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Rapid report

Properties of the apo-form of the NADP(H)-binding domain III of proton-pumping *Escherichia coli* transhydrogenase: implications for the reaction mechanism of the intact enzyme

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Abstract

Proton-translocating nicotinamide nucleotide transhydrogenases contain an NAD(H)-binding domain (dI), an NADP(H)-binding domain (dIII) and a membrane domain (dII) with the proton channel. Separately expressed and isolated dIII contains tightly bound NADP(H), predominantly in the oxidized form, possibly representing a so-called ''occluded'' intermediary state of the reaction cycle of the intact enzyme. Despite a K_d in the micromolar to nanomolar range, this NADP(H) exchanges significantly with the bulk medium. Dissociated $NADP^+$ is thus accessible to added enzymes, such as NADP-isocitrate dehydrogenase, and can be reduced to NADPH. In the present investigation, dissociated NADP(H) was digested with alkaline phosphatase, removing the 2′-phosphate and generating NAD(H). Surprisingly, in the presence of dI, the resulting NADP(H)-free dIII catalyzed a rapid reduction of 3-acetylpyridine-NAD^+ by NADH, indicating that 3-acetylpyridine-NAD^+ and/or NADH interacts unspecifically with the NADP(H)-binding site. The corresponding reaction in the intact enzyme is not associated with proton pumping. It is concluded that there is a 2′-phosphate-binding region in dIII that controls tight binding of NADP(H) to dIII, which is not a required for fast hydride transfer. It is likely that this region is the Lys424–Arg425–Ser426 sequence and loops D and E. Further, in the intact enzyme, it is proposed that the same region/loops may be involved in the regulation of NADP(H) binding by an electrochemical proton gradient.

Keywords: Transhydrogenase; NAD; NADP; Proton pump; Membrane protein

Nicotinamide nucleotide transhydrogenase (TH) from *Escherichia coli* is a proton pump in which the reduction of $NADP^+$ by NADH through a hydride ion is linked to the translocation of one proton from the periplasmic space to the cytosol in bacteria, according to the reaction

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

in which the number of protons translocated per NADPH generated is 1. In the presence of an electrochemical proton gradient, Δp, reaction (1) from left to right is activated some 5–10-fold and the apparent equilibrium is strongly shifted to the right.

The *E. coli* enzyme is composed of an α subunit (54.6 kD) and a β subunit (48 kD), containing the NAD(H)-binding domain I (dI) and the NADP(H)-binding domain III (dIII), respectively. Intact and active TH is a tetramer, $\alpha_2\beta_2$. Both subunits also contain transmembrane helices, of which helices 1–4 reside in the α subunit and helices 6–14 reside in the β subunit, together constituting the membrane domain (dII) which also houses the proton channel (Fig. 1). Physiologically, the role of TH is presumably to generate a high redox level of NADP(H) for detoxification and regulatory purposes (for reviews, see Refs. [1,2]). Despite the fact that both dI [3] and dIII [4,5] as well as the dI–dIII complex [6] have been structurally resolved by X-ray crystallography, and that dI–dIII interactions have been established by NMR [7,8], both approaches providing essential insights into the hydride transfer mechanism, the coupling mechanism of

Abbreviations: TH, transhydrogenase; dI, domain I of transhydrogenase; dII, domain II of transhydrogenase; dIII, domain III of transhydrogenase; ecI–ecIII, the corresponding domains of *Escherichia coli* transhydrogenase; rrI, dI of *Rhodospirillum rubrum* transhydrogenase; $AcPyAD^+$, 3-acetylpyridine-NAD^+; NADP-ICDH, NADP(H)-specific isocitrate dehydrogenase; ADH, alcohol dehydrogenase

