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Review and Hypothesis. New insights into the reaction mechanism of transhydrogenase: Swivelling the dIII component may gate the proton channel

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Review

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***Review and Hypothesis*. New insights into the reaction mechanism of transhydrogenase: swivelling the dIII component may gate the proton channel.**

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Author contributions. JBJ and RBG developed the mechanism model and wrote the manuscript. JHL, CDS and LAS-B contributed specialist analysis of, and information on, the structural biology.

Key words: transhydrogenase, membrane-protein structure, nicotinamide nucleotides, proton-pump, proton-gating

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Abstract

The membrane protein transhydrogenase in animal mitochondria and bacteria couples reduction of $NADP^+$ by NADH to proton translocation. Recent X-ray data on *Thermus thermophilus* transhydrogenase indicate a significant difference in the orientations of the two dIII components of the enzyme dimer (Leung, JH, Schurig-Briccio, LA, Yamaguchi, M, Moeller, A, Speir, JA, Gennis, RB and Stout, CD, 2015, *Science*, 347, 178-181). The character of the orientation change, and a review of information on the kinetics and thermodynamics of transhydrogenase, indicate that dIII swivelling might assist in the control of proton gating by the redox state of bound $NADP^+$/NADPH during enzyme turnover.

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Introduction

Proton-translocating transhydrogenase is an important enzyme found in the inner membranes of animal mitochondria and the cytoplasmic membranes of bacteria. In the forward direction it catalyses $NADP^+$ reduction by NADH coupled to inward proton translocation across the membrane. Thus, the enzyme is a proton motor in the same sense as the F_1F_O-ATP synthase.

Transhydrogenase in bacteria is one of several enzymes, often operating in parallel, which supply NADPH for biosynthesis. In *Escherichia coli*, for example, about one third of the NADPH needed for biomass production can be supplied by proton-translocating transhydrogenase [1, 2]. In some bacterial species over-expression of inserted transhydrogenase genes leads to commercially significant increases in the formation of fermentation products, such as lysine for animal feed, by increasing the rate of $NADP^+$ reduction [3].

Transhydrogenase is strongly expressed in the mitochondria of cells in a range of animal tissues [4]. Again, other enzymes participate in the reduction of $NADP^+$ in these cells and here even approximate estimates of the contribution of transhydrogenase to NADPH production are difficult to make [5, 6]. The NADPH produced by transhydrogenase may be particularly important in pathways responsible for the removal of damaging reactive oxygen species (ROS) [7]. Knockdown of the transhydrogenase gene in a human adrenocortical cell line resulted in elevated levels of ROS, and mutations of the gene were identified in individuals with familial glucocorticoid deficiency [8]. In an interesting parallel development it was reported that mouse strains carrying deletions in their transhydrogenase genes have impaired glucose-stimulated insulin secretion and glucose tolerance [9, 10]. It was proposed [11] that loss of transhydrogenase in mice prevents removal of superoxide radicals, and hence promotes activation of the mitochondrial uncoupling-protein 2 with consequent dissipation of the proton electrochemical gradient and inhibition of the ATP synthesis required for insulin secretion. Despite difficulties in properly establishing genetic backgrounds [12], the transhydrogenase-defective mouse strains may serve as useful models for understanding human type 2 diabetes.

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The dI, dII and dIII components of transhydrogenase.

Transhydrogenase has three structural components (Fig.1). The hydrophilic components dI and dIII protrude from the membrane on the cytoplasm side in bacteria, and dII spans the membrane. The dI binds NADH (and product NAD^+), dIII binds $NADP^+$ (and product NADPH), and dII conducts protons across the membrane. Transhydrogenase has two dI-dII-dIII protomers. The joining of the polypeptide chains within a protomer varies amongst species (Fig.2) but this is unlikely to significantly influence the enzyme mechanism. While dII is always covalently linked to dIII (through the "hinge region") the dI/dII and dIII/dI components may or may not be joined together through their polypeptide chains.

High-resolution structures of isolated dI [13-15] and dIII [16-18], and that of a dI-dIII complex [19-21], have been published during the last decade, and have afforded insights particularly into nucleotide binding and the hydride transfer step. The recent structure of the membrane-spanning dII at 2.8 Å resolution, and of the holo-enzyme at 6.9 Å from *Thermus thermophilus* [22], provide clues as to how hydride transfer from NADH to $NADP^+$ at the interface of dI and dIII is coupled to proton translocation through dII. Distances are such that coupling must be mediated by conformational changes across the protein.

Isolated dI of transhydrogenase is a stable dimer [23]. Bound NAD^+ (or NADH depending on crystallization conditions) is readily observed in the X-ray structures of isolated dI and dI-dIII complexes [13, 14, 19-21]. It occupies a classical nucleotide binding site in one of the two Rossmann folds that comprise the dI monomeric unit. Interactions between the protein and the ribose group of the nucleotide AMP moiety explain why the site strongly favours NAD^+/NADH binding over $NADP^+$/NADPH. The nicotinamide mononucleotide moiety of the NAD^+, which carries the redox-active nicotinamide ring, can adopt more than one conformation within the binding site [24]. Two conserved polypeptide loops of dI, the "mobile loop" and the "RQD loop" (Fig.1), interact with the bound nucleotide. The former, detectable in proton NMR spectra because of its segmental mobility [25], closes down on the surface of the protein following nucleotide binding. Mutations in the mobile loop of *Rhodospirillum rubrum* dI (*Rr*α_1M226F, *Rr*α_1T231C, *Rr*α_1G234A, *Rr*α_1Y235F, *Rr*α_1A236G, *Rr*α_1K237M – see Fig.2 for polypeptide nomenclature) lead to a decrease in NADH binding affinity and in the hydride-transfer rate [26]; its function may be to exclude water from the hydride-transfer site during catalysis. Mutations in the RQD loop of *R.rubrum* dI

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(*Rr*α_1Q132N, *Rr*α_1D135N, *Rr*α_1S138A) cause only small changes in NADH binding affinity (*Rr*α_1R127A has a more pronounced effect) but all lead to very strong inhibition of hydride transfer [27]. X-ray structures of these mutant dI proteins indicate that residues in the RQD loop are involved in positioning the dihydronicotinamide ring of NADH for hydride transfer to $NADP^+$ bound to dIII.

