The unusual transhydrogenase of *Entamoeba histolytica*

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Abstract We have expressed and purified a protein fragment from *Entamoeba histolytica*. It catalyses transhydrogenation between analogues of NAD(H) and NADP(H). The characteristics of this reaction resemble those of the reaction catalysed by a complex of the NAD(H)- and NADP(H)-binding subunits of proton-translocating transhydrogenases from bacteria and mammals. It is concluded that the complete *En. histolytica* protein, which, along with similar proteins from other protozoan parasites, has an unusual subunit organisation, is also a proton-translocating transhydrogenase. The function of the transhydrogenase, thought to be located in organelles which do not have the enzymes of oxidative phosphorylation, is not clear.

Key words: Transhydrogenase; Proton pump; Nucleotide binding; Hydride transfer; Protozoan parasite; *Entamoeba histolytica*

1. Introduction

Transhydrogenase couples the direct transfer of hydride-ion equivalents between the NC4*A*-position of NAD(H) and the NC4*B*-position of NADP(H) to the translocation of protons across a membrane:

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

The enzyme has been isolated and purified from the cytoplasmic membranes of bacteria and the inner membranes of mammalian mitochondria, and studied in detail (for reviews see [1,2]). Transhydrogenase has three components; dI, which has the binding site for NAD(H), and dIII, which has the binding site for NADP(H), protrude from the membrane, and dII spans the membrane. High-resolution structures of isolated dI [3] and dIII [1,4–6] were recently presented.

Genome sequences indicate that transhydrogenase is widely distributed in animal cells and many bacteria. It is absent from some bacteria, from *Saccharomyces cerevisiae* and probably from higher plants, though it is present in cyanobacteria and the green alga, *Acetabulum acetabularia*. In mammals, transhydrogenase has a single polypeptide, which runs dI–dII–dIII, N-terminus to C-terminus (Fig. 1). The intact mammalian protein is probably homodimeric [7,8]. In bacteria, gene sequences show that the dII component is always separated into dIIa and dIIb. Additionally, in some bacteria, dI is separated from dIIa. Thus, in *Escherichia coli*, *Salmonella typhimurium* and *Haemophilus influenzae*, for example, there are two polypeptides, PntA or α (comprising dI–dIIa) and PntB or β (comprising dIIb–dIII), whereas in *Rhodospirillum rubrum*, *Mycobacterium tuberculosis* and *Bordetella pertussis* and others, there are three polypeptides: PntAA (dI), PntAB (dIIa) and PntB (dIIb–dIII). Intact *E. coli* transhydrogenase is an $\alpha_2\beta_2$ dimer [9], but the oligomeric state of other bacterial species is not known.

Putative transhydrogenases have been identified from gene sequences of some protozoan parasites (*Entamoeba histolytica* [10,11], *Eimeria tenella* [12,13], *Plasmodium falciparum*) but they are unusual (Fig. 1). The predicted order of components in the single polypeptide is dIIb–dIII–dI–dIIa. Thus, the N-terminus of the protein corresponds to the N-terminus of the bacterial β polypeptide (PntB). An extra segment (38 amino acid residues in both *Ei. tenella* and *En. histolytica*) is predicted between dIII and dI and might simply serve as a linker. The location of the putative transhydrogenase in these organisms also raises interesting questions. Their protein pre-sequences are either grossly modified from the characteristic mitochondrial pre-sequence (*En. histolytica*), or they are entirely lacking (*Ei. tenella*). Immunochemical experiments suggest that the *Ei. tenella* dIIb–dIII–dI–dIIa protein is located, not in the mitochondria, but in 'refractile bodies' of unknown function [13]. In *En. histolytica* (which does not possess mitochondria), comparison of the protein pre-sequence with that of Hsp60 suggests that the dIIb–dIII–dI–dIIa protein is associated with 'mitosomes' or 'cryptons', small organelles which number only about one per cell [14–17]. Although the mitosomes/cryptons are mitochondrially derived, they are thought to lack the enzymes of oxidative phosphorylation.

Transhydrogenation activity in lysing, intact cells of *En. histolytica* was reported [18] but the protein(s) responsible for the activity were not characterised. In the present work, we have isolated DNA coding for the putative dIII–linker–dI region of *En. histolytica* transhydrogenase (henceforth called *eh*dIII–dI), and expressed and purified the protein. Its catalytic properties closely resemble those of complexes formed from mixtures of isolated dI and isolated dIII from transhydrogenases of bacteria and mammalian mitochondria [19–21]. This result indicates that the complete enzyme is probably a transhydrogenase with properties typical of those found in other species.

