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Mutations at tyrosine-235 in the mobile loop region of domain I protein of transhydrogenase from *Rhodospirillum rubrum* strongly inhibit hydride transfer

Tania Bizouarn [a], Rachel Grimley [a], Christine Diggle [a], Christopher M. Thomas [b], J. Baz Jackson [a,*]

[a] *Schools of Biochemistry, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK*
[b] *Biological Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK*

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Abstract

Transhydrogenase from mitochondrial and bacterial membranes couples proton translocation to hydride transfer between NAD(H) and NADP(H). The enzyme has three domains, of which domains I and III protrude from the membrane. These possess the NAD(H)- and NADP(H)-binding sites, respectively, whereas domain II spans the membrane. In domain I there is a mobile loop which emanates from the surface of the protein, but which closes down upon NAD(H) binding. In this report we show that the NADP(H)-dependent reduction of acetylpyridine adenine dinucleotide by NADH catalysed by *Rhodospirillum rubrum* transhydrogenase has 'ping-pong' kinetics, confirming that the reaction is cyclic. We then describe the kinetic and thermodynamic properties of mutants of recombinant domain I protein from the *R. rubrum* enzyme, in which Tyr-235 in the mobile loop has been substituted with Phe or Asn residues (dI.Y235F and dI.Y235N, respectively). (1) Equilibrium dialysis measurements show that dI.Y235F and dI.Y235N bind NADH more weakly than wild-type domain I protein (the K_d increases twofold and fourfold, respectively). (2) Reverse transhydrogenation rates (in steady state) of domain I-depleted membrane vesicles reconstituted with either dI.Y235F or dI.Y235N are inhibited by about 50% and 78%, respectively, relative to those obtained in reconstitutions with wild-type domain I protein. (3) Reverse transhydrogenation rates (in steady state) of mixtures of recombinant domain III protein and either dI.Y235F or dI.Y235N are inhibited only by about 10% and 20%, respectively, relative to those obtained in mixtures with wild-type protein. (4) Forward transhydrogenation rates (in both the complete enzyme and in domain I:III complexes) are inhibited even less by the mutations than the reverse reactions. (5) In contrast with (1), (2) and (3), cyclic transhydrogenation was strongly inhibited in both the reconstituted membrane system and in the recombinant domain I:III complexes (only 7–8% activity remains with dI.Y235F, and only 2–3% with dI.Y235N). It was recently established that, in contrast to forward and reverse transhydrogenation, the cyclic reaction is substantially limited by the rate of hydride transfer. It is therefore concluded that mutations at Tyr-235 in the mobile loop severely disrupt the hydride transfer step in the catalytic reaction of transhydrogenase.

Keywords: Transhydrogenase; Proton pump; NMR; Tyrosine-235; Domain I protein; (*Rhodosprirllum rubrum*)

Abbreviations: AcPdAD$^+$, acetylpyridine adenine dinucleotide

* Corresponding author. Fax: +44 121 414 3982; E-mail: j.b.jackson@bham.ac.uk

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1. Introduction

Transhydrogenase, an enzyme found in the inner membrane of animal mitochondria and the cytoplasmic membrane of bacteria, seems to be unique as a proton pump because the standard free energy of its chemical reaction is close to zero:

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

In vivo the reaction is driven from left to right by the free energy of the proton electrochemical gradient (Δp) generated by the action of respiratory or photosynthetic electron transport chains. The function of the enzyme is not known definitively, although a number of possibilities have been discussed (reviewed [1]). Perhaps the most plausible role for mitochondrial transhydrogenase is that it provides a controlling link between Δp and flux through the tricarboxylic acid cycle [1], while in bacteria it probably supplies NADPH for amino acid biosynthesis [2] and for the reduction of glutathione in methanol metabolism [3].

The enzyme has three domains. Domains I and III protrude from the membrane (on the matrix side in mitochondria and on the cytoplasmic side in bacteria). Domain I has the binding site for NAD^+ and NADH, domain III for $NADP^+$ and NADPH. The very hydrophobic domain II spans the membrane. Information on the structure of transhydrogenase is reviewed [4].

Transhydrogenase from *Rhodospirillum rubrum* has a unique subunit composition; domain I exists as a separate polypeptide (formerly called Th_s), which can be removed by washing from everted membrane vesicles (chromatophores). The enzyme can be reconstituted with full recovery of activity by mixing the domain I-depleted membranes, either with purified native [5], or purified recombinant [6], domain I protein.

Although domain III in all known H^+-transhydrogenases is on the same polypeptide as the membrane-spanning domain II, water-soluble recombinant domain III of the *R. rubrum* enzyme was prepared by incorporating a new start codon at the appropriate position in the gene [7]. A mixture of recombinant domain I and III proteins from *R. rubrum* catalyses transhydrogenation reactions in the absence of the membrane-spanning domain II [7].

