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The specificity of proton-translocating transhydrogenase for nicotinamide nucleotides

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ABSTRACT

In its forward direction, transhydrogenase couples the reduction of $NADP^+$ by NADH to the outward translocation of protons across the membrane of bacteria and animal mitochondria. The enzyme has three components: dI and dIII protrude from the membrane and dII spans the membrane. Hydride transfer takes place between nucleotides bound to dI and dIII. Studies on the kinetics of a lag phase at the onset of a "cyclic reaction" catalysed by complexes of the dI and dIII components of transhydrogenase from *Rhodospirillum rubrum*, and on the kinetics of fluorescence changes associated with nucleotide binding, reveal two features. Firstly, the binding of $NADP^+$ and NADPH to dIII is extremely slow, and is probably limited by the conversion of the occluded to the open state of the complex. Secondly, dIII can also bind NAD^+ and NADH. Extrapolating to the intact enzyme this binding to the "wrong" site could lead to slip: proton translocation without change in the nucleotide redox state, which would have important consequences for bacterial and mitochondrial metabolism.

1. Introduction

Proton-translocating nicotinamide nucleotide transhydrogenase, located in the cytoplasmic membrane of bacteria and the inner mitochondrial membrane of animal cells, catalyses the reaction,

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

Inward proton translocation through the enzyme, which can be driven by the proton electrochemical gradient (Δp), is coupled to transhydrogenation in its "forward" direction (left to right in the equation). The resulting NADPH can be used in biosynthesis reactions [1–3], and to reduce glutathione for protection against oxidative damage [4–6]. There also appears to be a role for transhydrogenase in glucose-dependent insulin secretion in the β-cells of mice [7,8], and the enzyme has the capacity to regulate the rate of the tricarboxylic acid cycle through interactions with the nucleotide substrates and products of the isocitrate dehydogenases [9]. Uncertainties about the function of the enzyme are not clarified by the very patchy distribution of transhydrogenase amongst species of both prokarotes and eukaryotes revealed by genome sequences [10].

Transhydrogenase has three components: dI, which has the binding site for NAD^+/NADH, and dIII, which has the binding site for $NADP^+$/NADPH, protrude from the membrane (into the matrix of mitochondria and the cytoplasm of bacteria), and dII spans the membrane. High resolution structures in different nucleotide-bound states are available for dI from *Rhodospirillum rubrum* [11,12] and *Escherichia coli* [13], and for dIII from *Bos taurus* [14], *Homo sapiens* [15] and *R. rubrum* [16]. There are also several X-ray structures of an asymmetric dI_2dIII_1 complex of the *R. rubrum* enzyme [17–20] that reveal features of the direct hydride transfer between bound nucleotides. There is, as yet, no high resolution structure of dII.

Following early experiments on the steady-state kinetics of transhydrogenase [21–23], the two classes of binding site on the enzyme were thought to be specific for their respective nucleotides. There is direct evidence for specific binding to isolated dI of NADH relative to NADPH from fluorescence [24] and NMR [25] experiments, but tight nucleotide binding and slow rates of exchange have made experiments with isolated dIII more difficult. There has been a tacit assumption of specificity because binding of a nucleotide into the "wrong" site would result in enzyme slip: for example, inward proton translocation, and hence dissipation of Δp, could take place without any net oxidation or reduction of the nucleotides.

The activity of transhydrogenase is usually measured using analogue nucleotides having distinctive absorbance spectra. Typically, the reduction of acetyl pyridine adenine dinucleotide ($AcPdAD^+$, an analogue of NAD^+) by NADPH is used to measure the rate of the "reverse reaction" (right to left in Eq. (1)). Experiments with $AcPdAD^+$ also reveal an interesting set of partial reactions [22,26,27]. The "cyclic reaction" is defined [28] as the combined reduction of $NADP^+$ by NADH, and oxidation of NADPH by $AcPdAD^+$, when both take place without either the $NADP^+$ or NADPH dissociating from their binding site on dIII (see Fig. 1a); the net reaction is the reduction of $AcPdAD^+$ by NADH, and is not accompanied by proton translocation [27,29,30].

Abbreviations: $AcPdAD^+$, acetyl pyridine adenine dinucleotide (oxidised form); dIII. E155W, the dIII component of transhydrogenase in which Glu155 has been substituted by Trp (etc.)

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The cyclic reaction is mechanistically very significant because $NADP^+$/NADPH binding to, and dissociation from, dIII appear to be coupled by long-distance conformational changes to proton translocation across the membrane through dII [27,31,32]. In dI_2dIII_1 complexes, hydride transfer between bound nucleotides [33,34], $AcPdAD^+$ binding and AcPdADH release, and NADH binding and NAD^+ release, to/from dI [35] are all fast processes but $NADP^+$ and NADPH dissociation from dIII [36–39] is extremely slow. This combination results in rapid cyclic but greatly depressed rates of forward and reverse transhydrogenation in dI_2dIII_1 complexes.

A slow rate of what we shall here call an "aberrant cyclic reaction" was observed in bacterial membrane fractions, and in purified transhydrogenase of *E. coli* [27,28,40–42]. It was manifested as $AcPdAD^+$ reduction by NADH ostensibly in the absence of $NADP^+$/NADPH, and it was attributed to the binding of either $AcPdAD^+$/AcPdADH or NAD^+/NADH into the wrong site on dIII [41,42]. Thus, either the $AcPdAD^+$/AcPdADH or the NAD^+/NADH, whilst remaining bound to dIII, are alternately reduced by NADH, and oxidised by $AcPdAD^+$, bound to dI. The form of aberrant cyclic in which NAD^+/NADH operates within the dIII site is shown in Fig. 1b. Typically, the aberrant cyclic reaction in intact transhydrogenase occurs at only a few percent of the rate of the cyclic reaction [28]. However, in an interesting series of experiments conducted by Pedersen et al. [43], tightly bound $NADP^+$ and NADPH were removed from isolated *E. coli* dIII by treatment with alkaline phosphatase (see below), and the resulting "apo-dIII" was used to produce hybrid complexes with *R. rubrum* dI (see also [39]). These complexes were found to catalyse remarkably rapid rates of the aberrant cyclic reaction, and thus raised questions about nucleotide specificity of the dIII site in transhydrogenase. In the current paper we repeat the experiments of Pedersen et al. but using dI_2dIII_1 complexes in which both components were derived from the *R. rubrum* enzyme. Equivalent results were obtained, although our analysis of the reaction kinetics establishes that NAD^+/NADH, but not $AcPdAD^+$/AcPdADH, can bind to the dIII site and support the aberrant cyclic reaction. Moreover, the experimental protocol enabled us to show that the binding of nucleotide (whether $NADP^+$ or NADH) into the dIII site of dI_2dIII_1 complexes is extremely slow. We have also isolated an R165A mutant of the dIII component of *R. rubrum* transhydrogenase. X-ray structures show that, in wild-type dIII, the guanidinium sidechain of Arg165 forms two hydrogen bonds to the 2′-phosphate of $NADP^+$ and NADPH [19]. Mutation of the equivalent residue (Arg425) and the adjacent Lys424 in *E. coli* dIII led to weaker binding of $NADP^+$/NADPH [44,45]. In the R165A mutant of *R. rubrum* dIII, we also find that $NADP^+$ binds more weakly, making it easier experimentally to prepare the apo-form of the protein without the need for alkaline phosphatase; again we find evidence of an aberrant cyclic reaction in which NADH but not $AcPdAD^+$ binds into the wrong site. Support for the low specificity, and for very slow binding of nucleotides, into the dIII site of dI_2dIII_1 complexes constructed using either wild-type apo-dIII or apo-dIII.R165A is also provided by fluorescence experiments. The physiological significance of NADH binding into the wrong site of transhydrogenase will be discussed.