Isolated dIII proteins are monomeric and have either tightly bound $NADP^+$ or NADPH depending on purification conditions. The dIII component comprises a single, classical Rossmann fold but the $NADP^+$ (or NADPH) is bound in a non-classical manner with a reverse nucleotide orientation [16, 17]. Favoured binding of $NADP^+$/NADPH relative to NAD^+/NADH is ensured by interaction between the 2' phosphate of the AMP moiety of the former and a conserved KRS motif in loop E. The central section of this loop arches over the pyrophosphate group of the bound nucleotide forming a "lid" (Fig.1). The protruding feature designated helix D/loop D is conformationally mobile [28], and along with the interacting loop E, is thought to have a central role in the transhydrogenase energy-coupling mechanism, perhaps in the crucial transition between the open and occluded states of the enzyme (see below). Mutations of *Ec*βK424, *Ec*βR425 and *Ec*βY431 in helix E and of *Ec*βD392 in helix D/loop D in the intact *E.coli* enzyme lead to inhibition of transhydrogenation activity [29-32]. Studies on these and other mutants in loop E and helix D/loop D of isolated *E.coli* dIII reveal further consequences of interactions of the protein with $NADP^+$ and NADPH [33, 34]. In crystal structures the equivalent residues to *Ec*βD392 form hydrogen bonds with the pyrophosphate and a ribose hydroxyl group of bound $NADP^+$/NADPH [16, 17]. Its unusually high pK_a, sensitive to the redox state of the bound nucleotide [35-37], and the fact that its substitution results in a failure in $NADP^+$/NADPH binding [33] to isolated dIII, indicate an important catalytic role for this residue.

Until recently information on the structure of the transmembrane dII component of transhydrogenase was reliant upon amino acid sequence and biochemical analysis (see ref [38]. Thus, the single-subunit enzyme from animal mitochondria was thought to have 14 transmembrane helices (TM) per protomer, the two-subunit enzyme from *E.coli*, 13, and the three subunit enzyme from *R.rubrum*, 12 TM (Fig.2). Gene fusions during evolution were thought to increase the number of TM in the *E.coli* and mitochondrial-type enzymes [39, 40]. Consistent with these views, the new X-ray structure of dII from *T.thermophilus* transhydrogenase (a three-subunit enzyme) has 12 TM per protomer, 3 in the α_2 subunit, and

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9 in the β subunit [22]. The most highly conserved of the transmembrane helices are TM2, TM3, TM4, TM9, TM10, and especially, TM13 and TM14 (for the TM numbering system, see Fig.2). The proton channel through dII (one per protomer) is thought to comprise the hexagram of TM3, TM4, TM9, TM10, TM13 and TM14 seen in the *T.thermophilus* crystal structure. Within the hexagram TM3, TM9 and TM13 form a central three-helix bundle enclosing a "tunnel", perhaps the proton-translocation pathway itself, and the bundle is surrounded by TM4, TM10 and TM14. Six layers of amino acid residues are distinguished along the channel. Probably necessary for H^+ transport, pairs of residues in layers 3 and 4 are hydrogen bonded. There are no ordered water molecules within the proton channel. The two protomers of the dII dimer interact through the respective TM2. In the holoenzyme crystal structure one dII protomer is slightly displaced relative to the other. Conservative single-site substitutions of only *Ec*αH450 (in TM3), *Ec*βH91 (TM9), *Ec*βS139 (TM10), *Ec*βD213 (in the cytoplasmic loop between TM12 and 13), *Ec*βN222 (TM13), *Ec*βG252 (TM14) and in a run of residues from *Ec*βK261-βR265 (in the hinge at the C-terminus of TM14 which connects dII to dIII – see Fig.1) of many examined in *E.coli* dII lead to strong inhibition of transhydrogenase activity [30, 31, 41-48]. *Ec*βH91 has often provoked interest in that it is the only conserved protonatable residue close to the centre of the membrane dielectric whose substitution significantly deactivates transhydrogenation. However, in many species (including *T.thermophilus*) the His is replaced by an Asn (*Tt*βN89); in some of these species the lost His is accompanied by the appearance of another in TM3 (as in *Tt*βH42) though this is not always the case (we may speculate that transhydrogenases in this last category do not translocate H^+ but perhaps Na^+). Low concentrations of Zn^{++} block proton translocation through the *E.coli* dII channel; both FTIR [49] and X-ray absorption studies [50] implicate a His residue in the metal-ion binding but the role of βHis91 in the process is not yet clear. The conserved salt bridge between *Tt*βD202 and *Tt*βR254 in the hinge, which very likely has an important role in catalysis [44], is located next to the proton channel at the membrane surface [22]. Differences in electron density of the hinge region suggest different conformations in the two protomers. Despite extensive mutagenesis experiments on the dII sequence the roles of individual amino acid residues in proton translocation and energy transduction have remained unclear. In Fig.3 the amino acid residue(s) responsible for proton binding in the dII channel, and the subsequent coupling to hydride transfer (see below), is labelled "*X*".

The dIII component of transhydrogenase swivels during turnover

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A most unexpected feature of the holo-enzyme X-ray structure confirmed by cross-linking inserted cysteine residues is that the dIII component of one protomer has a "face-up" orientation, in which its bound $NADP^+$ is located close to the NAD^+/NADH binding site of dI, but the other dIII has a "face-down" orientation in which the bound $NADP^+$ would be brought into the short cytoplasmic loops linking the TM of dII (Fig.1) [22]. Transhydrogenase is thus asymmetric, and its dIII components evidently swivel between face-up and face-down orientations during turnover. Modelling studies show that neither the holo-enzyme [22] nor dI-dIII complexes [19] can simultaneously accommodate two dIII components in the face-up orientation. The emerging view is that during operation transhydrogenase undergoes an alternation of sites: the reaction sequence in one protomer runs approximately 180° out-of-phase with that in the other (Fig.3).

Below we discuss the function of the dIII swivel in the context of the transhydrogenation mechanism. In accordance with earlier suggestions dIII in the face-up orientation is positioned for the enzyme to catalyse hydride transfer from NADH to $NADP^+$ but in the light of the the new holo-enzyme structure we now propose that in its face-down orientation dIII is responsible for gating the proton channel in dII to give H^+ access to one side of the membrane or the other.