The rates of 'reverse' and 'forward' transhydrogenation (Eqn. 1) catalysed by the complete, membrane-bound enzyme from *R. rubrum* are limited by slow release of product $NADP^+$ and NADPH, respectively [8]. In mixtures of the domain I/III proteins, the rates of the reverse and forward transhydrogenation reactions are even slower (about 1000-fold) than in the complete enzyme because, in the absence of domain II, the release of $NADP^+$ and NADPH are retarded [7].

Transhydrogenase will also perform a rapid 'cyclic reaction', the reduction of $AcPdAD^+$ (an analogue of NAD^+) by NADH in the presence of either $NADP^+$ or NADPH [9–13]: the NADPH/$NADP^+$ remain permanently bound to the domain III site of the protein, and are alternately oxidized by $AcPdAD^+$ and then reduced by NADH in the domain I site [11,12]. Because the binding of $AcPdAD^+$ and NADH, and the release of AcPdADH and NAD^+, are relatively fast, the reaction is mainly limited by the rate of hydride transfer between nucleotides [11,12]. *R. rubrum* domain I:III complexes catalyse the cyclic reaction at about the same rate as the complete enzyme, indicating that the apparatus for the hydride transfer step is located entirely within domains I and III, and does not require domain II [7]. The $NADP^+$ and NADPH that are tightly bound to recombinant domain III even after purification are sufficient to support the cyclic reaction at high rates in the I:III complexes.

The facility with which components of *R. rubrum* transhydrogenase can be separated and recombined with restoration of activity, promises to be valuable in the study of the mechanism of action of the enzyme. Parallel investigations of the forward, reverse and cyclic reactions are revealing because these processes share some components (hydride transfer) but not others (NADP(H) release, H^+ release), and they are characterized by different rate-limiting steps. This rationale was first used to establish that dicyclohexylcarbodiimide treatment of purifed transhydrogenases from *Rhodobacter capsulatus* [14] and from *E. coli* [11] leads to a decrease in the rate of $NADP^+$ (or proton) release, but has little effect on the rate of hydride transfer. Subsequently, a similar argument was applied to mutants of the *E. coli* enzyme that

were more inhibited in reverse, than in cyclic transhydrogenation [15–17]. In this report we demonstrate that mutations at Tyr-235 (in the NMR-visible 'mobile loop' region [18–20] of domain I of *R. rubrum* transhydrogenase) fall into a different category.

Sequence comparisons show that the mobile loop is a conserved feature in transhydrogenases but is absent from alanine dehydrogenase, which is otherwise very similar in sequence to domain I [5]. On the basis of NMR experiments, it was proposed that the loop closes down on the surface of domain I following the binding of NADH [18–20]. Tyr-235 is conserved in all transhydrogenase sequences as part of a short, Gly-Tyr-Ala motif, and at least in *R. rubrum* domain I protein makes close contact with bound nucleotide [19]. Substitution of Tyr-235 with either Phe or Asn led to changes in the nucleotide binding characteristics of the recombinant domain I protein, but rates of reverse transhydrogenation in domain I-depleted chromatophores reconstituted with mutant proteins were only depressed by about 50% and 80%, respectively, relative to wild-type [20]. Light-driven forward transhydrogenation rates in these reconstituted systems were inhibited only by about 60% and 80%, respectively. However, since forward and reverse transhydrogenation are limited by the rate of product release, it was difficult, on the basis of those observations, either to define the position of the inhibited step, or to estimate the degree of inhibition of that step. In this report an analysis of forward, reverse and cyclic transhydrogenation in reconstituted complete enzyme and in recombinant I:III complexes shows that these mutations lead to specific and pronounced inhibition of hydride transfer.

2. Materials and methods

R. rubrum wild-type strain S1, and the overexpressing strain, RTB2, were grown phototrophically, and chromatophores, and chromatophores depleted of domain I protein were prepared as described [5,21]. The bacteriochlorophyll content of these membranes was assayed using the in vivo extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ [22].

The generation of the Tyr-235 mutants of the *R. rubrum* transhydrogenase domain I protein (dI.Y235F and dI.Y235N) was described [7]. Recombinant domain I and domain III proteins from *R. rubrum* transhydrogenase (wild-type and mutants) were expressed in *E. coli*, and purified by column chromatography as described [6,7], except that, following a suggestion of Dr. Svetlana Sedenlikova of the University of Sheffield, the Reactive Green 19 column used in the preparation of domain I protein was replaced with a 6×3 cm column of Butyl Toyopearl (Tosohaas). The sample was loaded on to the column in 10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (TD buffer) plus 1.5 M $(NH_4)_2SO_4$, washed with 40 ml 1.05 M $(NH_4)_2SO_4$ in TD buffer, and eluted with a gradient (400 ml) of 1.05 M to 0.6 M $(NH_4)_2SO_4$ in TD buffer. A similar column was also included as the last step in the preparation of domain III protein (see [7]). In this case the sample was loaded in 10 mM Tris-HCl, pH 8.0, 10% glycerol (TG buffer) plus 0.41 M $(NH_4)_2SO_4$, and washed with 60 ml 0.41 M $(NH_4)_2SO_4$ in TG buffer, then 40 ml 0.29 M $(NH_4)_2SO_4$ in TG buffer, and eluted with a gradient (400 ml) of 0.29 M to 0.16 M $(NH_4)_2SO_4$ in TG buffer. The proteins were more than 95% pure according to analysis by SDS-PAGE [6,7]. Protein concentration was estimated by the microtannin procedure [23].