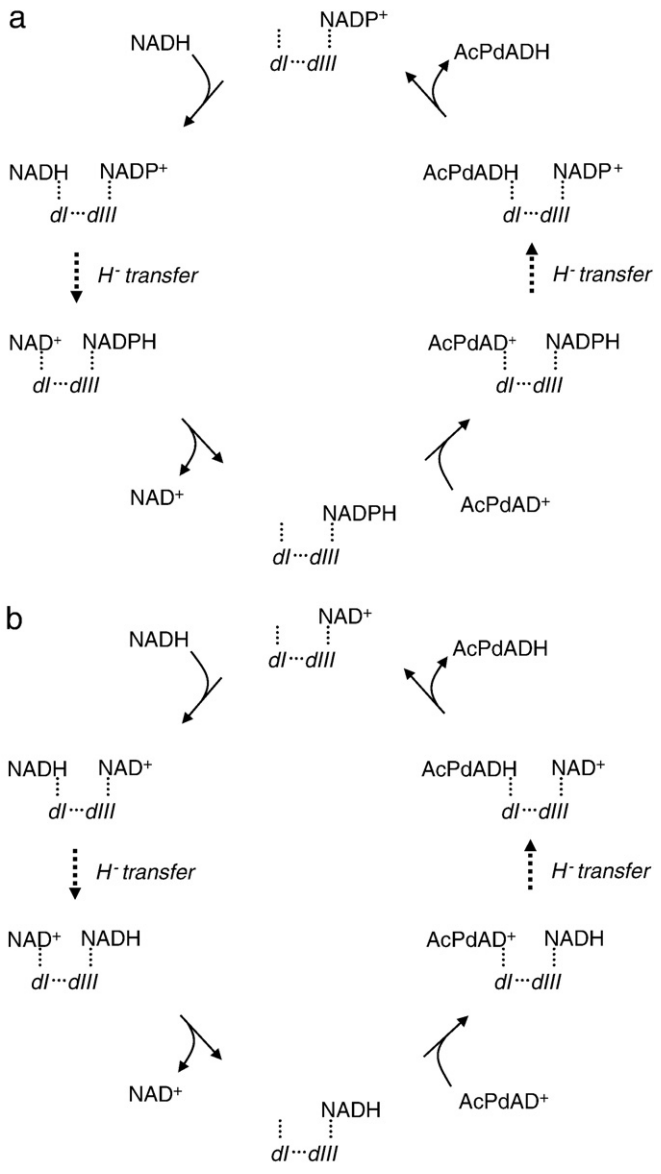

Fig. 1. Partial reactions of transhydrogenase. (a) The cyclic reaction (only NAD^+, NADH, $AcPdAD^+$ and AcPdADH occupy the dI site, and only $NADP^+$ and NADPH occupy the dIII site). (b) An aberrant cyclic reaction (with NAD^+ and NADH occupying the dIII site). The schemes apply to dI_2dIII_1 complexes, and in a limited way, to the intact enzyme (see text). The dotted lines indicate interactions between dI and dIII, and between the protein components and bound nucleotides. The dotted arrows show the two hydride-transfer steps in the cyclic reaction, and in the aberrant cyclic reaction. Only one of the two dI subunits is depicted.

2. Materials and methods

Recombinant dI, dIII and the mutant, dIII.E155W, of *R. rubrum* transhydrogenase were expressed from plasmids pCD1 [24], pNIC2 [46] and pJDV1 [47], respectively, in appropriate strains of *E. coli* after growth in LB medium at 37 °C and induction with isopropyl thiogalactoside. The proteins were purified from harvested cells by column chromatography, and stored as described in the earlier reports (and see [48]). Protein concentrations were determined using the microtannin assay [49]. The $NADP^+$ content of some dIII preparations was assayed as described [36,50]. In attempts to lower the $NADP^+$ content of dIII, purified protein (3.0 ml) was passed twice through two sequentially mounted 5 ml HiTrap desalting columns (GE Healthcare) pre-equilibrated with 20 mM Tris–HCl, pH 8.0. $NADP^+$ was more thoroughly removed from dIII by the procedure described for the *E. coli* protein [43]. *R. rubrum* dIII was first dialysed against 20 mM CHES-KOH, pH 9.0 for 16 h at 4 °C. Aliquots (typically 200 μl of 20 μM protein) were then incubated with calf-intestinal alkaline phosphatase (40 U) from New England Biolabs for 10 min at 37 °C, plunged into ice, and used within 30 min. To confirm that this procedure led to removal of $NADP^+$, the protein aliquots (before and after phosphatase treatment) were added to 1.8 ml 20 mM MOPS-KOH, pH 7.0, 5 mM $MgCl_2$, 2 mM isocitrate, and the fluorescence change resulting from the addition of 1 U of porcine $NADP^+$-linked isocitrate dehydrogenase (Sigma Life Science) was recorded using 340 nm excitation and 460 nm emission on a PTI Quantamaster fluorimeter. To confirm the generation of NAD^+ from alkaline phosphatase treatment of dIII, similar protein aliquots were added to 1.8 ml

20 mM MOPS-KOH, pH 7.0, 5 mM $MgCl_2$, 17 mM ethanol, and the fluorescence changes upon addition of 10 U of yeast alcohol dehydrogenase (Sigma Life Science) were recorded.

Plasmid pCD1 was subjected to site-directed mutagenesis using the Stratagene Quikchange kit and DNA primers from AltaBiosciences to give the plasmid pLH4 harbouring the gene encoding dI.W72F. This plasmid was used to transform cells of *E. coli* C600, and the mutant dI protein was expressed and purified as described for wild-type dI (see above).

Plasmid pNIC2 was subjected to site-directed mutagenesis by Yorkshire Biosciences Ltd. to give the construct, pLH1, harbouring the gene encoding dIII.R165A. This plasmid was used to transform cells of *E. coli* BL21(DE3), and the mutant protein was expressed as described for wild-type dIII (see above). However, unlike wild-type dIII the mutant protein was predominantly found in the inclusion-body fraction after cell breakage, and a new procedure was therefore devised for purification. Cells were harvested from 3.2 l of culture, and washed and sonicated in 50 mM Tris–HCl, pH 8.0, 1.0 mM EDTA, 100 μM $NADP^+$, 500 μM phenylmethanesulfonyl fluoride, as described for the wild-type protein (see above). The resulting extract was centrifuged at 150,000×*g* for 1 h, and the pellet was resuspended in 80 ml 20 mM Tris–HCl, pH 8.0, 6.0 M guanidine-HCl, 4 μM $NADP^+$, and incubated on ice for 30 min. After subsequent centrifugation at 90,000×*g*, the supernatant was diluted 35-fold in 20 mM Tris–HCl, pH 8.0, 6.0 M guanidine-HCl, 4 μM $NADP^+$, and stored at −20 °C. Approximately 40 ml of the stored material was thawed on ice and dialysed against 4 l 30 mM HEPES-KOH, pH 8.0, 10 μM $NADP^+$, 15% (v/v) glycerol at 4 °C for 1 h. The dialysed material was concentrated by loading on to a 5 ml QHP HiTrap column (GE Healthcare) pre-equilibrated with 20 mM Tris–HCl, pH 8.0, 4 μM $NADP^+$ and eluting with 20 mM Tris–HCl, pH 8.0, 4 μM $NADP^+$, 2.0 M NaCl. Analysis of this material by SDS-PAGE established that the dIII.R165A protein was >95% pure. $NADP^+$ was removed from the purified protein (3.0 ml) by passing twice through two sequentially mounted 5 ml HiTrap desalting columns pre-equilibrated with 20 mM Tris–HCl, pH 8.0.