The binding-change mechanism of coupling to proton translocation in transhydrogenase

The strongest evidence on the mechanism of action of transhydrogenase relates to the hydride-transfer step. This takes place with the dIII component in an "occluded state" in which both the binding and release of $NADP^+$ and NADPH are extremely slow relative to enzyme turnover [51-53]. Crystal structures show that with dIII in the occluded state the *pro-R* hydrogen atom on C4 of the dihydronicotinamide ring of NADH (on dI) can be brought into close apposition with the *si* face of C4 of the nicotinamide ring of $NADP^+$ (on dIII) to effect direct, stereo-specific and rapid hydride transfer [21]. The dIII component can also adopt an "open state", where $NADP^+$ and NADPH can rapidly bind and dissociate, respectively, but where hydride transfer between bound $NADP^+$ and NADH is blocked [36, 54]. X-ray structures reveal nucleotide-binding conformations in which the block may be achieved; essentially the nicotinamide and dihydronicotinamide rings are held apart by the enzyme [24]. The pH dependences of transhydrogenation reactions in bacterial membranes

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suggest that protonation of the enzyme converts open dIII into its occluded state and, subsequent to the hydride transfer step, deprotonation then converts occluded dIII back into its open state [51, 54, 55]. Thus, the basic mechanism is (i) substrate nucleotides, $NADP^+$ and NADH, associate with their binding sites in an enzyme protomer having both dIII and dI in open states, (ii) protonation of dII from the outside aqueous phase converts the open dIII to occluded, (iii) hydride-ion equivalents are transferred from bound NADH to $NADP^+$, (iv) deprotonation of dII on the cytoplasmic side regenerates the open state of dIII, and (v) the nucleotide products, NADPH and NAD^+, are released. The binding affinities of NAD^+ and NADH to dI are not significantly altered during the catalytic cycle; this enzyme component remains open except perhaps during the brief ($< 10^{-3}$ s) period of hydride transfer [23, 56]. The proposed mechanism affords two important features. First, by confining hydride transfer to a form of the enzyme in which the nucleotides $NADP^+$ and NADPH remain tightly bound, it prevents the redox step (potentially a facile reaction) from taking place without proton translocation ("slip"). Second, it permits the enzyme to adjust the binding properties of dIII for $NADP^+$ and NADPH differently in the open and occluded states: in the open state to encourage substrate binding and product release, and in the occluded state to favour a large equilibrium constant for hydride transfer [57]. The consequent change in $NADP^+$/NADPH binding energy necessitates compensating changes in the equilibrium constants of steps associated with proton binding and proton release.

Gating of the proton channel

To provide directionality, for example to enable the enzyme to utilize a transmembrane proton electrochemical gradient and drive nucleotides to an elevated ratio of [NADPH][NAD^+]/[$NADP^+$][NADH], the H^+-translocation reaction has to be appropriately gated: proton binding/release steps on either side of the membrane must be coordinated with the progress of the reaction [54]. In principle, the redox state of either NAD^+/NADH or $NADP^+$/NADPH bound to reaction intermediates could provide the trigger to allow proton access to or from one side of the membrane or the other. However, the dI component remains essentially in an open state during turnover of the enzyme (see above). Thus, NAD^+ and NADH can rapidly bind to and be released from dI throughout the transhydrogenase reaction – they would be a poor trigger for controlling proton gating. $NADP^+$/NADPH are a much better proposition. In the framework of the proposed mechanism, gating would be effectively achieved if elements in the proton channel were to sense the redox state of

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NADP$^+$/NADPH bound to dIII, and adjust the local conformation to allow or disallow the passage of protons to or from the solvent. When NADP$^+$ is bound, the channel is able to access protons only from the outside, and when NADPH is bound, only from the cytoplasmic side. Thus, with NADP$^+$ in its dIII site H$^+$ binds into the channel from the outside (at relatively high proton electrochemical potential in respiring bacteria) generating the occluded state and, following reduction to NADPH, H$^+$ is released from the channel into the bacterial cytoplasm (at relatively low proton potential) regenerating the open state. A difficulty with this view was that previous predictions of the holo-enzyme structure based only on X-ray data on dI-dIII complexes had dIII in a face-up orientation [19, 20]. Thus, bound NADP$^+$ and NADPH were thought to be held some 20 Å or more from the proton channel. This arrangment is essential for direct hydride transfer from NADH (see above) but is not so well suited for gating: the "signal" relaying information on the redox state of the dIII nucleotide would have to be transmitted a considerable distance to the proton channel to control the gating.

The new structure of the *T.thermophilus* holo-enzyme reveals that the dIII component of one protomer is in the face-up orientation appropriate for hydride transfer; the other has a face-down orientation in which bound NADP$^+$ or NADPH would be brought into the short cytoplasmic loops linking the TM of dII [22]. In this latter conformation nucleotide influence on the structure of the proton channel is much easier to envisage. The (dihydro-) nicotinamide end of the bound nucleotide is proximal to the inter-TM loops. The positive charge on the nicotinamide ring of NADP$^+$ relative to the uncharged dihydronicotinamide ring of NADPH might thus be sufficient to switch the channel gates.

In Fig.3 the view that dIII swivelling regulates proton gating has been incorporated into the basic and well-supported transhydrogenase mechanism outlined above. At the end of catalytic turnover in a single protomer, dIII is face-down and in its open state (intermediate 1, either red or blue). Release of products NAD$^+$ and NADPH are followed by substrate NADH and NADP$^+$ binding. NADP$^+$ newly-bound to the face-down dIII (intermediate 2) triggers access of the proton channel to the outside aqueous phase. Protonation of *X* from the outside causes the dIII to become occluded and swivel into the face-up orientation to enable hydride transfer to its bound NADP$^+$ from NADH bound to dI (intermediate 3). The still-occluded dIII, now bearing NADPH (intermediates 4 and 5), then swivels back into the face-down orientation (intermediate 6). The bound NADPH in its face-down dIII triggers access of the

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proton channel to the inside aqueous phase where H^+ is released from *X*, and the dIII is converted back into the open state.

Alternation of sites

It is recalled that the *T.thermophilus* holo-enzyme structure has one dIII in the face-up orientation and the other face-down (Fig.1), and that there is strong evidence that isolated dI-dIII complexes from *R.rubrum* have their dIII fixed in the face-up orientation, are occluded and are capable of rapid hydride transfer between bound nucleotides [19, 57, 58]. The events taking place during operation of the dimer then fall into place (Fig.3). The two protomers run approximately 180° out of phase: while one is completing the hydride-transfer step with dIII in an occluded state (intermediate 3 → 4), the other is preparing for the replacement of bound product NADPH with substrate $NADP^+$ in dIII in an open state (intermediate 6 → 1). During the period in which proton binding from the outside aqueous phase is converting the open to the occluded state of dIII in one protomer (intermediate 2), proton release to the cytoplasm is converting dIII in the other from the occluded to the open state (intermediate 6).