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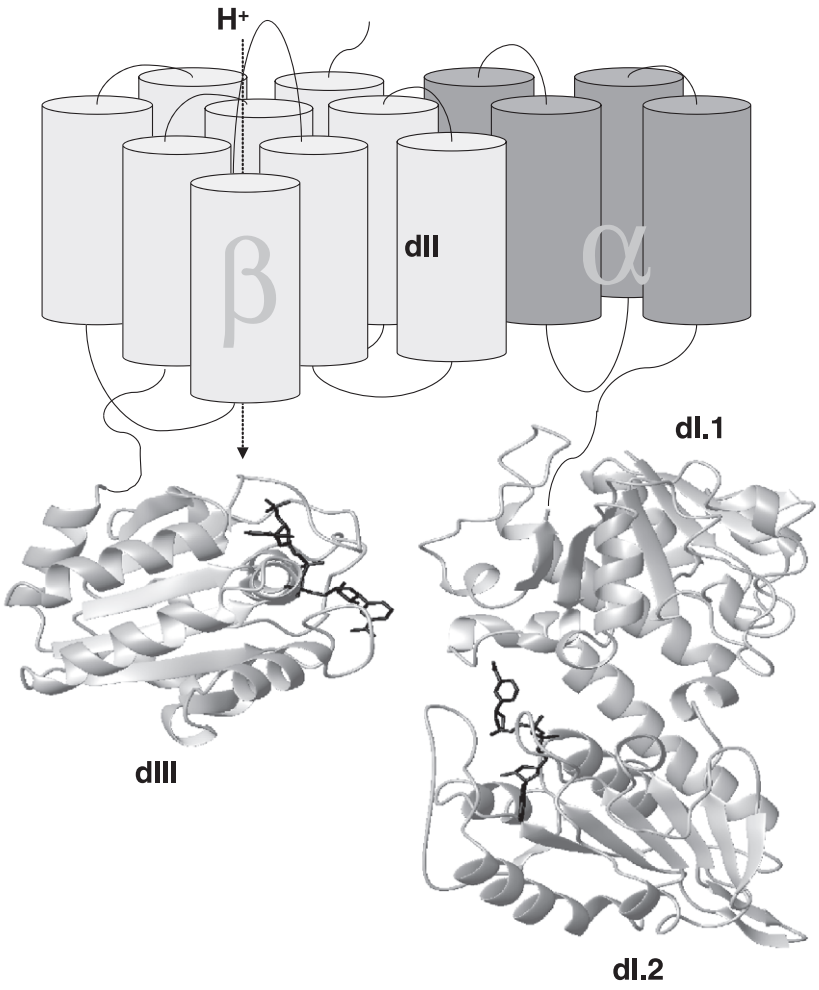

Fig. 1. A cartoon of the *E. coli* transhydrogenase and its domains, shown as the αβ-monomer.

intact transhydrogenase is unknown. Even though Δ*p*-dependent alterations cannot be excluded in dI, the general view is that dissociation of NADP(H) from dIII constitutes the limiting factor in both directions of reaction [1] including the Δ*p*-stimulated forward reaction (left to right) [1,2]. One essential observation in this context is the fact that isolated dIII contains tightly bound NADP(H), assumed to represent an ''occluded'' state in the overall reaction mechanism [2]. Thus, in the presence of isolated dI and substrates, dIII catalyzes very slow forward and reverse reactions, but a high rate of cyclic reduction of 3-acetylpyridine-NAD^+ by NADH, mediated by the bound NADP(H). An important question concerns what the structural differences are between dIII and the corresponding domain in the intact enzyme that has no bound NADP(H). To this end we have generated the apo-form of dIII from *E. coli* (ecIII) and characterized its properties. Fig. 2 shows a close-up of the NADP(H)-binding site in dIII.

EcIII was expressed and purified as described [7,9]. $NADP^+$ release from ecIII was monitored by fluorescence as described previously [9]. All measurements were made with a SPEX model FL1T1 t2 spectrofluorometer with both excitation and emission slits set to 2.5 nm. The excitation and emission wavelengths used were 340 and 460 nm, respectively. Alkaline phosphatase from bovine intestinal mucosa was used to remove the 2′-phosphate from NADP(H). Each measurement was preceded by incubation of 120 μg of ecIII with 20 U alkaline phosphatase in 10 mM Tris–HCl, pH 9, at 37 °C for 30 min in a total volume of 100 μl. Prior to activity measurement, the incubation mix was diluted to 2 ml with 20 mM MOPS, 5 mM $MgCl_2$, pH 7.0. $NADP^+$ was assayed with isocitrate dehydrogenase in the presence of isocitrate and $MgCl_2$ [10] and NADH formation was assayed using alcohol dehydrogenase (ADH) in the presence of semicarbazide and ethanol according to [10]. Cyclic activities were measured optically using *Rhodospirillum rubrum* domain I (rrI) as described [9] in a medium composed of 20 mM CHES, 20 mM MES, 20 m MOPS, 20 mM Tris, 50 mM NaCl (pH 7.0). Incubations of 18 μg ecIII in 10 mM Tris–HCl, pH 9, at 37 °C for 10 min either with or without 10 U of alkaline phosphatase were made prior to activity measurements. The total incubation volume was always 50 μl. Due to its stabilising effect on activity, 300 μM NADH was added during the incubation.