Plasmid pLH1 was subjected to another round of site-directed mutagenesis using the Stratagene Quikchange kit and AltaBiosciences DNA primers to give the plasmid pLH2 harbouring the gene encoding the double mutant, dIII.E155W.R165A. This plasmid was used to transform cells of *E. coli* BL21(DE3), and the double-mutant protein was expressed and purified as described above for dIII.R165A.

$AcPdAd^+$ reduction was measured from the absorbance change at 375 nm recorded on a Shimadzu UV-2401 spectrophotometer with an absorbance coefficient of 6.1 mM^{-1} cm^{-1}. The standard assay for cyclic transhydrogenation contained 50 mM MOPS-KOH, pH 7.2, 50 mM KCl, 2 mM $MgCl_2$, 100 μM NADH, 1.0 μM dI and 30 nM dIII (either in its $NADP^+$-bound state or supplemented with 100 μM $NADP^+$) at 25 °C; after 2 min pre-incubation, the reaction was initiated by addition of 200 μM $AcPdAD^+$. The standard assay for reverse transhydrogenation was performed in the same way but using 200 μM NADPH instead of NADH, 5.0 μM dI and 150 nM dIII without added $NADP^+$. Trp-fluorescence changes associated with nucleotide binding were recorded on a PTI Quantamaster fluorimeter.

3. Results

3.1. Nucleotide binding to the apo-dIII component of *R. rubrum* transhydrogenase

The isolated dIII components of *R. rubrum* [36], *E. coli* [38,39] and human [51] transhydrogenase have a high affinity for $NADP^+$/NADPH at their single nucleotide-binding sites; an interpretation of published data [52] indicates that the same is true for bovine dIII [36]. Buffer solutions used in the purification procedure for *R. rubrum* dIII contain 4 μM $NADP^+$ to maintain the site occupancy [53]. Despite its relatively low molecular weight (765 Da), we found $NADP^+$ to be extremely slow to permeate through either Visking or Spectra/Por dialysis membrane (from Medicell), both of which have a reported 12–14 kDa cut-off. We therefore used gel-filtration chromatography in an attempt to remove $NADP^+$ from the dIII protein. After two passages down the column (see Materials and methods), the $NADP^+$ content was still high, at ~0.8 mol nucleotide mol^{-1} dIII (Table 1). Further $NADP^+$ was therefore removed by treatment with alkaline phosphatase, as described for *E. coli* dIII [43]. Although the degree of dissociation of $NADP^+$ from dIII is slight, the high concentration of alkaline phosphatase used in the treatment ensures gradual cleavage of the 2′-phosphate from the nucleotide released into solution, and eventual complete conversion to NAD^+, which binds only very weakly to the protein (see below). To confirm this reasoning, nucleotide fluorescence changes were recorded during addition of iso-citrate (IC) and $NADP^+$-dependent iso-citrate dehydrogenase (ICDH) to solutions of the *R. rubrum* dIII (see Materials and methods and [43]). Before treatment of the dIII with alkaline phosphatase, slow reduction of $NADP^+$ by the IC/ICDH was evident from the fluorescence increase (data not shown); after treatment, the fluorescence change was decreased by >90%. The conversion of bound $NADP^+$ to NAD^+ was checked by measuring the fluorescence change upon addition of NAD^+-dependent alcohol dehydrogenase to the phosphatase-treated samples of dIII (see Materials and methods).

In agreement with earlier results [36], reverse transhydrogenation catalysed by *R. rubrum* dI_2dIII_1 complexes formed from dI and untreated dIII was extremely slow: the rate of $AcPdAD^+$ reduction by NADPH in current experiments was approx 1.4 mol $AcPdAD^+$ reduced mol^{-1} dIII min^{-1}. Relative to the much faster reverse reaction in the intact enzyme (>1000 fold), that in dI_2dIII_1 complexes is heavily limited by very slow release of product $NADP^+$ from dIII [36]. Also in agreement with the previous results, cyclic transhydrogenation in dI_2dIII_1 complexes prepared from dIII untreated with phosphatase was very fast—it takes place by way of the tightly bound $NADP^+$ on dIII (Fig. 1a): the rate of $AcPdAD^+$ reduction by NADH in current preparations in standard assays (see Materials and methods) was 2710 ± 570 mol $AcPdAD^+$ reduced mol^{-1} dIII min^{-1} (mean value from four preparations each of dI and dIII).

The low level of bound $NADP^+$ and NADPH in the phosphatase-treated dIII enables us to measure separately the rates of cyclic and aberrant cyclic transhydrogenation in dI_2dIII_1 complexes. In the experiment shown in Fig. 2a, 30 nM phosphatase-treated dIII was first mixed with excess dI (1 μM) to ensure that essentially all the former was in complex ($K_d \leq 60$ nM [37]), and the proteins were then incubated with NADH for 2 min before addition of $AcPdAD^+$. Reduction of the $AcPdAD^+$ began promptly at a rapid rate (2280 mol $AcPdAD^+$ reduced mol^{-1} dIII min^{-1}) and continued almost linearly for ~30 s before it began to decline through substrate depletion and product accumulation. As was concluded in equivalent experiments with the hybrid *E. coli*/*R. rubrum* dI_2dIII_1 complexes [43], this $AcPdAD^+$ reduction can be attributed, in the absence of $NADP^+$/NADPH, to an aberrant cyclic reaction with either NAD^+/NADH (Fig. 1b) or $AcPdAD^+$/AcPdAdH on the dIII (see also [41,42]). In the experiment shown in Fig. 2d, a similar mixture of dI and phosphatase-treated dIII was incubated with $NADP^+$ and $AcPdAD^+$ for 2 min before addition of NADH. Again rapid $AcPdAD^+$ reduction then began promptly (2360 mol $AcPdAD^+$ reduced mol^{-1} dIII min^{-1}) and

Table 1
$NADP^+$ content of wild-type dIII and dIII.R165A from *R. rubrum* transhydrogenase.

Protein	$NADP^+$ before GF[a] (mol $NADP^+$ mol^{-1} protein)[b]	$NADP^+$ after GF[a] (mol $NADP^+$ mol^{-1} protein)
Wild-type dIII	0.71, 0.79, 0.73	0.75, 0.78, 0.78
dIII.R165A	0.28, 0.35, 0.12	0.00, 0.01, 0.04

[a] GF = gel filtration, see Materials and methods.
[b] The $NADP^+$ content was determined as described in Materials and methods. Values are shown for three separate protein preparations each of wild-type and mutant dIII.