The available experimental evidence on transhydrogenase would be satisfied if the two protomers in the figure operate independently of one another, only occasionally and briefly arrested to prevent both dIII components simultaneously adopting the clashing face-up orientation. However, this independent operation seems unlikely since then the enzyme would function more effectively as isolated protomers. It is more probable that events in the two protomers are conformationally linked to secure a kinetic advantage in the operation of the dimer. A conformational linkage between the protomers is most likely at steps 2 → 3 and 5 → 6 where proton binding/release, interconversion of the open/occluded states, and dIII swivelling all take place. Thus, the swivelling of face-up → face-down orientations in one protomer could be compulsorily linked to face-down → face-up swivelling in the other; and/or conformational changes resulting from proton binding in one protomer could be linked to conformational changes resulting from proton release in the other. These possibilities are not distinguished in the figure. In general, however, there are clear indications that structural changes in one protomer are linked to structural changes in the other – differences in the orientation of dIII, in the structure of the dII proton channel, and in the conformations of the hinge region, of the two protomers were all referred to above. Even in the isolated dI dimer the two NAD^+/NADH binding sites of the monomers adopt slightly different conformations,

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one appropriately poised for hydride transfer, the other not [13]. It seems likely that cooperative interactions between the two protomers of transhydrogenase are central to its mechanism of action. It may also be noted that it has not been ruled out that the dimer could accommodate both dIII components in the face-down orientation, which might simplify our understanding of the transition, 5 → 6, and that protonation/deprotonation of dIII itself is the cause of the occluded/open transition of this component.

The development of procedures for purifying and crystallizing the holoenzyme and its isolated components from the *T.thermophilus* transhydrogenase opens up ways to test predictions of the above model. Effort will be directed towards improving the X-ray resolution of the holoenzyme. Crucially, it is predicted that the open state of dIII will be stabilized in the face-down orientation of this component, and this will be reflected in changes in the structure of the $NADP^+$/NADPH binding pocket. Interactions of the (dihydro)nicotinamide group of the bound $NADP^+$/NADPH with amino acid residues in dII will test the prediction that the redox state of the nucleotide is involved in gating the proton channel. Changes in the structure of the hinge region are expected to have a central role in the swivelling of dIII. It is anticipated that working with low concentrations of Zn^{++} and the construction of mutants in the hinge, in the dII proton channel and in the conformationally mobile region of dIII (for example at the equivalent of *Ec*βD392 – see above) might be required to lock the conformational state of the enzyme. The possibility of introducing specific mutations into just one protomer of the dimer should help to refine our understanding of the alternating-sites mechanism and identify points of conformational linkage. Changes in the binding of $NADP^+$/NADPH are at the heart of the proposed mechanism; methods for the fluorescence detection of these changes have been developed for isolated dIII and should be adaptable for use in the holoenzyme.

The different mechanisms of coupling to the proton electrochemical gradient in bioenergetic machines

Although many features of the mechanism of coupling the proton electrochemical gradient to the chemical reaction catalyzed by transhydrogenase are far from clear, it is evident that the enzyme utilizes mechanisms unlike those of the other bioenergetic machines present in the mitochondrial inner membrane or in the bacterial membrane: NADH:quinone oxidoreductase (Complex I); quinol:cytochrome *c* oxidoreductase (Complex III or the bc_1

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Complex); cytochrome *c* oxidase (Complex IV); and the F_1F_O-ATP synthase (Complex V). The F_1F_O-ATP synthase couples proton flux across the membrane through two half-channels to conformational changes linked to rotatory motion and a three-fold alternating sites mechanism of ATP synthesis [59]. Complex I also conveys protons across the membrane via four sets of half-channels whose opening/closing conformations are gated by the chemistry occuring at the single distant quinone binding site [60]. The chemistry of oxygen reduction by cytochrome *c* oxidase is used to gate proton channels and alter proton affinities resulting in proton pumping, but conformational changes must be subtle compared to either Complex I or the ATP synthase [61]. Complex III has a unique "Q-cycle" to move charges across the membrane and a large conformational change of the Rieske Fe-S subunit to assure the two electrons from quinol are bifurcated, i.e., directed to different electron acceptors [62]. The enzymology of each of these systems is distinct from the others and each is a remarkable testament to the different ways that evolution has solved the problem of both generating and utilizing the proton motive force across a biological membrane. We can now add the transhydrogenase to this list of amazing molecular machines at the heart of bioenergetics.

Figure Legends

Fig.1. The three-dimensional structure of transhydrogenase from T.thermophilus.

Modified from ref [22]. The gray bars indicate the position of the membrane. The dashed lines delineate the short polypeptide hinges of presently unknown structure that link dII and dIII. NAD(H) and NADP(H) correspond to nucleotides bound in the NAD^+/NADH and $NADP^+$/NADPH sites, respectively. See text for comments on secondary-structure features and on the orientation change of dIII.

Fig.2. The four types of polypeptide organization of transhydrogenases from representative species.

Tt, *Thermus thermophilus*; *Rr*, *Rhodospirillum rubrum*; *Ec*, *Escherichia coli*; *Bt*, *Bos taurus*; *Eh*, *Entamoeba histolytica*. The horizontal lines correspond to polypeptide chains. The labels α, α_1, α_2 and β are names of the polypeptide chains (but sometimes also called PntA, PntAA, PntAB and PntB, respectively). The components dI, dII and dIII, and the

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hinge region, are common to all 4 types of polypeptide organization. The small black rectangles represent transmembrane helices (TM) numbered according to predictions on bovine transhydrogenase but to date only visualised in X-ray structures of the *T.thermophilus* enzyme [22]: question marks adjacent to the number of TM in other species show that estimation is based only on prediction and limited biochemical experiments.

Fig.3. Proposed mechanism of coupling to proton translocation in transhydrogenase.

Each panel diagramatically shows an intermediate in the transhydrogenase dimer as it runs in the forward direction. One protomer of the dimer is coloured red, the other blue. The relative positions of the dI, dII and dIII components correspond to those seen in the X-ray structure of the *T.thermophilus* holo-enzyme (PDB, 4O9U) – see Fig.1. The dI component is shown as a rectangle, dII as a rounded rectangle spanning the membrane, and dIII as a trapezoid with its $NADP^+$/NADPH binding site located at the broader end. In the face-up orientation of dIII (see text) the $NADP^+$/NADPH binding site is located next to the NAD^+/NADH binding site of dI; in the face-down orientation the $NADP^+$/NADPH binding site is directed into the cytoplasmic loops between the TM of dII . The dI and dIII components and *X*, a proton binding site in the dII channel (see text), are shown bounded by dashed lines in open states, and solid lines in occluded states. The pairs of parallel dashed lines in dII show allowed proton access along the channel between *X* and the aqueous phase on one side of the membrane or the other. The six reaction intermediates (1-6) run approximately 180° out-of-phase in the two protomers. Thus, intermediates 1, 2, 3, 4, 5 and 6 in one protomer are paired with intermediates 4, 5, 6, 1, 2 and 3, respectively, in the other. Hydride transfer from NADH to $NADP^+$, only possible with dIII in its occluded face-up orientation, is highlighted with a green arrow. For simplicity, NAD^+ is shown as dissociating, and NADH as binding, only to intermediate 1; in fact, NAD^+ and NADH can probably reach equilibrium binding with all intermediates on the reaction path (see text). The reverse transhydrogenation reaction, oxidation of NADPH by NAD^+ coupled to H^+ efflux, is described essentially by a simple reversal of the reaction sequence shown in this figure.