Reverse transhydrogenation (the reduction of $AcPdAD^+$ by NADPH), forward transhydrogenation (the reduction of thio-$NADP^+$ by NADH) and cyclic transhydrogenation (reduction of $AcPdAD^+$ by NADH in the presence of added or endogenous $NADP^+$ or NADPH) were measured as described [24], using absorbance coefficients given [25] in the conditions described in the figure legends.

Equilibrium dialysis was carried out using the procedures given [26] in the apparatus described [27].

3. Results

3.1. The effect of mutation at Tyr-235 on the rate of reverse and forward transhydrogenation

In confirmation of earlier results [20], when domain I-depleted chromatophores were reconstituted with dI.Y235F or with dI.Y235N, the V_{max} of 'reverse transhydrogenation' ($AcPdAD^+$ reduction by NADPH) was decreased by about 50% and 78%, respectively, relative to membranes reconstituted with

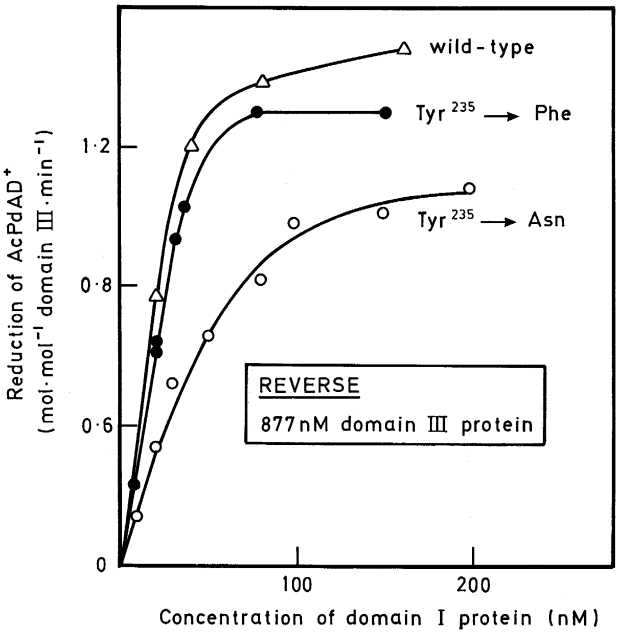

Fig. 1. Reverse transhydrogenation in mixtures of recombinant domain III protein plus wild-type domain I (△) or dI.Y235F (●) or dI.Y235N (○). Experiments were performed in 50 mM Mops, pH 7.2, 50 mM KCl, 2 mM $MgCl_2$, 200 μM NADPH and 200 μM $AcPdAD^+$.

wild-type domain I (results not shown). Note that the rate of this reaction is mainly limited by the release of product $NADP^+$ [8].

Reverse transhydrogenation by a mixture of recombinant domain I and domain III proteins from *R. rubrum* transhydrogenase is even more strongly rate limited by the slow release of $NADP^+$ from domain III [7]. Fig. 1 shows that dI.Y235F and dI.Y235N in mixtures with domain III protein catalyse $AcPdAD^+$ reduction by NADPH at steady-state rates that are only slightly less than those with wild-type domain I. The experiment was performed as a titration at fixed domain III concentration and variable domain I (wild-type or mutant). The results show (see [7]) that the reaction saturates when the domain I concentration is substantially less than that of domain III. Relative to wild-type, only slightly more of the mutant domain I proteins were needed to obtain saturation.

The rate of 'forward transhydrogenation' (reduction of thio-$NADP^+$ by NADH) by a mixture of domain I and III proteins is extremely slow [7]. It is limited by the release of thio-NADPH from domain III. Within experimental error the rates of forward transhydrogenation in mixtures of domain III protein, and either the wild-type or mutant domain I proteins (dI.Y235F and dI.Y235N) were similar, at approximately 0.045 $mol \cdot mol^{-1}$ domain $III \cdot min^{-1}$ (saturated with the respective domain I) — data not shown.