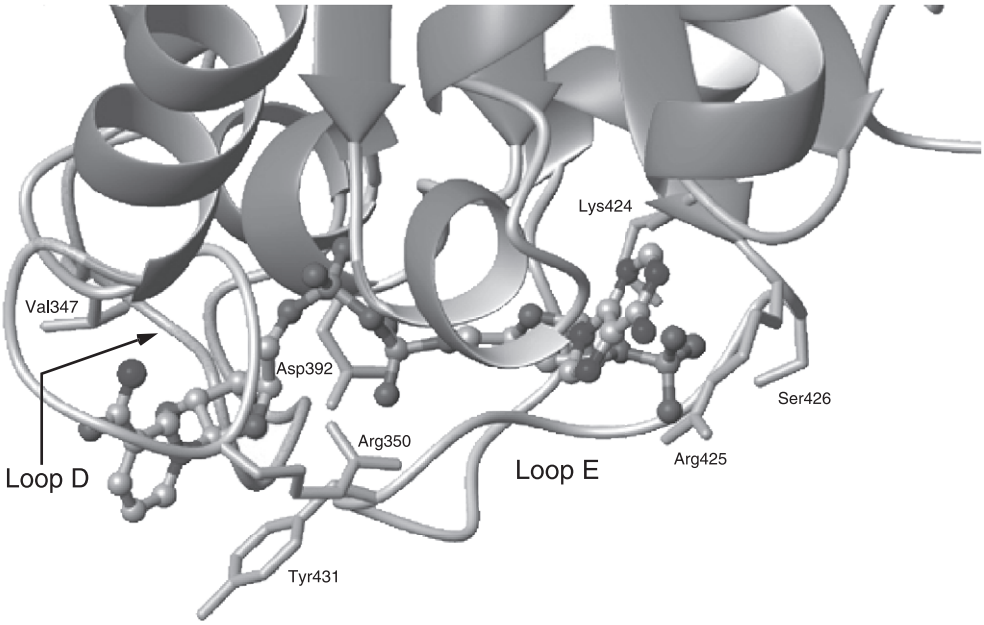

Fig. 2. The NADP(H)-binding site in the dIII crystal structure of the bovine mitochondrial TH (PDB code 1D4O). Bound NADP(H) is shown as a ball and stick structure with the nicotinamide moiety to the left and the adenine moiety to the right. Residues Lys424–Arg425–Ser426 binding the 2′-phosphate of NADP(H), residues Val347 and Tyr431 binding the nicotinamide ring, and residues Arg350 and Asp392 binding the pyrophosphate moiety of NADP(H), are indicated and numbered according to the equivalent *E. coli* residues. Loops D and E are also indicated. The figure was created with the software MOLMOL [19].

Wild-type ecIII contains about 8% apo-form and 92% NADP(H), the latter distributed as 87% $NADP^+$ and 5% NADPH [9]. Despite the fact that $NADP^+$ and NADPH are bound to dIII with dissociation constants in the micromolar and nanomolar range, respectively [7,9,11], both exchange sufficiently fast to react with externally added reducing and oxidizing enzymes [11]. This is exemplified in Fig. 3A, where bound $NADP^+$ is reduced by NADP-isocitrate dehydrogenase (NADP-ICDH) at a rate which is related to K_d, whereas, as expected, the NAD-alcohol dehydrogenase in the presence of semicarbazide and ethanol had no effect. Because of the significant dissociation rate for NADP(H), it was assumed that the 2′-phosphate of NADP(H) could be hydrolyzed by added alkaline phosphatase, thereby removing NADP(H) from the binding site. The latter enzyme is known to hydrolyze the 2′-phosphate of NADP(H) [12]. Indeed, following incubation of ecIII with alkaline phosphatase at pH 9.0 for 30 min and readjustment of the pH to 7.0, $NADP^+$ was no longer detectable by the NADP-ICDH assay (Fig. 3C). Instead, ADH gave a fluorescence change, indicating that $NADP^+$ had been converted to NAD^+ which then was reduced. Shorter times of incubation at pH 9.0 gave a larger amount of NADH (not shown), indicating that the small amounts of NAD^+ formed at pH 9.0 were unstable, in agreement with the known instability of $NAD(P)^+$ at high pH. Added free NAD^+ gave the expected additional increase in absorbance due to the NADH formed (Fig. 3C). That the incubation and pH shift in themselves had little or no effect on the $NADP^+$ content or apparent K_d is shown in Fig. 3B.

Bound NADPH dissociates some 50–100-fold slower from ecIII than $NADP^+$ [9]. Following phosphatase treatment, the low amount of NADPH bound to ecIII was no longer detectable enzymatically by, e.g. glutathione reductase in the presence of oxidized glutathione, suggesting that it too had been degraded (not shown).