References

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1. Fuhrer, T. & Sauer, U. (2009) Different biochemical mechanisms ensure network-wide balancing of reducing equivalents in microbial metabolism, *Journal of Bacteriology.* **197**, 2112-2121.
2. Chou, H.-H., Marx, C. J. & Sauer, U. (2015) Transhydrogenase promotes the robustness and evolvability of E.coli deficient in NADPH production, *PLOS Genetics.* **11**.
3. Kabus, A., Georgi, T., Wendisch, V. F. & Bott, M. (2007) Expression of the *Escherichia coli pntAB* genes encoding a membrane-bound transhydrogenase in *Corynebacterium glutamicum* improves L-lysine formation, *Applied Microbiology and Biotechnology.* **75**, 47-53.
4. Arkblad, E. L., Egorov, M., Shakhparonov, M., Romanova, L., Polzikov, M. & Rydstrom, J. (2002) Expression of proton-pumping nicotinamide nucleotide transhydrogenase in mouse, human brain and *C.elegans*, *Compative Biochemistry and Physiology.* **133**, 13-21.
5. Sazanov, L. A. & Jackson, J. B. (1994) Proton-translocating transhydrogenase and NAD- and NADP-linked isocitrate dehydrogenase operate in a substrate cycle which contributes to fine regulation of the tricarboxylic acid cycle in mitochondria, *FEBS Letters.* **344**, 109-116.
6. Gameiro, P. A., Laviolette, L. A., Kelleher, J. K., Iliopoulos, O. & Stephanopoulos, G. (2013) Cofactor balance by nicotinamide nucleotide transhydrogenase (NNT) coordinates reductive carboxylation and glucose catabolism in the tricarboxylic acid (TCA) cycle, *Journal of Biological Chemistry.* **288**, 12967-12977.
7. Arkblad, E. L., Tuck, S., Pestov, N. B., Dmitriev, R. I., Kostina, M. B., Stenvall, J., Tranberg, M. & Rydstrom, J. (2005) A *Caenorhabditis elegans* mutant lacking functional nicotinamide nucleotide transhydrogenase displays increased sensitivity to oxidative stress, *Free Radical Biology and Medicine.* **38**, 1518-1525.
8. Meimaridou, E., Kowalczyk, J., Guasti, L., Hughes, C. R., Wagner, F., Frommolt, P., Nurnberg, P., Mann, N. P., Banerjee, R., Saka, H. N., Chapple, J. P., King, P. J., Clark, A. J. L. & Metherell, L. A. (2012) Mutations in *NNT* encoding nicotinamide nucleotide transhydrogenase cause familial glucocorticoid deficiency, *Nature Genetics.* **44**, 740-742.
9. Toye, A., Lippiat, J. D., Proks, P., Shimomura, K., Bentley, L., Hugill, A., Mijat, V., Goldsworthy, M., Moir, L., Haynes, A., Quarterman, J., Freeman, H. C., Ashcroft, F. M. & Cox, R. D. (2005) A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice, *Diabetologia.* **48**, 675-686.
10. Aston-Mourney, K., Wong, N., Kebede, M., Zraika, S., Balma, L., McMahon, J. M., Fam, B. C., Favaloro, J., Proietto, J., Morahan, G. & Andrikopoulos, S. (2007) Increased nicotinamide nucleotide transhydrogenase levels predispose to insulin hypersecretion in a mouse strain susceptible to diabetes, *Diabetologia.* **50**, 2476-2485.
11. Freeman, H. C., Shimomura, K., Cox, R. D. & Ashcroft, F. M. (2006) Nicotinamide nucleotide transhydrogenase: a link between insulin secretion, glucose metabolism and oxidative stress, *Biochemical Society Transactions.* **34**, 806-810.
12. Fergusson, G., Ethier, M., Guevremont, M., Chretien, C., Attane, C., Joly, E., Fioramonti, X., Prentki, M., Poitout, V. & Alquier, T. (2014) Defective insulin secretory response to intravenous glucose in C57Bl/6J compared to C57Bl/6N mice, *Molecular Metabolism.* **3**, 848-854.
13. Buckley, P. A., Jackson, J. B., Schneider, T., White, S. A., Rice, D. W. & Baker, P. J. (2000) Protein-protein recognition, hydride transfer and proton pumping in the transhydrogenase complex, *Structure.* **8**, 809-815.
14. Prasad, G. S., Wahlberg, M., Sridhar, V., Sundaresan, V., Yamaguchi, M., Hatefi, Y. & Stout, C. D. (2002) Crystal structures of transhydrogenase domain I with and without bound NADH, *Biochemistry.* **41**, 12745-12754.
15. Johansson, T., Oswald, C., Pedersen, A., Tornroth, S., Okvist, M., Karlsson, B. G., Rydstrom, J. & Krengel, U. (2005) X-ray structure of domain I of the proton-pumping membrane protein transhydrogenase from *Escherichia coli*, *Journal of Molecular Biology.* **352**, 299-312.
16. Prasad, G. S., Sridhar, V., Yamaguchi, M., Hatefi, Y. & Stout, C. D. (1999) Crystal structure of transhydrogenase domain III at 1.2 A resolution, *Nature Structural Biology.* **6**, 1126-1131.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