3.2. Cyclic transhydrogenation as a measure of the rate of the hydride transfer step

The rate of cyclic transhydrogenation is mainly limited by hydride transfer (see Section 1). Thus, the

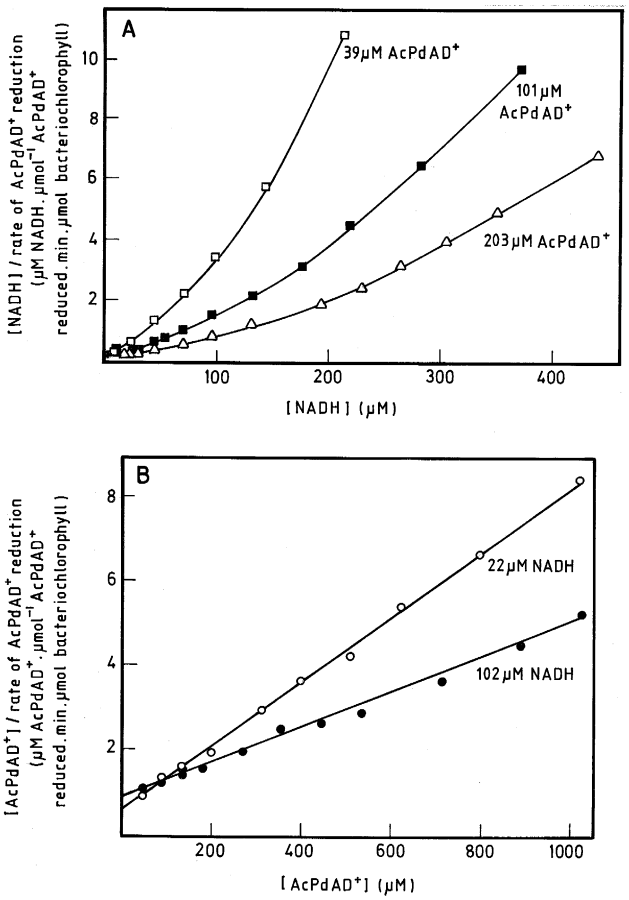

Fig. 2. The cyclic reaction catalysed by chromatophore transhydrogenase is 'ping-pong' in NADH and $AcPdAD^+$. Domain I-depleted chromatophore membranes from a transhydrogenase over-expressing strain of *R. rubrum* (see Section 2) were resuspended in 50 mM Mops, pH 7.0, 50 mM NaCl, 19 μM $NADP^+$, to a final bacteriochlorophyll of 0.7 μM, together with 33nM recombinant domain I protein, at 30°C. The other nucleotide concentrations are given in the figure. The reaction was initiated by addition of $AcPdAD^+$. A; fixed $AcPdAD^+$, variable NADH. B: fixed NADH, variable $AcPdAD^+$.

effects of mutagenesis on the rate of the cyclic reaction should provide a good indication as to the role of individual amino acid residues on the hydride transfer step. A kinetic analysis of the nucleotide substrate dependence of the cyclic reaction of wild-type *R. rubrum* transhydrogenase has not previously been presented, but is necessary to provide a basis for understanding the effects of mutation. In Fig. 2 the reactions were performed with domain I-depleted chromatophore membranes reconstituted with wild-type domain I — by carrying out appropriate controls in the absence of domain I, we can ensure using this