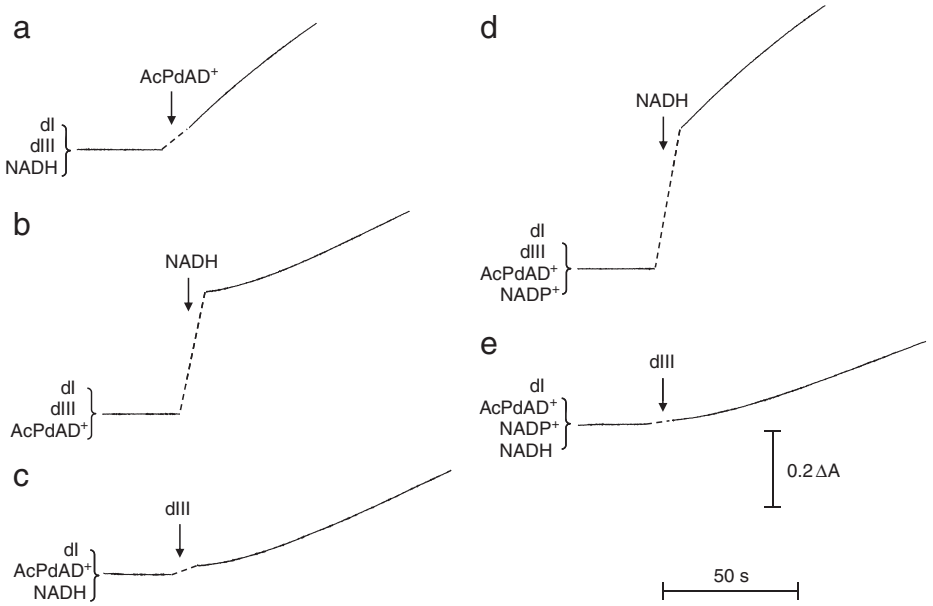

Fig. 2. AcPdAD$^+$ reduction by dI_2dIII_1 complexes from *R. rubrum* transhydrogenase. AcPdAD$^+$ reduction was measured from the absorbance change at 375 nm. Each assay contained 50 mM MOPS-KOH, pH 7.2, 50 mM KCl and 2 mM $MgCl_2$. $T = 25$ °C. Nucleotides and transhydrogenase protein components, present at the start of the experiment, are shown behind the curly brackets; recording began approx 2 min after their mixing by addition of either nucleotide or protein as indicated by arrows. For clarity, disturbances caused by the mixing procedure are replaced by the dashed lines; the instantaneous change upon mixing is due to the absorbance of added nucleotide and protein. Where shown, dI was added to give 1.0 μM, and dIII, 30 nM. The dIII used in all these experiments was the wild-type form pre-treated with alkaline phosphatase, as described in Materials and methods. Where shown in (a), (b) and (d), the nucleotide additions were: NADH, 200 μM; AcPdAD$^+$, 200 μM; and NADP$^+$, 200 μM. Where shown in (c) and (e), they were: NADH, 100 μM; AcPdAD$^+$, 200 μM; and NADP$^+$, 100 μM.

continued at an almost linear rate for ~30 s. In principle, this could have arisen from a combination of cyclic and aberrant cyclic transhydrogenation depending on whether NADP$^+$/NADPH occupied the dIII site (cyclic) or either NAD$^+$/NADH or AcPdAD$^+$/AcPdADH (aberrant cyclic).

A study of the kinetics of AcPdAD$^+$ reduction helps to identify the nucleotides bound to dIII. Firstly, we investigated the aberrant cyclic reaction. When the experiment described in Fig. 2a was repeated with a shorter exposure of the dI_2dIII_1 complexes to NADH (~30 s) before adding the AcPdAD$^+$, the reaction began with a slight lag, and at even shorter exposures, the duration of the lag phase increased (data not shown). This mixing protocol was too awkward for reliable measurement but the experiment suggests that a period of incubation of the protein with NADH is needed before the full rate of the aberrant cyclic reaction is reached. In support of this, the lag phase was pronounced when the order of nucleotide additions was reversed, and the dI_2dIII_1 complexes were pre-incubated with AcPdAD$^+$ before initiating the reaction with NADH (Fig. 2b). The duration of the lag phase in this experimental format (arbitrarily defined as the time taken for the reaction to reach half its maximal rate—see also Fig. 3) was 15–20 s, and was independent of the time of exposure (5 to 60 s) to the AcPdAD$^+$ (data not shown). In a different protocol (Fig. 2c), dI was pre-incubated with NADH and AcPdAD$^+$, and the reaction was then initiated by addition of dIII. Again the lag was pronounced; the time taken for the reaction to reach half its maximal rate was 15–20 s, and the lag-phase duration did not depend on the time of pre-incubation of the dI with NADH and AcPdAD$^+$. These results suggest that the lag phase is the result of a slow interaction between NADH and dIII. Note that in dI_2dIII_1 complexes formed from dIII untreated with phosphatase (not shown), there was no lag in equivalent experiments to those shown in Fig. 2b and c because the dIII site was already loaded with NADP$^+$ (thus leading to a prompt cyclic reaction, Fig. 1a). Stopped-flow experiments have established that *R. rubrum* dI and dIII very rapidly associate to generate dI_2dIII_1 complexes [53], and that NAD$^+$, NADH, AcPdAD$^+$, AcPdADH binding to, and release from, dI are all very fast relative to the duration of the lag phase seen in the current experiments [35]. The lag phase must arise from a slow rate of binding of NADH to the dIII. Once bound, the NADH on dIII is rapidly oxidised by AcPdAD$^+$ on dI to give NAD$^+$ which remains bound to the dIII. This NAD$^+$ is then re-reduced by NADH on dI in an aberrant cyclic reaction, as shown in Fig. 1b. Note that NADH and NAD$^+$ remain bound in the dIII site during the cycle, and that NAD$^+$, NADH, AcPdAD$^+$ and

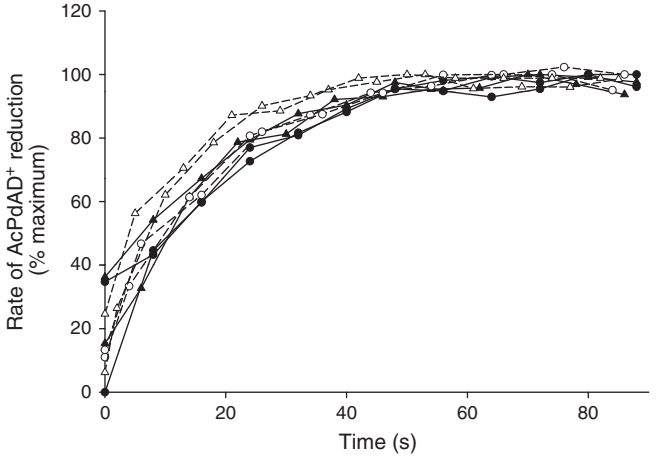

Fig. 3. Rates of AcPdAD$^+$ reduction by dI_2dIII_1 complexes from *R. rubrum* transhydrogenase during the lag phase at the onset of cyclic and aberrant cyclic transhydrogenation. The apo-form of wild-type dIII was prepared by phosphatase treatment, and of dIII.R165A, by two passages down a gel-filtration chromatography column (see Materials and methods). Rates of AcPdAD$^+$ reduction were measured from experiments: ●, with complexes of wild-type dI and wild-type apo-dIII, using the protocol shown in Fig. 2a; ○, from similar experiments but using complexes formed from wild-type dI and apo-dIII.R165A; ▲, from experiments using wild-type dI and wild-type apo-dIII, and initiating the reaction with a mixture of 200 μM NADH plus 200 μM NADP$^+$; △, from similar experiments using wild-type dI and apo-dIII.R165A. Rates were calculated from tangents to the absorbance change at 375 nm, and were plotted as a function of time after initiating the reaction. Results of duplicate experiments from each experimental condition are presented to illustrate scatter on the data.