17. White, S. A., Peake, S. J., McSweeney, S., Leonard, G., Cotton, N. P. J. & Jackson, J. B. (1999) The high-resolution structure of the NADP(H)-binding component (dIII) of proton-translocating transhydrogenase from human heart mitochondria, *Structure.* **8**, 1-12.
18. Jeeves, M., Smith, K. J., Quirk, P. Q., Cotton, N. P. J. & Jackson, J. B. (2000) Solution structure of the NADP(H)-binding component (dIII) of proton-translocating transhydrogenase from *Rhodospirillum rubrum*, *Biochimica et Biophysica Acta.* **1459**, 248-257.
19. Cotton, N. P. J., White, S. A., Peake, S. J., McSweeney, S. & Jackson, J. B. (2001) The crystal structure of an asymmetric complex of the two nucleotide binding components of proton-translocating transhydrogenase, *Structure.* **9**, 165-176.
20. Sundaresan, V., Chartron, J., Yamaguchi, M. & Stout, C. D. (2005) Conformational diversity in NAD(H) and interacting transhydrogenase nicotinamide nucleotide binding domains, *Journal of Molecular Biology.* **346**, 617-629.
21. Bhakta, T., Whitehead, S. J., Snaith, J. S., Dafforn, T. R., Wilkie, J., Rajesh, S., White, S. A. & Jackson, J. B. (2007) Structures of the dI_2dIII_1 complex of proton-translocating transhydrogenase with bound, inactive analogues of NADH and NADPH reveal active site geometries, *Biochemistry.* **46**, 3304-3318.
22. Leung, J. H., Schurig-Briccio, L. A., Yamaguchi, M., Moeller, A., Speir, J. A., Gennis, R. B. & Stout, C. D. (2015) Division of labor in transhydrogenase by alternating proton translocation and hydride transfer, *Science.* **347**, 178-181.
23. Venning, J. D., Rodrigues, D. J., Weston, C. J., Cotton, N. P. J., Quirk, P. Q., Errington, N., Finet, S., White, S. A. & Jackson, J. B. (2001) The heterotrimer of the membrane-peripheral components of transhydrogenase and the alternating-site mechanism of proton translocation, *Journal of Biological Chemistry.* **276**, 30678-30685.
24. Mather, O. C., Singh, A., van Boxel, G. I., White, S. A. & Jackson, J. B. (2004) Active-site conformational changes associated with hydride transfer in proton-translocating transhydrogenase, *Biochemistry.* **43**, 10952-10964.
25. Diggle, C., Grimley, R. L., Quirk, P. Q., Thomas, C. M. & Jackson, J. B. (1995) Conformational dynamics of a mobile loop in the NAD(H)-binding subunit of proton-translocating transhydrogenases from *Rhodospirillum rubrum* and *Escherichia coli*, *European Journal of Biochemistry.* **228**, 719-726.
26. Gupta, S., Quirk, P. Q., Venning, J. D., Slade, J., Bizouarn, T., Grimley, R. L., Cotton, N. P. J. & Jackson, J. B. (1998) Mutation of amino acid residues in the mobile loop region of the NAD(H)-binding domain of proton-translocating transhydrogenase, *Biochimica et Biophysica Acta.* **1409**, 25-38.
27. Brondijk, H. C., van Boxel, G. I., Mather, O. C., Quirk, P. Q., White, S. A. & Jackson, J. B. (2006) The role of invariant amino acid residues at the hydride-transfer site of proton-translocating transhydrogenase, *Journal of Biological Chemistry.* **281**, 13345-13354.
28. Sundaresan, V., Yamaguchi, M., Chartron, J. & Stout, C. D. (2003) Conformational change in the NADP(H) binding domain of transhydrogenase defines four states, *Biochemistry.* **42**, 12143-12153.
29. Olausson, T., Hultman, T., Holmberg, E., Rydstrom, J., Ahmad, S., Glavas, N. A. & Bragg, P. D. (1993) Site-directed mutagenesis of tyrosine residues at nicotinamide nucleotide binding sites of *Escherichia coli* transhydrogenase, *Biochemistry.* **32**, 13237-13244.
30. Meuller, J., Hu, X., Olausson, T. & Rydstrom, J. (1996) Indentification of an aspartic acid residue in the beta subunit which is essential for catalysis and proton pumping by transhydrogenase from *Escherichia coli*, *Biochimica et Biophysica Acta.* **1273**, 191-194.
31. Hu, X., Zhang, J., Fjellstrom, O., Bizouarn, T. & Rydstrom, J. (1999) Site-directed mutagenesis of charged and potentially proton-carrying residues in the beta subunit of the proton-translocating nicotinamide nucleotide transhydrogenase from *Escherichia coli*, *Biochemistry.* **38**, 1652-1658.
32. Fjellstrom, O., Axelsson, M., Bizouarn, T., Hu, X., Johansson, C., Meuller, J. & Rydstrom, J. (1999) Mapping of residues in the NADP(H)-binding site of proton-translocating nicotinamide nucleotide transhydrogenase from *Escherichia coli*, *Journal of Biological Chemistry.* **274**, 6350-6359.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

33. Bergkvist, A., Johansson, C., Rydstrom, J. & Karlsson, B. G. (2000) Interactions of the NADP(H)-binding domain III of proton-translocating transhydrogenase from *Escherichia coli* with NADP(H) and the NAD(H)-binding domain I studied by NMR and site-directed mutagenesis, *Biochemistry.* **39**, 12595-12605.
34. Johansson, C., Pedersen, A., Karlsson, B. G. & Rydstrom, J. (2002) Redox-sensitive loops D and E regulate NADP(H) binding in domain III and domain I-domain III interactions in proton-translocating transhydrogenase, *European Journal of Biochemistry.* **269**, 4505-4515.
35. Quirk, P. Q., Jeeves, M., Cotton, N. P. J., Smith, K. J. & Jackson, J. B. (1999) Structural changes in the recombinant, NADP(H)-binding component of proton-translocating transhydrogenase revealed by NMR spectroscopy, *FEBS Letters.* **446**, 127-132.
36. Jackson, J. B., White, S. A., Quirk, P. Q. & Venning, J. D. (2002) The alternating site, binding change mechanism for proton translocation by transhydrogenase, *Biochemistry.* **41**, 4173-4185.
37. Pedersen, A., Johansson, T., Rydstrom, J. & Karlsson, B. G. (2005) Titration of *E. coli* transhydrogenase domain III with bound $NADP^+$ or NADPH studied by NMR reveals no pH-dependent conformational change in the physiological pH range, *Biochimica et Biophysica Acta.* **1707**, 254-258.
38. Meuller, J. & Rydstrom, J. (1999) The membrane topology of proton-pumping *Escherichia coli* transhydrogenase determined by cysteine labeling, *Journal of Biological Chemistry.* **274**, 19072-19080.
39. Studley, W. K., Yamaguchi, M., Hatefi, Y. & Saier, M. H. (1999) Phylogenetic analyses of proton-translocating transhydrogenases, *Microbial and Comparative Genomics.* **4**, 173-186.
40. Wilson, R., Obiozo, U. M., Quirk, P. Q., Besra, G. S. & Jackson, J. B. (2006) A hybrid of the transhydrogenase from *Rhodospirillum rubrum* and *Mycobacterium tuberculosis* catalyses rapid hydride transfer but not the complete, proton-translocating reaction, *Biochimica et Biophysica Acta.* **1757**, 215-223.
41. Yamaguchi, M. & Hatefi, Y. (1995) Proton-translocating transhydrogenase of *Escherichia coli*. Involvement of aspartate 213 in the membrane-intercalating domain of the beta subunit in energy transduction, *Journal of Biological Chemistry.* **270**, 16653-16659.
42. Yamaguchi, M., Stout, C. D. & Hatefi, Y. (2002) The proton channel of the energy-transducing nicotinamide nucleotide transhydrogenase of *Escherichia coli*, *Journal of Biological Chemistry.* **277**, 33670-33675.
43. Yamaguchi, M. & Stout, C. D. (2003) Essential glycine in the proton channel of *Escherichia coli* transhydrogenase, *Journal of Biological Chemistry.* **278**, 45333-45339.
44. Althage, M., Bizouarn, T. & Rydstrom, J. (2001) Identification of a region in the communication between the NADP(H) binding domain and the membrane domain in proton pumping *E.coli* transhydrogenase, *Biochemistry.* **40**, 9968-9976.
45. Karlsson, J., Althage, M. & Rydstrom, J. (2003) Roles of individual amino acids in helix 14 of the membrane domain of proton-translocating transhydrogenase from *Escherichi coli* as deduced from cysteine mutagenesis, *Biochemistry.* **42**, 6575-6581.
46. Bragg, P. D. & Hou, C. (1996) The role of conserved histidine residues in the pyridine nucleotide transhydrogenase of *Escherichia coli European Journal of Biochemistry.* **241**, 611-618.
47. Bragg, P. D. & Hou, C. (2001) Characterization of mutants of beta histidine91, beta aspartate213, and beta asparagine222, possible components of the energy transduction pathway of the proton-translocating pyridine nucleotide transhydrogenase of *Escherichia coli*, *Archives of Biochemistry and Biophysics.* **388**, 299-307.
48. Bragg, P. D. & Hou, C. (1999) Mutation of conserved polar residues in the transmembrane domain of the proton-pumping pyridine nucleotide transhydrogenase of *Escherichia coli*, *Archives of Biochemistry and Biophysics.* **363**, 182-190.
49. Whitehead, S. J., Iwaki, M., Cotton, N. P. J., Rich, P. & Jackson, J. B. (2009) Inhibition of proton-transfer steps in transhydrogenase by transition metal ions, *Biochimica et Biophysica Acta.* **1787**, 1276-1288.