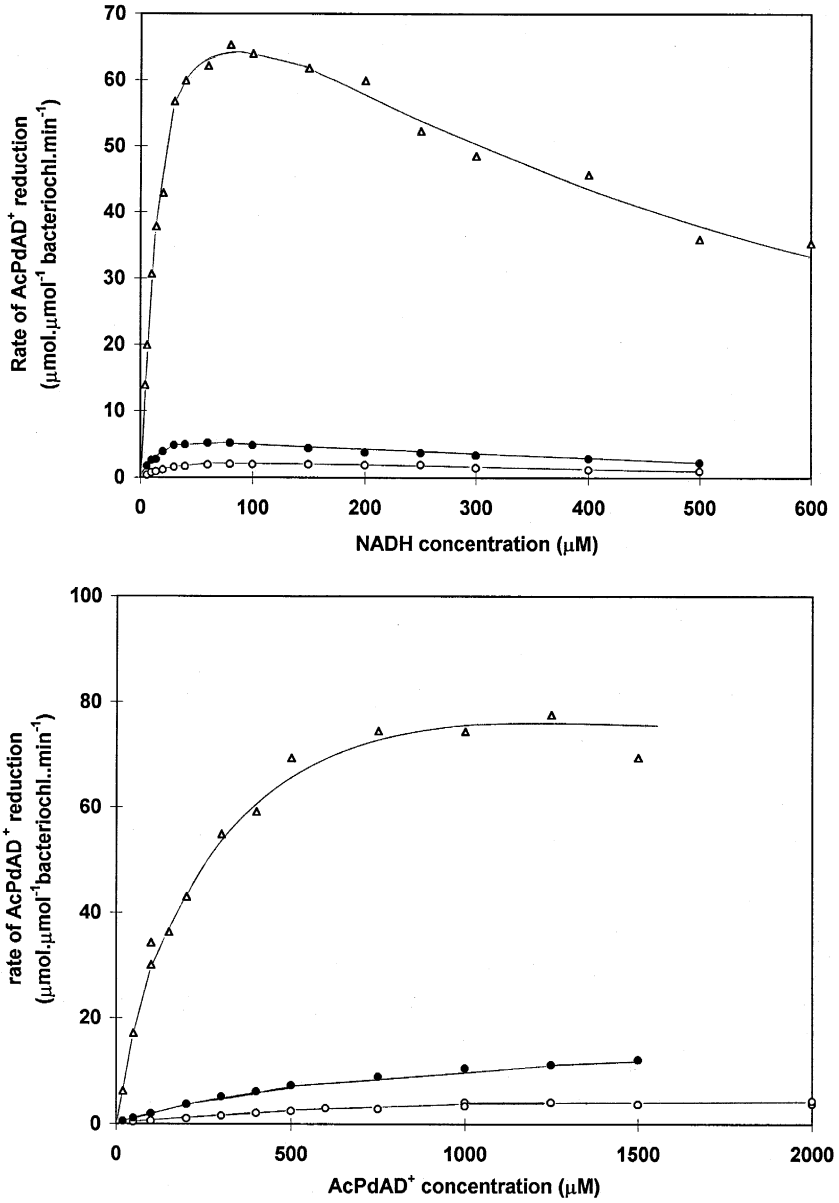

Fig. 3. The effect of mutations at Tyr235 in domain I on the rate of cyclic transhydrogenation in reconstituted domain I-depleted membranes. Experiments were performed as in Fig. 2, except that, for the wild-type, the domain I concentration was 30 nM and the bacteriochlorophyll concentration of the depleted membranes was 0.3 μM. For the mutants, the domain I concentration was 90 nM and the bacteriochlorophyll concentration of the depleted membranes was 3.2 μM. In all experiments, the concentration of $NADP^+$ was 25 μM. Top: fixed $AcPdAD^+$ (200 μM), variable NADH. Bottom: fixed NADH (85 μM), variable $AcPdAD^+$. △, wild-type domain I; ●, dI.Y235F; ○, dI.Y235N.

system, that AcPdAD$^+$ reduction arises only from proton-translocating transhydrogenase. The data are presented as s/v against s plots of the dependence of the rate of AcPdAD$^+$ reduction by NADH, in the presence of NADP$^+$, (A) on the concentration of NADH at different fixed concentrations of AcPdAD$^+$, and (B) on the concentration of AcPdAD$^+$ at different fixed concentrations of NADH. As was described in detail for the detergent-dispersed *E. coli* enzyme, the reaction is 'ping-pong' relative to AcPdAD$^+$ and NADH, with the two nucleotides competing for the same binding site. Thus, (a) the curves converge at $s \geq 0$, and (b) high NADH concentrations give rise to 'substrate inhibition', indicated by the upward curvature at high values of s. These observations confirm that AcPdAD$^+$ and NADH interact with the same