AcPdADH on dI rapidly exchange with nucleotides in solution. In contrast to NADH, $AcPdAD^+$ is unable to bind to dIII and participate in an aberrant cyclic reaction: if $AcPdAD^+$ had bound to dIII during the 60 s incubation period in the experiment of Fig. 2b, then the reaction would have begun without a lag after the addition of NADH. Thus, the participation of $AcPdAD^+$ in the experiments shown in Fig. 2 is only through its binding to dI.

Further study of the $AcPdAD^+$-reduction kinetics showed that the binding of $NADP^+$ to apo-dIII prior to a cyclic reaction is also a slow process. When the experiment of Fig. 2d (see above) was repeated with a shorter exposure (~30 s) of the dI_2dIII_1 complexes to $NADP^+$ and $AcPdAD^+$ before adding NADH, the reaction began with a slight lag. At even shorter exposures, the duration of the lag phase increased but this mixing protocol was again too awkward for reliable measurement. However, in a modified protocol (Fig. 2e), dI was preincubated with $NADP^+$, $AcPdAD^+$ and NADH, and reaction then initiated by addition of phosphatase-treated dIII. In this case the lag phase at the onset of the reaction was pronounced; the time taken for the reaction to reach half its maximal rate was again 15–20 s. It is concluded that the lag in this experiment arises from slow binding of $NADP^+$ to the dIII before the onset of cyclic transhydrogenation (Fig. 1a).

The steady-state rate of the aberrant cyclic reaction was similar to that of the cyclic reaction (compare the experiments of Fig. 2a and d, respectively). That is, the rate of $AcPdAD^+$ reduction was similar when NAD^+/NADH ("wrong" nucleotides, Fig. 1b) as distinct from $NADP^+$/NADPH ("right" nucleotides, Fig. 1a) occupied the dIII site.

We have used the analysis developed above to determine the kinetics of binding of the wrong and right nucleotides into the dIII site. In the first set of experiments, a protocol similar to that shown in Fig. 2b for aberrant cyclic transhydrogenation was adopted. The instantaneous rate of $AcPdAD^+$ reduction (the first derivative of the absorbance change) during the lag phase was plotted against time in Fig. 3. Since the rate of NADH binding is slow, and the rates of other component reactions are fast (see above), the rate of $AcPdAD^+$ reduction during the lag phase is proportional to the degree of occupancy of the dIII site. The plots therefore show the filling of the dIII site with NADH. In a second set of experiments, the protocol was modified by adding $NADP^+$ simultaneously with the NADH; again the instantaneous rate of $AcPdAD^+$ reduction during the lag phase was plotted against time (Fig. 3). Here, the NADH and $NADP^+$ compete for binding to dIII to produce a population of complexes catalysing aberrant cyclic, and a population catalysing cyclic transhydrogenation. Fig. 3 plots were normalised to the same maximum rate (at the end of the lag phase), and show that representative experiments from the two sets gave similar binding kinetics.

The protocols of the experiments used in Fig. 2 were also extended to compare the concentration dependences for NADH and $NADP^+$. The dependence of the rate of the aberrant cyclic reaction on the NADH concentration, and of the rate of the cyclic reaction on the $NADP^+$ concentration, are shown in Fig. 4. In both sets of experiments, time was given to allow the NADH and the $NADP^+$ to reach binding equilibrium with the dIII site before initiating the reaction. The nucleotide concentration needed to reach the respective half-maximal rate was 40 μM for NADH, and 5 μM for $NADP^+$. The former value is probably dominated by the relatively low binding affinity of NADH for dI ($K_d \approx 20$ μM) [37]; the latter might partly reflect the higher affinity of the $NADP^+$ for dIII. However, analyses of these data are complicated by a small contribution to the intial rate of the cyclic reaction from the aberrant cyclic reaction at low concentrations of $NADP^+$ even during the first few seconds after adding the NADH, and also from the kinetic complexities of these reactions, including the substrate inhibition arising because NADH and $AcPdAD^+$ both compete for the same binding site on dI—observe the decrease in rate at high concentrations of NADH in the top panel of Fig. 4, and see [54].

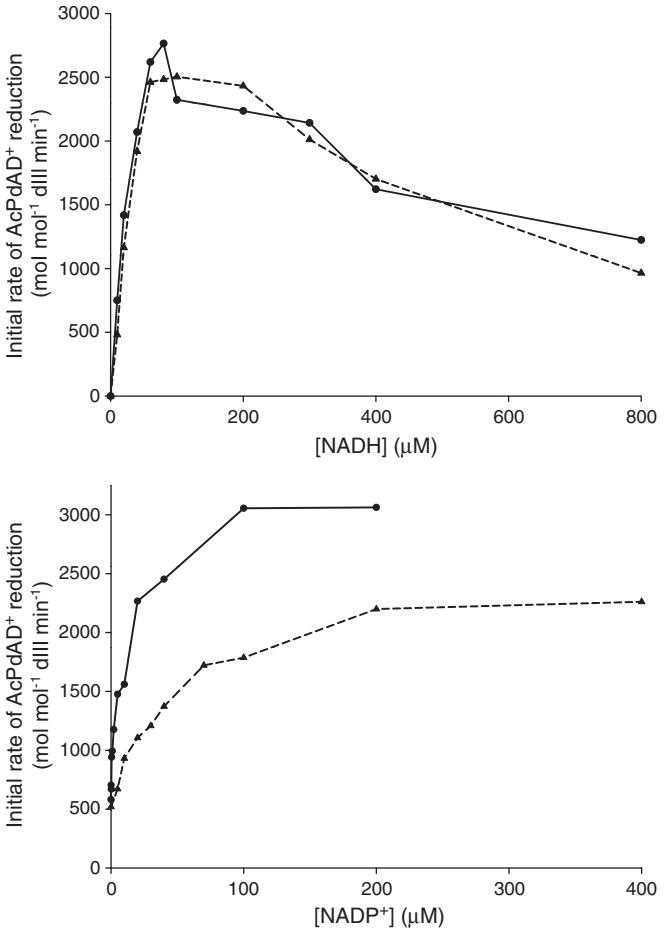

Fig. 4. The dependence of the rate of $AcPdAD^+$ reduction upon NADH concentration during aberrant cyclic, and upon $NADP^+$ concentration, during cyclic transhydrogenation. Experiments were performed with the apo-form of either wild-type dIII, prepared by phosphatase treatment, or dIII.R165A, prepared by two passages down a gel-filtration chromatography column (see Materials and methods). The buffer and protein concentrations were as described in Fig. 2. ● (and solid lines), complexes formed from wild-type dI and wild-type apo-dIII; ▲ (and dashed lines), complexes formed from wild-type dI and apo-dIII.R165A. Top panel: the buffer, together with dI and the appropriate dIII, were incubated with NADH (at the concentration shown) for 5 min, and the aberrant cyclic reaction was then initiated with 200 μM $AcPdAD^+$. Bottom panel: the buffer, together with dI and the appropriate dIII, were incubated with 200 μM $AcPdAD^+$ and $NADP^+$ (at the concentration shown) for 5 min and the reaction was then initiated with 100 μM NADH. Rates were measured immediately after disturbances from the mixing had subsided.