The rates of cyclic transhydrogenation catalyzed by untreated and phosphatase-treated ecIII in the presence of rrI and $NADP^+$ were comparable to previous assessments of rrI–ecIII V_{max}, [9,11], i.e., about 5000 mol AcPyADH mol $ecIII^{-1}$ min^{-1} (Fig. 4, traces A–C). The remarkably high activity of phosphatase-treated, and thus NADP(H)-free, ecIII in the presence of rrI but in the *absence* of added $NADP^+$ (Fig. 4, trace D) demonstrates that ecIII was capable of catalyzing an unspecific cyclic transhydrogenation between NADH and $AcPyAD^+$ at approximately 75% of V_{max}, corresponding to 3770 mol AcPyADH mol $ecIII^{-1}$ min^{-1}. The pH dependence of the phosphatase-treated ecIII in the presence of rrI was not significantly different from that of nontreated ecIII [13] (not shown).

Isolation of stable apo-forms of wild-type ecIII has not been reported previously, the main problem being instability and aggregation (M. Althage, O. Fjellström and J. Rydström, unpublished). However, two mutants, D392C [7] and R425C [14], showed 100% apo form as well as high reverse and low cyclic activities, consistent with a markedly decreased binding of NADP(H). Indeed, this was expected since Asp392 is essential for activity in intact TH [15]. Asp392 forms H-bonds with the pyrophosphate moiety of NADP(H), and

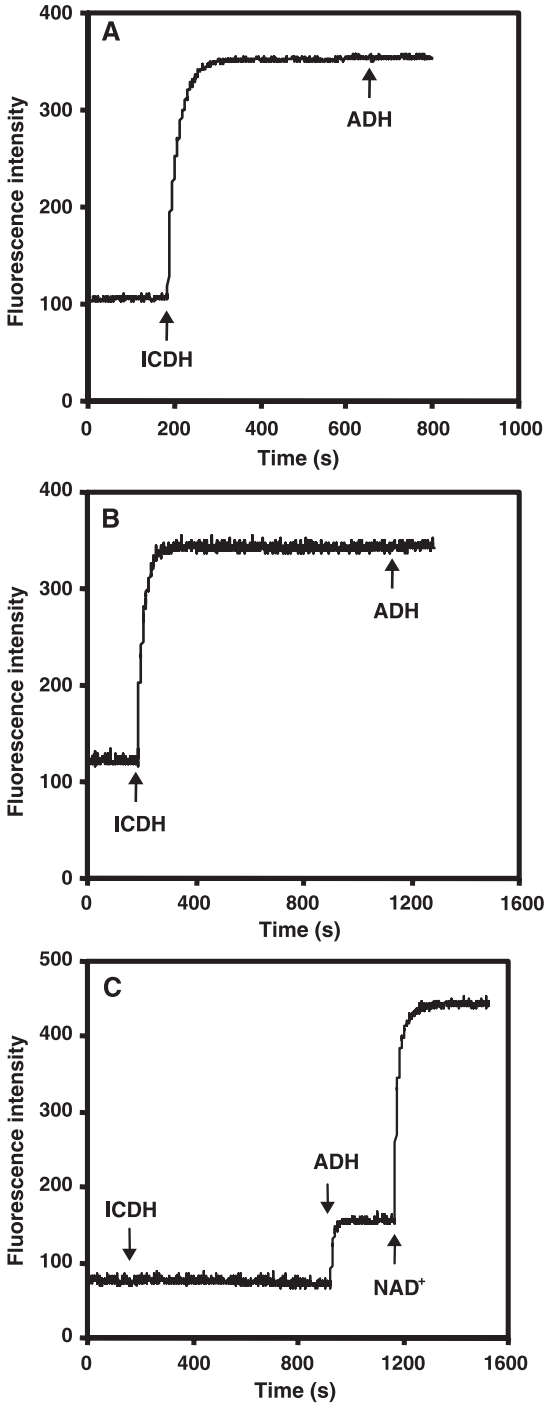

Fig. 3. Removal of 2′-phosphate of $NADP^+$ bound to ecIII with alkaline phosphatase. (A) Reduction of $NADP^+$ bound to untreated ecIII by NADP-ICDH. (B) Conditions were as in (A) except that ecIII was exposed to pH 9.0 followed by a readjustment to pH 7.0. In both (A) and (B), ADH was added to reduce any NAD^+ formed. (C) Reduction of $NADP^+$ and NAD^+ bound to phosphatase-treated ecIII by NADP-ICDH and ADH, respectively; NAD^+ was subsequently added. Reduction of $NADP^+$ and NAD^+ was followed fluorimetrically. The concentrations of ethanol, semicarbazide and isocitrate were 0.1%, 50 mM and 2 mM, respectively. The amounts of isocitrate dehydrogenase (ICDH) and alcohol dehydrogenase (ADH) added in each measurement were 1 and 10 U, respectively.