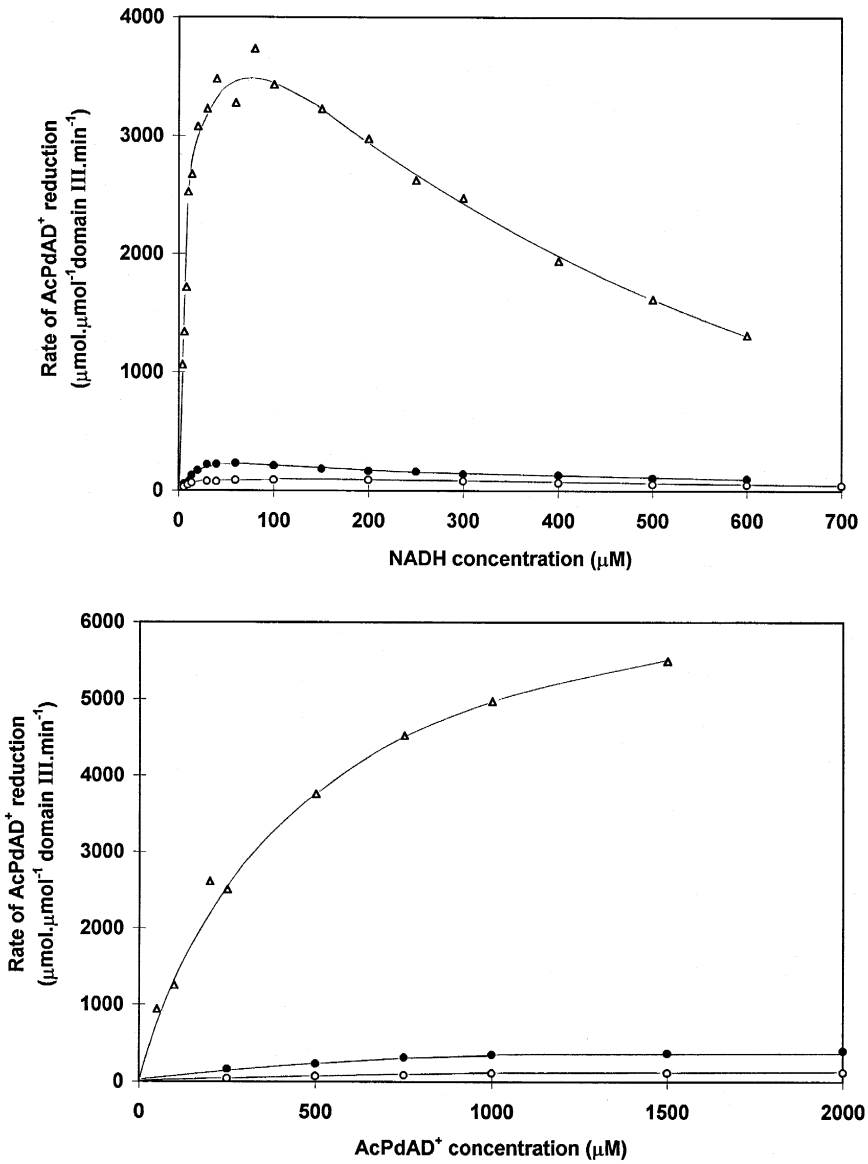

Fig. 4. The effect of mutations at Tyr235 in domain I on the nucleotide concentration dependence of the rate of cyclic transhydrogenation in domain I:III complexes. Domain I protein (30 nM for wild-type, and 150 nM for the mutants) plus domain III protein (4 nM when using wild-type domain I, and 39nM, when using mutant domain I) were mixed in 50 mM NaCl, 50 mM Mops, pH 7.0. Top: fixed AcPdAD$^+$ (200 μM), variable NADH. Bottom: fixed NADH (85 μM), variable AcPdAD$^+$. △, wild-type domain I; ●, dI.Y235F; ○, dI.Y235N.

site on the enzyme (the nucleotide binding site on domain I), and that $NADP^+$ and NADPH remain tightly bound during the reaction (the binary complexes E.$NADP^+$ and E.NADPH remain stable during turnover, the former being reduced by NADH, and the latter being oxidized by $AcPdAD^+$). Substrate inhibition at high concentrations of $AcPdAD^+$ was not evident from the data, presumably because domain I binds this nucleotide rather more weakly than NADH [19,26].

Fig. 3 compares the untransformed plots of substrate dependences of the cyclic reaction as catalysed by domain I-depleted chromatophores reconstituted with wild-type domain I, with dI.Y235F and with dI.Y235N. Fig. 4 shows the same but using mixtures of domain III with wild-type domain I, with dI.Y235F and with dI.Y235N. Note firstly, with respect to wild type domain I, that the profiles of the data were similar for both the complete enzyme and for the domain I:III complex. This suggests that the nucleotide-binding character of domain I is unaltered by the presence of transmembrane domain II. We showed previously that the absolute rates of the cyclic reaction in the complete enzyme are similar to those in the I:III complex [7]. The k_{cat} for the complex is somewhat greater than 100 s^{-1} — the precise value can not be estimated because of the substrate inhibition referred to above. Secondly, observe that the mutant proteins had a similar effect on cyclic transhydrogenation in the two systems. In both they severely inhibited the cyclic reaction (dI.Y235F by 92–93%, and dI.Y235N by 97–98%), i.e., considerably more than they inhibited forward and reverse transhydrogenation (see above). Despite the extensive inhibition, for both the complete, membrane-bound system and for the I:III complex, the nucleotide concentration dependences were very similar with wild-type domain I and with dI.Y235F and dI.Y235N. This is reflected in the similar 'K_m^{app}' and 'K_i^{app}' values for the wild-type and mutants (where 'K_m^{app}' is the nucleotide concentration needed to give 50% of the observed maximal rate and and 'K_i^{app}' is the nucleotide concentration giving rise to 50% inhibition; the true Michaelis parameters are difficult to determine because of the uncertainty in the value of k_{cat}), and probably indicates that for both mutants, the binding affinities for NADH and for $AcPdAD^+$ are decreased to a similar extent.