3.2. Nucleotide binding to the R165A mutant of R. rubrum dIII

Although wild-type dIII was mostly expressed in the soluble fraction of host cells (e.g. [36]), the dIII.R165A mutant was expressed predominantly in inclusion bodies. A new procedure was therefore devised to purify dIII.R165A involving solubilisation of the protein followed by refolding (see Materials and methods). Control experiments established that wild-type dIII recovered full activity when subjected to the same procedure (results not shown). Table 1 reveals that in contrast to wild-type dIII, bound $NADP^+$ originating from preparation buffers was almost completely removed from dIII.R165A by two steps of gel-filtration chromatography (see Materials and methods)—this therefore provides a simple procedure for preparing apo-dIII.R165A; phosphatase treatment of the mutant protein is not required. The rate of reverse transhydrogenation catalysed by complexes of dI and dIII.R165A in the standard assay conditions (see Materials and methods) was approx 13 mol $AcPdAD^+$ reduced mol^{-1} dIII min^{-1}—about 9-fold greater than the rate obtained with wild-type protein. An increase in the rate of reverse transhydrogenation

was also observed for the equivalent mutation in *E. coli* dIII in complex with *R. rubrum* dI [45]. The increase is attributable to the enhanced rate of $NADP^+$ release from the mutant proteins. Note that, in complexes of *R. rubrum* dI and dIII.R165A, the rate of reverse transhydrogenation is still about 100-fold lower than the rate of the cyclic reaction (see below): evidently, $NADP^+$ release from dIII.R165A still heavily restricts the reverse reaction.

Experiments were performed using protocols identical to those described in Fig. 2a and d but with mixtures of dI and apo-dIII.R165A. The steady-state rate of the aberrant cyclic reaction (protocol of Fig. 2a) was about 20% lower, and of the cyclic reaction (protocol of Fig. 2d), about 35% lower, than those observed with the mixtures of dI and phosphatase-treated wild-type dIII (data not shown). Importantly however, the absence of a lag at the onset of $AcPdAD^+$ reduction in equivalent experiments to those described in Fig. 2a and d, and the presence of a lag in equivalent experiments to Fig. 2b, c and e, were also a feature of the complexes prepared from dI and apo-dIII.R165A (data not shown). By the same reasoning, the lags are a consequence of slow NADH binding to apo-dIII.R165A at the onset of the aberrant cyclic reaction, and of slow $NADP^+$ binding to apo-dIII.R165A at the onset of the cyclic reaction. The lag kinetics, representing the filling of the site with nucleotide (see above), were similar for NADH and $NADP^+$ binding to dI complexes with apo-dIII, and with apo-dIII.R165A (Fig. 3).

The dependence of the rate of the aberrant cyclic reaction on the NADH concentration, and of the rate of the cyclic reaction on the $NADP^+$ concentration, in mixtures of dI and apo-dIII.R165A are shown in Fig. 4. The NADH profile is not significantly different from that determined with complexes of dI and apo-dIII but the $NADP^+$ profile shows that a higher concentration of the nucleotide is needed to achieve the half-maximal rate than with the wild-type protein complex (~30 μM). Although there are difficulties in the analysis of these experiments (see above), the results indicate that the R165A mutation may have a greater effect on the dIII affinity for $NADP^+$ than for NADH, which is consistent with the specific interaction of Arg165 and the 2′-phosphate group of the bound nucleotide seen in X-ray structures [19].

Note that the experiments with apo-dIII.R165A described above were all performed at similar protein concentrations to those with wild-type apo-dIII. Separate experiments using a similar protocol to that in Fig. 2d, were undertaken to determine the dependence of the rate of cyclic transhydrogenation on dI concentration at fixed dIII (30 nM). The concentration of dI required for a half-maximal rate of the cyclic reaction was ~40 nM with wild-type apo-dIII and ~100 nM with apo-dIII.R165A. This suggests a slightly lower affinity of dI for the mutant but confirms that the experiments described above and below were all performed with dIII essentially in its fully complexed form with dI.

3.3. Nucleotide binding to dI_2dIII_1 complexes determined by protein fluorescence

In the dIII.E155W mutant of *R. rubrum* transhydrogenase, the Glu residue at position 155 is substituted by a Trp, which becomes the only Trp in the protein. The mutant protein has very similar catalytic properties to those of wild-type dIII but fluorescence from Trp155 provides a valuable probe with which to monitor nucleotide binding; emission is more intense when $NADP^+$ is bound to dIII than when NADPH is bound [34,35,37,47,55] and see [38,39]. In the present work, we have used a dIII.E155W.R165A double mutant, and phosphatase-treated dIII.E155W, to examine nucleotide binding to the apo forms of the protein using fluorescence. The rate of cyclic transhydrogenation in complexes formed from dI and dIII.E155W.R165A (1770 ± 130 mol mol^{-1} min^{-1}, the mean for three preparations) was similar to that formed from dI and dIII.R165A (1750 ± 380 mol mol^{-1} min^{-1} for three preparations) in the standard assay—as expected from earlier results with the wild-type protein, the introduction of the E155W mutation does not significantly affect catalytic activity.

We have also isolated a mutant of dI (designated dI.W72F) in which Trp72, the only Trp in dI, was substituted with a Phe. The mutant dI protein was fully active in complex with wild-type dIII, the preparation having a cyclic transhydrogenation rate of 3170 mol $AcPdAD^+$ reduced mol^{-1} dIII min^{-1} in standard assay conditions. The intense and unusually short-wavelength emission at 308 nm of wild-type dI, studied in depth [56,57], was decreased 8-fold in dI.W72F with 275 nm excitation, and 26-fold with 290 nm excitation (not shown); the residual fluorescence ($\lambda_{max} = 303$ nm, probably due to Tyr emission) was not changed upon NADH binding (compare with wild-type dI fluorescence [24])—data not shown. The availability of dI.W72F has therefore enabled us to investigate fluorescence changes (from the now single Trp155) that result from nucleotide binding to the dIII component of generic dI_2dIII_1 complexes without any interference from fluorescence changes due to Trp72 in the dI.

Fig. 5 summarises results from two sets of experiments: complexes were generated from dI.W72F and either apo-dIII.E155W (nucleotide removed by phosphatase-treatment) or apo-dIII.E155W.R165A (nucleotide removed by gel filtration). The Trp155 fluorescence was then measured during addition of various nucleotides, all at 20 μM. In each case, nucleotide addition led to a prompt artefactual decrease in fluorescence due to an "inner-filtering" effect (the absorption of excitation and/or emission light by the nucleotide). Following the inner-filtering effect, $NADP^+$ gave rise to a slow increase in fluorescence (Fig. 5b and e), and the reduced nucleotides, NADPH (Fig. 5a and d) and NADH (Fig. 5c and f), a slow decrease in fluorescence. These fluorescence changes signify nucleotide binding to the dIII component of the complex. Analysis of replicate experiments established that NAD^+ addition led to a small increase (~1%) in fluorescence, but that $AcPdAD^+$ addition did not cause any significant fluorescence change. Although the fluorescence changes produced by the nucleotides were small (<10% of the total emission), and subject to rather large error on the long time scale of the experiments, the kinetics of the changes were approximately the same for oxidised and for reduced nucleotides, and for both types of protein complex. Analysis of several replicate experiments indicated a half time for the changes of approximately 20 s—similar to the half-time for the increase in the rate of cylic and aberrrant cyclic transhydrogenation attributed to $NADP^+$ and NADH binding, respectively, during the lag phase in Fig. 3. Experiments at different nucleotide concentrations with dI_2dIII.E155W.R165A indicated that NADPH bound tightly to the dIII component of the complex ($K_d \approx 0.1$ μM), that $NADP^+$ and NADH bound with K_d values in the region of 5 μM, and that NAD^+ bound only weakly.