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50. Veronesi, J., Whitehead, S. J., Frankia, F., Gianchi, L., Boscherini, F., Venturoli, G., Cotton, N. P. J. & Jackson, J. B. (2010) X-ray absorption studies of Zn^{++}-binding sites in *Escherichia coli* transhydrogenase and its betaH91K mutant, *Biochimica et Biophysica Acta.* **1797**, 494-500.
51. Hutton, M., Day, J. M., Bizouarn, T. & Jackson, J. B. (1994) Kinetic resolution of the reaction catalysed by proton-translocating transhydrogenase from *Escherichia coli* as revealed by experiments with analogues of the nucleotide substrates, *European Journal of Biochemistry.* **219**, 1041-1051.
52. Diggle, C., Cotton, N. P. J. & Jackson, J. B. (1996) Properties of the purified, recombinant, NADP(H)-binding domain III of the proton-translocating transhydrogenase from *Rhodospirillum rubrum*, *European Journal of Biochemistry.* **241**, 162-170.
53. Pedersen, A., Karlsson, B. G. & Rydstrom, J. (2008) Proton-translocating transhydrogenase: an update of unresolved and controversial issues, *Journal of Bioenergetics and Biomembranes.* **40**, 463-473.
54. Jackson, J. B. (2012) A review of the binding-change mechanism for proton-translocating transhydrogenase, *Biochimica et Biophysica Acta.* **1817**, 1839-1846.
55. Bizouarn, T., Stilwell, S., Venning, J. D., Cotton, N. P. J. & Jackson, J. B. (1997) The pH dependences of reactions catalysed by the complete proton-translocating transhydrogenase from *Rhodospirillum rubrum*, and the complex formed from its recombinant nucleotide-binding domains, *Biochimica et Biophysica Acta.* **1322**, 19-32.
56. Venning, J. D., Peake, S. J., Quirk, P. Q. & Jackson, J. B. (2000) Stopped-flow reaction kinetics of recombinant components of proton-translocating transhydrogenase with physiological nucleotides, *Journal of Biological Chemistry.* **275**, 19490-19497.
57. Venning, J. D. & Jackson, J. B. (1999) A shift in the equilibrium constant at the catalytic site of proton-translocating transhydrogenase: significance for a binding-change mechanism, *Biochemical Journal.* **341**, 329-337.
58. Pinhero, T. J. T., Venning, J. D. & Jackson, J. B. (2001) Fast hydride transfer in proton-translocating transhydrogenase revealed in a rapid mixing continuous flow device, *Journal of Biological Chemistry.* **276**, 44757-44761.
59. Martin, J., Hudson, J., Hornung, T. & Frasch, W. D. (2015) Fo-driven rotation in the ATP synthase direction against the force of F1ATPase in the FoF1 ATP synthase, *Journal of Biological Chemistry.* **290**, 10717-10728.
60. Sazanov, L. A. (2014) The mechanism of coupling between electron transfer and proton translocation in respiratory complex 1, *Journal of Bioenergetics and Biomembranes.* **46**, 247-253.
61. Brzezinski, P. & Gennis, R. B. (2008) Cytochrome c oxidase: exciting progress and remaining mysteries, *Journal of Bioenergetics and Biomembranes.* **40**, 521-531.
62. Crofts, A. R., Lhee, S., Crofts, S. B., Cheng, J. & Rose, S. (2006) Proton pumping in the bc1 complex: a new gating mechanism that prevents short circuits, *Biochimica et Biophysica Acta.* **1757**, 1019-1034.