Fig. 5 shows the results of an experiment in which cyclic transhydrogenation was measured as a function of the concentration of domain I protein (wild-type, dI.Y235F and dI.Y235N) at a fixed concentration of domain III (note the differences in scale on the ordinate). As noted [7], and also in marked contrast to the results on reverse transhydrogenation (e.g., Fig. 1), the ratio of wild-type domain I/III required for

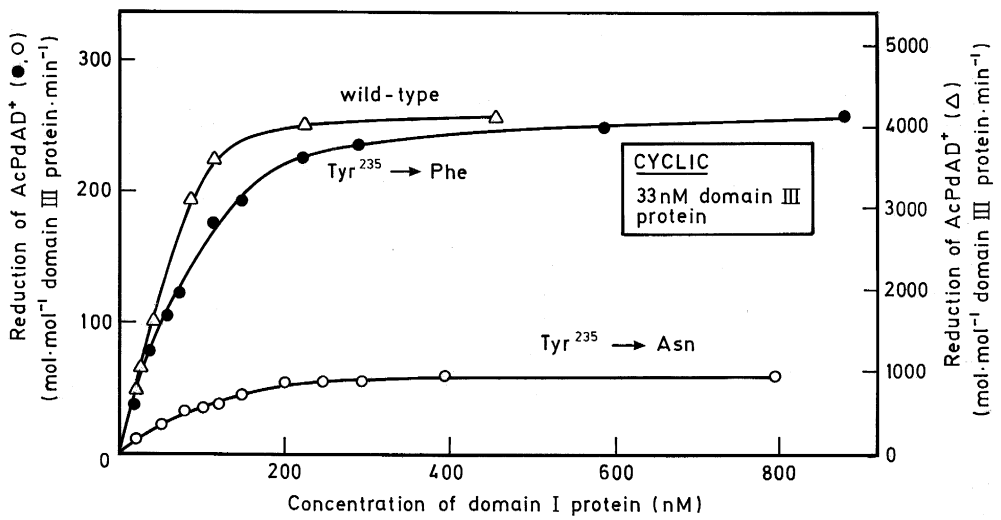

Fig. 5. The effect of mutations at Tyr235 in domain I on the protein concentration dependence of the rate of cyclic transhydrogenation in domain I:III complexes. Conditions as in Fig. 1, except that 200 μM NADH was present, and the NADPH concentration was 20 μM. △, wild-type; ●, dI.Y235F; ○, dI.Y235N. Note the difference in the scales for wild-type and mutants.

Table 1
NADH binding affinities and stoichiometries of domain I proteins

Domain I protein	K_d(μM)	Number of sites
Wild-type	30	1.1
dI.Y235F	65 ± 11	1.0
dI.Y235N	116 ± 15	1.2

Parameters were determined by equilibrium dialysis, as described in [26]. Wild-type data are taken from that report.

half-saturation of the cyclic reaction was about one, but, despite the strong inhibition of the rates of reaction, that ratio was only slightly higher for the two mutant domain I proteins.

3.3. Direct measurement of the nucleotide binding affinity of domain I proteins carrying the mutations Tyr-235 → Phe and Tyr-235 → Asn

Two sets of observations suggested that mutations at Tyr-235 in the mobile loop region of domain I of *R. rubrum* transhydrogenase result in a decreased affinity of nucleotide binding. (a) NMR experiments indicated that NAD^+ exchanges more rapidly with dI.Y235F and with dI.Y235N than with wild-type protein [20]. (b) In a reconstituted system of recombinant domain I protein plus domain I-depleted chromatophore membranes, the K_m for $AcPdAD^+$ during simple reverse transhydrogenation with NADPH was substantially increased (10-fold for dI.Y235F, and 13-fold for dI.Y235N) [20]. Measurements of the quenching of the fluorescence of Trp72 by NADH [6] were not sensitive enough to reveal differences in nucleotide binding affinity of domain I protein. However, a decrease in the binding affinity for NADH caused by mutations at Tyr-235 is now confirmed by equilibrium dialysis measurements using a recently published procedure (Table 1). The K_d increased from approximately 30 μM in the wild type protein to approximately 65 μM in dI.Y235F and 116 μM in dI.Y235N. In wild-type [26] and both mutants (Table 1) the stoichiometry was consistent with one nucleotide binding site per monomer.

4. Discussion

In this report the effects of substitution of Tyr-235 in the mobile loop of domain I of *R. rubrum* transhydrogenase on several key reactions have been investigated. Substitutions of Tyr-235 with either Phe or Asn had a more pronounced inhibitory effect on reverse and forward transhydrogenation in the complete enzyme, than they did in the domain I:III complex. The rates of $NADP^+$ and NADPH release are thought to be much slower, and thus contribute more completely to rate limitation, in the I:III complex than in the complete enzyme [7]. Thus, it may be concluded that mutations at Tyr-235 have little effect on NADP(H) release, or the associated protonation/deprotonation that accompany this process.