If the binding of nucleotide to dIII were a simple, second-order reaction, then the initial rate of the fluorescence change (following the inner-filtering effect) would be directly proportional to the nucleotide concentration. However, at least with $NADP^+$, NADPH and NADH, in dI_2dIII.E155W.R165A complexes, this was not the case: increase in nucleotide concentration led to a less than proportionate increase in initial rate. Because of its high affinity, this was most readily seen with NADPH. Thus, above 1 μM nucleotide, where the binding sites were fully occupied at equilibrium, and below 50 μM nucleotide, where the inner-filtering effect was still managably small, the initial rate of the fluorescence change was independent of nucleotide concentration (data not shown). The experiments reveal that the nucleotide-binding reaction is not a simple second-order process but that binding is likely to be associated with a protein conformational change.

In earlier work (with complexes containing wild-type dI) it was shown that exchange of $NADP^+$ for NADPH led to a decrease in dIII.E155W fluorescence, and exchange of NADPH for $NADP^+$ to an increase in fluorescence [37,47,55] and see [38]. The change in fluorescence could have been explained by resonance energy transfer from Trp155 to the NADPH. However, the current finding that $NADP^+$ itself enhances the fluorescence of apo-dIII indicates that local changes in the environment around Trp155 resulting from nucleotide binding, rather than energy

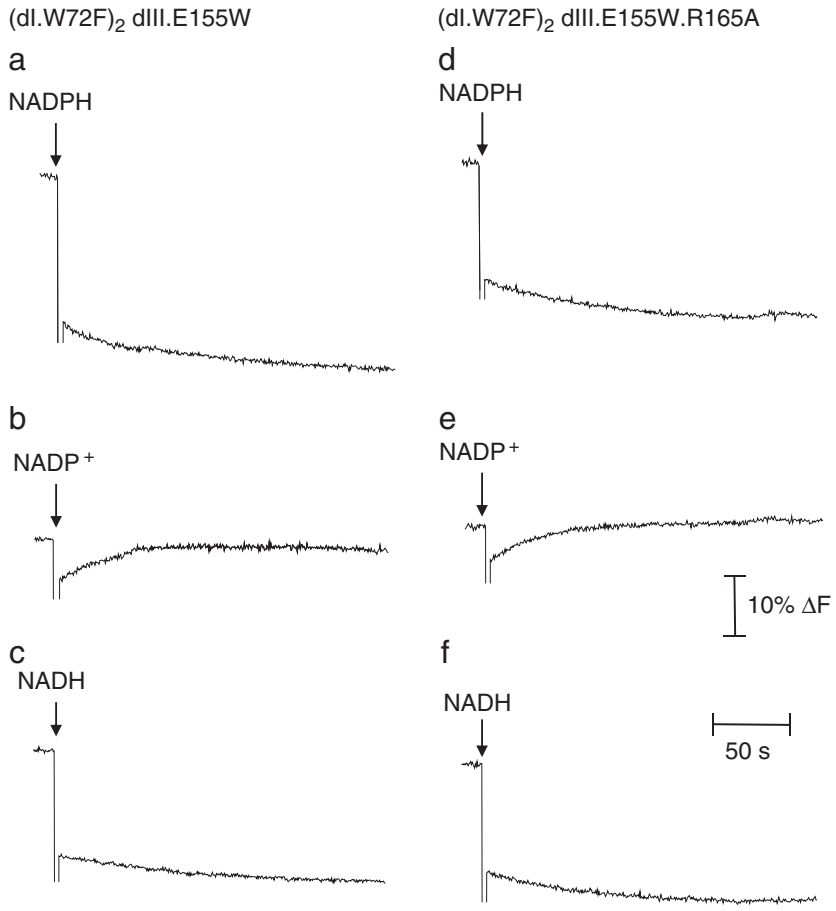

Fig. 5. The fluorescence change of Trp155 in the dIII component of dI_2dIII_1 complexes from *R. rubrum* transhydrogenase caused by addition of nucleotides. Experiments on the left were performed with apo-dIII.E155W, prepared by phosphatase treatment, and those on the right with apo-dIII.E155W.R165A, prepared by two passages down a gel-filtration chromatography column (see Materials and methods). The dIII proteins (400 nM), together with dI.W72F (800 nM), were incubated in 20 mM MOPS-KOH, pH 7.0, for approximately 5 min to record a baseline before addition of nucleotides (all 20 μM) at the arrows. The initial prompt fluorescence decrease in all experiments is the result of the inner-filtering effect of the nucleotide. Excitation, 280 nm; emission, 340 nm; half band widths, 4 nm; $T = 25$ °C.

transfer, cause alterations in the fluorescence emission: changes in the Trp155 environment caused by reduced nucleotides are different from those caused by oxidised nucleotides.

4. Discussion

Studies on high-resolution structures and the reaction kinetics of transhydrogenase have provided a framework for understanding the mechanism of action of the enzyme [31,32] (but see [58] for some unresolved considerations). Central to the mechanism is that, in the forward direction (Eq. (1)), inward proton translocation through dII drives the protein through an "open" state, in which dIII releases the product NADPH and re-binds fresh $NADP^+$, and an "occluded" state, in which the hydride-transfer step takes place. The dI component can bind and release nucleotides in both states. Isolated dI_2dIII_1 complexes, in the absence of the membrane-spanning dII, behave as though they are locked in the occluded state [31]. Thus, they display very rapid rates of hydride transfer between bound nucleotides [33,34] and, as measured in exchange reactions [47], extremely slow rates of release of bound NADPH and $NADP^+$ from their dIII sites. These properties explain why forward and reverse transhydrogenation reactions in dI_2dIII_1 complexes are slow, but the cyclic reaction is fast [36]. The rapid rate of direct hydride transfer is reflected by the close apposition of the C4 atoms of the two nicotinamide rings of, for example, tetrahydro-NADH and $NADP^+$ in crystal structures [19]. NADPH and $NADP^+$ release from dIII in dI_2dIII_1 complexes is thought to be restricted as a result of the loop E "lid" being closed down over the bound nucleotide in the occluded state [15], and see [59].

The present experiments show that the *binding* of nucleotides ($NADP^+$, NADPH and the special cases, NAD^+, NADH) to the dIII site in dI_2dIII_1 complexes is also very slow. The new data, and the results described in previous work, are readily explained by the scheme for nucleotide binding and release shown in Fig. 6. In this scheme, isolated dI_2dIII_1 complexes, whether they are in the apo- or nucleotide-bound forms, are maintained predominantly in the occluded state: the equilibrium constant, K', is small, and K''' is large. The slow rates of $NADP^+$, NADPH, NAD^+ and NADH binding seen in the fluorescence experiments (Fig. 5), and of $NADP^+$ and NADH binding in the analysis of the cyclic and aberrant cyclic assays (Figs. 2 and 3), are all similar. The observed nucleotide binding does not fit to second order reaction kinetics (see Results). In terms of the model, these findings are explained by the notion that the rate-limiting step in nucleotide binding to apo-dIII in dI_2dIII_1 complexes is the conversion of the occluded to the open states, which is governed by k_1; the true second order rate constant k_3 is expected to be large, and to give rise to rapid rates of binding only to the open state. Thus, the kinetics of the lag phase (Fig. 3), and of the fluorescence change resulting from nucleotide binding (Fig. 5), give the value of k_1 (~0.03 s^{-1}). In the intact enzyme this is increased to at least the turnover number of the enzyme (~50 s^{-1}), and the step is a candidate for one whose rate is modulated by remote interaction with H^+ during translocation through dII.