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Figure 1

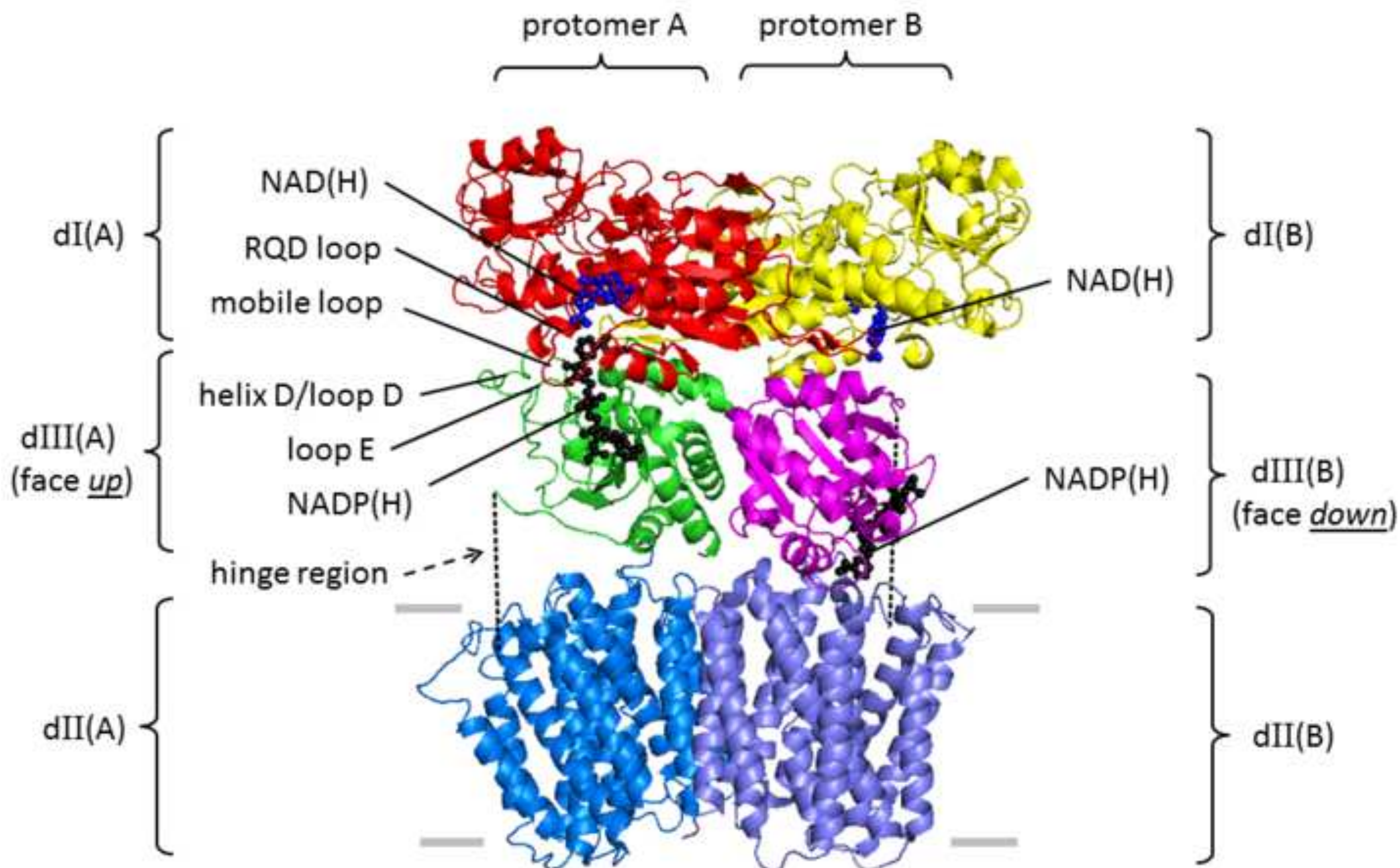

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Figure 2

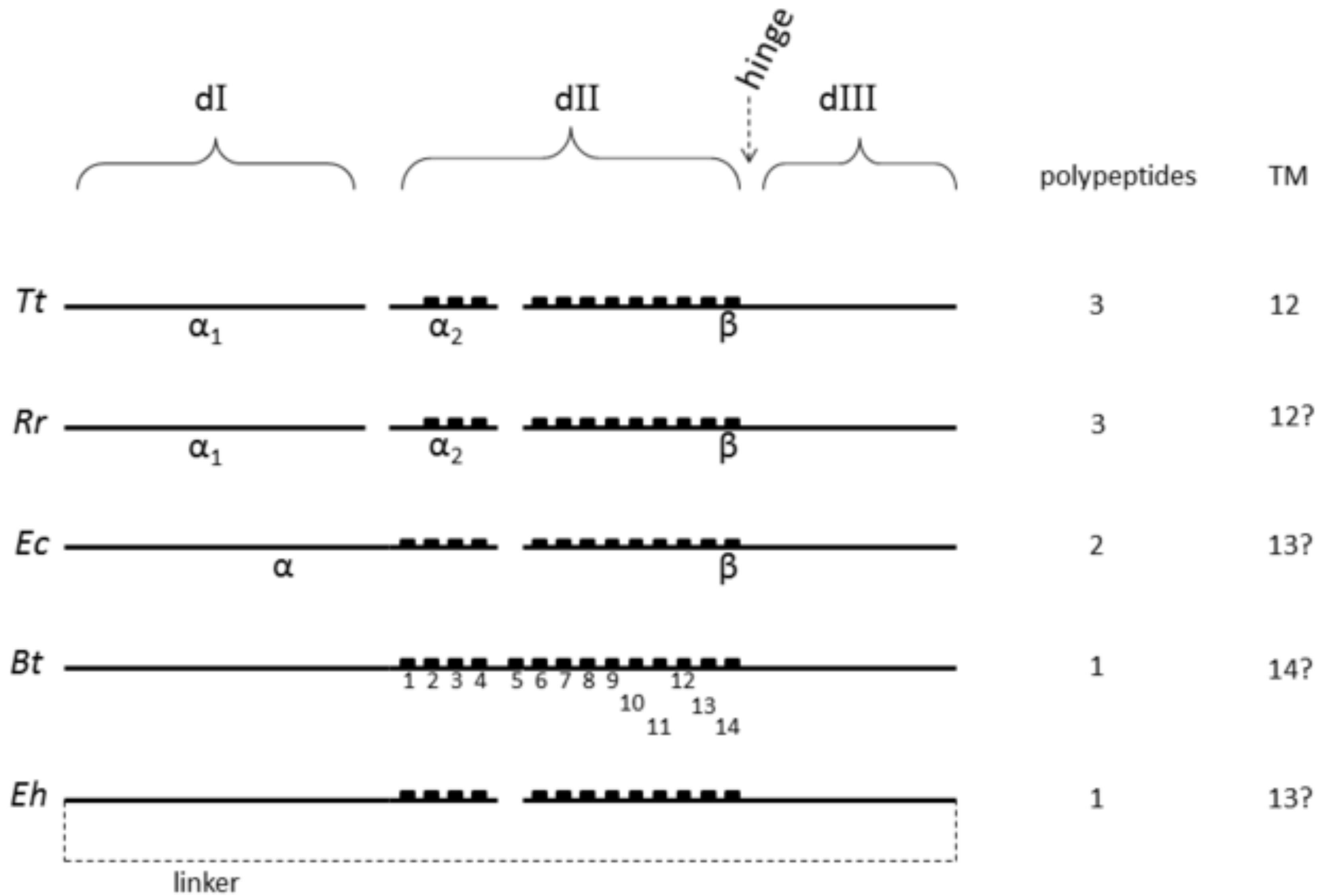

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Figure 3