proton binding from the *p*-phase, either to dII, or to dIII via dII) leads to an 'occluded' conformation in which $NADP^+$ (and NADPH) are prevented from dissociation, in which $NADP^+$ is destabilized relative to NADPH, and in which rapid hydride transfer is permitted. Note that the process of occlusion is necessary in this mechanism to permit the Δp-induced switch in the NADP(H)-binding affinity whilst preventing nucleotide expulsion from the site. It might involve an increase in hydrogen bond contact between the nicotinamide ring, or its ribose, and the protein, or the movement of a protein segment over the nucleotide. The C4 position on the *pro-S* face of the nicotinamide ring must, however, remain accessible to nucleotide bound to the dI protein to allow H^- transfer. Following the redox reaction between the bound nucleotides, a further input of energy from Δp (e.g. H^+ release to the *n*-phase) leads to regeneration of the 'open' state from which NADPH can dissociate. Isolated dIII, and the dI:dIII complex, are permanently locked into the 'occluded' state, where we see that, even though NADH binds much more tightly than NAD^+ (which, in itself, would *lower* the equilibrium constant), NADPH binds *very* much more tightly than $NADP^+$. This means that, whilst the hydride transfer step is not directly coupled to proton translocation, the differences in the nucleotide binding energies, and the rapid reaction rate, ensure that the dI.NAD^+:dIII.NADPH state is more populated than dI.NADH:dIII.$NADP^+$. Assuming that similar arguments also apply in the intact enzyme, this device, since it immediately precedes the rate-limiting step, would kinetically favour the reaction in the 'forward' direction.

Acknowledgements

This work was supported by grants from the Biotechnology and Biological Sciences Research Council and the British Heart Foundation. We are very grateful to Mark Jeeves and Philip Quirk for discussion.

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