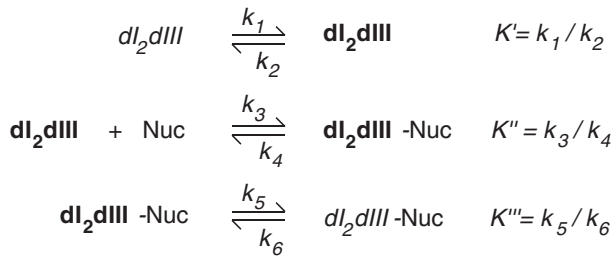

Fig. 6. Nucleotide binding to the dIII component of the dI_2dIII_1 complex. The dI_2dIII_1 complex can adopt either an occluded state (shown in italics) or an open state (shown in bold). In isolated complexes, the occluded state is more stable (K' is small and K''' is large). Nucleotide ("Nuc") binding to dIII can only take place when the complex is in the open state. Binding of nucleotide to the dI component is not shown but it can occur in either the open or the occluded states. Hydride-transfer reactions can only take place in the occluded state. The subscript "1" is omitted from dIII in the dI_2dIII_1 complex for clarity.

$NADP^+$ dissociates much more readily from the R165A mutant of dIII than from the wild-type protein (Table 1). However, the rates of nucleotide binding to complexes of dI and dIII.R165A, from the kinetics of the lag in cyclic and aberrant cyclic transhydrogenation, and from the kinetics of fluorescence changes, were not significantly different from those observed in complexes with wild-type dIII. It is therefore concluded that the affinity for $NADP^+$ of the open state of dIII in the complexes ($K_d = 1/K''$ in Fig. 6) is decreased by the mutation but that the value of k_1 is unaffected. The decreased affinity is not unexpected because, in crystal structures of wild-type dIII, Arg165 (together with Lys164 and Ser166) participates in a network of H-bond interactions with the 2′-phosphate of $NADP^+$/NADPH [15,19,60]. The three amino acid residues are located at the N-terminus of loop E; the unchanged k_1 value indicates that the R165A mutation does not greatly affect the rate of opening of loop E to expose the nucleotide-binding site during the transition between the open and occluded states.

The somewhat unexpected finding that NADH/NAD^+ (or, it was thought, AcPdADH/$AcPdAD^+$) can bind into the dIII component of hybrid (*R. rubrum*/*E. coli*) dI_2dIII_1 complexes and support high rates of an aberrant cyclic reaction [43], is confirmed by the experiments described in this report with complexes from the *R. rubrum* enzyme; in fact, the reaction kinetics show that only NADH/NAD^+ (and not AcPdADH/$AcPdAD^+$) are effective at the concentrations used. The data from the reaction kinetics and fluorescence experiments suggest that the affinities of dIII for nucleotides are in the order, $NADPH > NADP^+ \approx NADH > NAD^+ > AcPdAD^+$. Structural differences between the NADPH- and $NADP^+$-bound forms of dIII are detected as chemical shift changes in NMR experiments [61,62] but these differences have not been identified in X-ray data at current resolution [18,60]. It seems that, also with NAD^+/NADH, the reduced nucleotide has the higher affinity for dIII. Weaker binding of NADH than of NADPH (and of NAD^+ than $NADP^+$) is presumably a consequence of the replacement of the 2′-phosphate group with a hydroxyl, leading to a loss of some or all of the H-bonds with Lys164, Arg165 and Ser168, to local structural re-organisation, and thus to a decrease in binding energy. The still weaker binding of $AcPdAD^+$ to dIII is the result of the replacement of the carboxamide of the nicotinamide ring with an acetyl group. Depending on species, the NH_2 of the $NADP^+$ carboxamide makes potential H-bonds with either or both of the peptide carbonyls of the highly conserved, Ala88 and Val87 [14,15,19]. The weaker binding of $AcPdAD^+$ may be a consequence of the fact that its acetyl group is unable to make equivalent H-bonds. Because of difficulties in interpreting the kinetics, the conclusion that the decrease of affinity for $NADP^+$ appears to be more pronounced than that for NADH in dIII.R165A (see Fig. 4) should be treated with caution but it can be understood if, as expected, binding with the latter nucleotide relies less on interactions with the guanidinium group of Arg165.

An interesting question remains as to the possibility that, in some metabolic conditions in the cell, nucleotides might occupy the wrong sites of transhydrogenase. Of course, the cyclic and aberrant cyclic reactions of the enzyme are only observed experimentally with the non-physiological nucleotide, $AcPdAD^+$. The equivalents of the two reactions with physiological nucleotides have no metabolic consequence; neither results in any change in nucleotide redox state (both entail the reduction of NAD^+ by NADH), and neither leads to proton translocation across the membrane. Moreover, the cyclic and aberrant cyclic reactions would probably only take place at relatively low rates in the living cell: in contrast to dI_2dIII_1 complexes, the occluded state of the intact enzyme rapidly interconverts with the open state to permit exchange of the dIII-bound nucleotides with those in the solvent—and this breaks the cycling. Of possible metabolic significance is the finding that the specificity of the dIII site for $NADP^+$/NADPH relative to NAD^+/NADH is not as great as was once thought. The lack of information on specificity constants for nucleotides at this site makes analysis difficult. However, if we assume that dI is highly specific for NAD^+/NADH, which is supported experimentally [24,25], then two situations may be considered. Firstly, if NADH binds to dIII and NAD^+ to dI, then hydride transfer (not resulting in any change in overall nucleotide redox state) will lead to outward proton translocation. Since there is no free energy available from this redox reaction, it will only take place if proton transfer through transhydrogenase is downhill, i.e. if the proton electrochemical potential inside the bacterial cell is positive relative to that outside ($\Delta p > 0$)—perhaps an unlikely situation under physiological conditions. Secondly, if NAD^+ binds to dIII and NADH to dI, then hydride transfer (again not resulting in any overall change in nucleotide redox state) will lead to inward proton translocation; this will only take place if $\Delta p < 0$. We should expect a single proton to be translocated for each hydride-ion equivalent transferred from NADH to NAD^+, equivalent to the experimentally determined, $H^+/H^- = 1$, during $AcPdAD^+$ reduction by NADPH [63]. In this situation transhydrogenase would effectively dissipate Δp and "uncouple", for example, the respiratory chain from ATP synthesis. Transhydrogenase would behave as an outwardly directed proton diode gated by the redox states of the nicotinamide nucleotides. The process could be metabolically important if NAD^+/NADH becomes predominantly oxidised, and if the free concentrations of $NADP^+$/NADPH are low. Our knowledge of the redox states of the nicotinamide nucleotides in bacterial cells, and even in the mitochondrial matrix of animal cells, is very limited, and it is therefore very difficult to assess whether such conditions are ever met, and whether this slip reaction is likely to impinge on metabolism. The alternative possibility, that $NADP^+$/NADPH concentrations are too high ever to allow NAD^+/NADH binding to dIII, and that transhydrogenase always behaves as described by Eq. (1), cannot therefore be ruled out.

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