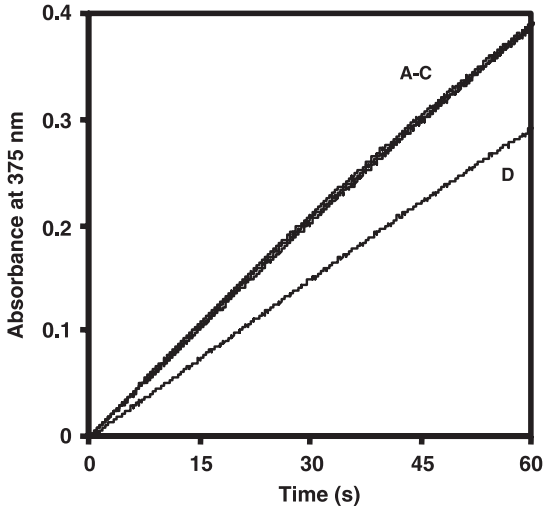

Fig. 4. Cyclic activities of untreated (A and C) and phosphatase-treated (B and D) ecIII. For incubation conditions, see Materials and methods. The concentrations of ecIII and rrI were 12.5 nM and 1 μM, respectively. The concentrations of $AcPyAD^+$ and NADH were 400 and 300 μM, respectively. Measurements were carried out either in the presence (A and B) or in the absence (C and D) of 200 μM $NADP^+$. Additions were made in the same order, i.e., ecIII, rrI, $NADP^+$, $AcPyAD^+$ and NADH.

Arg425 forms H-bonds with the 2′-phosphate group of NADP(H) [4,5].

That phosphatase-treated ecIII devoid of bound NADP(H) showed a high cyclic activity with only $AcPyAD^+$ and NADH indicates that, in the absence of NADP(H), $AcPyAD^+$ and/or NADH interacts unspecifically with the NADP(H)-binding site in dIII. This observation is of profound importance for the mechanism as to how binding of NADP(H) may be regulated. First, an unspecific interaction of dIII with NAD(H) is sufficient for proper orientation of the nicotinamide ring of NAD(H) in dIII relative to that in dI, and fast hydride transfer. Thus, the Lys424–Arg425–Ser426 region that normally stabilizes the 2′-phosphate through H-bonds is not required for fast hydride transfer. Second, the 2′-phosphate group of NADP(H) is responsible for the tight binding to dIII, most likely via the Lys424–Arg425–Ser426 region, and indirectly by loops D and E (Fig. 2). The closing effect of the latter loops is thus assumed to contribute to NADP(H) binding only *after* the interaction between the 2′-phosphate and the Lys424–Arg425–Ser426 region has been established. Third, it may be inferred that the Δp-dependent increase in the dissociation of NADPH from the intact TH involves a decreased binding of the 2′-phosphate of NADP(H) to Lys424–Arg425–Ser426, as well as a concomitant increased competition with bulk NAD(H), i.e. a specificity-change mechanism. The communication between the protonation events in dII and the Lys424–Arg425–Ser426 region in dIII remains to be established.

A cyclic activity between $AcPyAD^+$ and NADH catalyzed by intact TH [16], cysteine-free TH [17] and some mutants of the "hinge" region, e.g. R265C and S266C [18],

has indeed been reported previously that involves an unspecific interaction of $AcPyAD^+$ and/or NADH with the NADP(H)-binding site. In addition, transfer of hydride ions between $AcPyAD^+$ and NADH in the absence of $NADP^+$ does not involve proton pumping [16], suggesting that it is indeed a cyclic reaction or, alternatively, that it is a ''reverse'' reaction where NADH has replaced NADPH in reaction (1). Unfortunately, it is presently not possible to distinguish between a cyclic reaction and a reverse reaction in this context.

In conclusion, tightly bound NADP(H) in ecIII has been successfully removed by treatment with alkaline phosphatase, producing the apo-form of ecIII that binds $AcPyAD^+$/NADH unspecifically but with a retained high activity for a cyclic reaction. This suggests that primarily the 2′-phosphate-binding Lys424–Arg425–Ser426 region, and secondarily loops D and E, are involved in the ''occluded'' state, as well as the Δp-dependent binding changes in the NADP(H) site of the intact enzyme.

Acknowledgements

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