In contrast, in both the complete reconstituted enzyme, and in domain I:III complexes, cyclic transhydrogenation was strongly inhibited by the mutations. Because the binding and release of AcPdAD(H) and NAD(H) are fast [19], and because NADP(H) remains bound to the protein during the cyclic reaction [8,11,12], it is likely that the hydride transfer step is rate limiting. The strong inhibition of the cyclic reaction in the Tyr-235 mutants is therefore explained by the notion that their activities are blocked either at the hydride transfer step or at an associated conformational rearrangement.

In this context, it is also instructive to compare the profiles of reverse and cyclic transhydrogenation in the titrations of domain III protein with wild-type domain I, with dI.Y235F and dI.Y235N (Fig. 1 and Fig. 5). (1) Although in measurements of the cyclic reaction, the rates for the dI.Y235F and dI.Y235N mutants were depressed by an order of magnitude or more (see above), the protein titration profiles (relative to the maximum rate at saturation) were barely changed from the wild-type (Fig. 5). This is consistent with the argument [7] that the titration represents the binding curve for the I:III complex (the rate of the cyclic reaction is proportional to the concentration of the complex), and with the conclusion that these amino acid substitutions do not affect the affinity between domains I and III. From the data the K_d values of the I:III complexes for the wild-type and for the mutant domain I proteins are all in the range 30–50 nM. (2) It was noted that the titration profiles of the wild-type I/III proteins for reverse transhydrogenation differ markedly from those for the cyclic reaction [7]. Calculation shows that, during the reverse reaction, a single domain I protein can produc-

tively visit about 60 domain III proteins during the time taken for domain III to release $NADP^+$, and this is reflected in the fact that only a very small amount of the domain I protein is required to saturate domain III (Fig. 1). The estimated k_{off} value for the dissociation of the I:III complex is approximately 1.2 s^{-1} [7], much slower than the hydride transfer rate (a little more than 100 s^{-1}, see above), and it is clear, therefore, that the rate at which a wild-type domain I protein can react with an NADPH-loaded domain III protein depends more critically on the time taken for the two proteins to dock and undock, than upon the time of the hydride transfer reaction per se. This explains why, for the dI.Y235F and dI.Y235N mutants (whose hydride transfer rates in the I:III complex are decreased to approximately 7 s^{-1} and 2 s^{-1}, respectively), the amounts of domain I protein required for half-saturation of domain III protein were only slightly greater than the amount of wild-type domain I needed for half-saturation. Thus, although hydride transfer was strongly inhibited, its rate in the mutants was depressed to a value which only just approached the rate of the docking/undocking processes.

Parenthetically, it may be noted that, although some of the component reactions in the process of docking/undocking in the I:III complex might occur in the complete enzyme during turnover (i.e., they might represent catalytically important conformational changes), it is evident from the above discussion that, in its entirety, the process is too slow (at around 1.2 s^{-1}) to be an intermediate in transhydrogenation (the k_{cat} for reverse transhydrogenation was around 27 s^{-1} for the complete *R. rubrum* enzyme [7]). A similar conclusion was reached from observations on the rate of rebinding of isolated domain I to depleted chromatophore membranes, a reaction which proceeds on a time scale of a few seconds [28].

The results of this analysis show that the conserved Tyr at position 235 in the domain I protein of *R. rubrum* transhydrogenase is important specifically in the hydride transfer step of the enzyme, more important than was thought on the basis of measurements of forward and reverse transhydrogenation in the membrane-reconstituted system [20]. Substitution with an Asn residue is more inhibitory than substitution with a Phe; it seems that retention of the aromatic character in the latter mutation reduces the degree of inhibition. This Tyr is conserved in all known transhydrogenase sequences, but though important, it is evidently not essential. The residue is located in a segment of the domain I protein that is highly mobile in the absence of nucleotide, but which appears to close down and lose mobility as nucleotide binds. NMR experiments show that, in the bound state, Tyr-235 interacts with the adenosine moiety of the nucleotide [19]. The much decreased activities of the dI.Y235F and dI.Y235N mutants, and their lower affinities for NADH (Table 1), reinforces our view [18–20] that the 'mobile loop' has a function in maintaining the NAD(H) in an appropriate hydrophobic environment and conformation in domain I to effect the hydride transfer to or from the NADP(H) bound in domain III.

Fluorosulphonylbenzoyladenosine reacted covalently with Tyr-245 in mitochondrial transhydrogenase (equivalent to Tyr-235 of *R. rubrum* domain I protein), and caused pronounced inhibition of reverse transhydrogenation [29]. Different explanations for this inhibition, and for the reported protection by NADH, have been proposed [18,29,30]. Mutation of the equivalent αTyr-226 in *E. coli* transhydrogenase led to only 33–51% inhibition [30]. However, the experiments with *E. coli* transhydrogenase were probably performed under conditions (see [11]) in which the release of $NADP^+$ partly limited the rates of reaction. It is predicted from our experiments that cyclic transhydrogenation by the *E. coli* αTyr-226 mutant enzymes will be considerably more inhibited than the reverse reaction.

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