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Abbreviations: dI, the NAD(H)-binding component of transhydrogenase; dIII, the NADP(H)-binding component; *eh*dIII–dI, the tethered complex of dIII and dI of the putative transhydrogenase from *Entamoeba histolytica*; $AcPdAD^+$, acetyl pyridine nicotinamide dinucleotide (oxidised form)

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2. Materials and methods

DNA coding for *eh*dIII–dI (from Met236–Lys931 of the complete protein) was isolated by polymerase chain reaction (PCR) with a construct supplied by Dr. C.G. Clark, of the London School of Hygiene and Tropical Medicine, as target. Primers were synthesised by Alta Bioscience. The 44-base sense-primer, constructed from nucleotide residues 975–1006, incorporated a *Bsp*HI restriction site. The 40-base anti-sense-primer, constructed from nucleotide residues 2765–2792, incorporated a stop codon (at position 2790–2792) and a *Bam*HI restriction site. The PCR product was cloned into pPCR-Script using the pPCR-Script Amp SK(+) cloning kit (Stratagene). The *Bsp*HI and *Bam*HI fragment was then subcloned into the expression vector pET21d (Novagen), to give pCJW3, which was transformed into *E. coli* BL21(DE3). Plasmid preparations from selected clones were sequenced using an Applied Biosystems 373A.

Expression of *eh*dIII–dI was induced by addition of 1 mM isopropyl β-D-thiogalactoside to mid-log phase cells grown at 25°C in NZCYM media [22]. Cells were harvested 14 h post-induction by centrifugation, washed in 50 mM Tris–HCl pH 8.0, 1 mM EDTA, 2 mM dithiothreitol (TED buffer) and stored at −20°C. The cells were thawed, resuspended in TED buffer supplemented with 0.5 mM phenylmethylsulphonyl fluoride and 100 μM $NADP^+$, and disrupted by sonication. Soluble cell extract, obtained by centrifugation at $105\,000\times g$ for 120 min, was applied to a 5×30 cm column of Q-Sepharose Fast Flow (Pharmacia Biotech) equilibrated with 20 mM Tris–HCl pH 8.0, 2 mM dithiothreitol, 4 μM $NADP^+$ (buffer A) and the column was developed with a gradient of 0–0.5 M NaCl. Active fractions were pooled, dialysed (1:15) in buffer A supplemented with 10% glycerol for 2 h, and then brought to 10% saturation with ammonium sulphate and incubated overnight at 4°C. Precipitate was removed by filtration, and the protein solution was applied to a 2.6×18 cm column of Phenyl-Sepharose HP (Pharmacia Biotech) equilibrated in buffer A supplemented with 10%-saturated ammonium sulphate. The column was developed with a reverse gradient of ammonium sulphate (10 to 0% saturation) followed by a 0–20% glycerol gradient. Recombinant *eh*dIII–dI was eluted in the glycerol wash. Active fractions were pooled, concentrated to ~3 ml (Vivascience centrifugal filters, 10 kDa cut-off) and subjected to a final purification step on a 2.6×62 cm column of Hiload Superdex 200 (Pharmacia Biotech) equilibrated in buffer A supplemented with 150 mM NaCl and 5% glycerol. The protein was subsequently stored in 25% glycerol at −80°C in 2 ml thin-walled cryovials (Nalgene). SDS–PAGE was performed using a discontinuous buffer system as described [23]. Protein concentration was measured by the microtannin assay [24] with bovine serum albumin as standard.

Reverse and cyclic transhydrogenase activities were measured spectrophotometrically by following the reduction of acetyl pyridine nicotinamide dinucleotide (oxidised form) ($AcPdAD^+$) at 375 nm, 25°C, using a Perkin Elmer Lambda 16 spectrophotometer, and an absorbance coefficient of 6.10 mM^{-1} cm^{-1} [25]. Forward transhydrogenation rates were measured by following the reduction of thio-$NADP^+$ at 395 nm, using an absorbance coefficient of 11.3 mM^{-1} cm^{-1} [26]. The reaction buffer was 50 mM MOPS–KOH, pH 7.2, 50 mM KCl, 2 mM $MgCl_2$. For pH-dependence measurements the buffer was 20 mM MES, 20 mM MOPS, 20 mM HEPES, 20 mM CHES, 50 mM KCl and 2 mM $MgCl_2$, set to an appropriate pH with HCl or NaOH. Prior to all experiments, the stored protein was thawed on ice, concentrated in centrifugal filters (10 kDa cut-off) and washed with reaction buffer supplemented with 2 mM DTT and 4 μM $NADP^+$.

3. Results

There are 10 differences between the two published nucleotide sequences of the gene coding for the putative dIIb–dIII–dI–dIIa transhydrogenase of *En. histolytica* [10,11]. In our determinations, the sequences of the DNA insert coding for dIII–dI in pCJW3 (this work), and of the parent genomic clone, were identical to that in [11], except for a single base change to an A at position 2567; this base is an A also in [10].

Induction of cells of *E. coli* BL21(DE3) bearing pCJW3 with isopropyl β-D-thiogalactoside led to the expression of a protein, whose M_r of 66 kDa on SDS–PAGE corresponds to that of *eh*dIII–dI. The protein was purified by column chromatography. Typically, ~100 mg of the protein (>90% pure according to staining intensity on SDS–PAGE) were recovered from 3.2 l of *E. coli* culture. The purified protein eluted with an $M_r \approx 155$ kDa during non-denaturing gel-filtration chromatography; the predicted M_r of the monomer is 65.8 kDa.

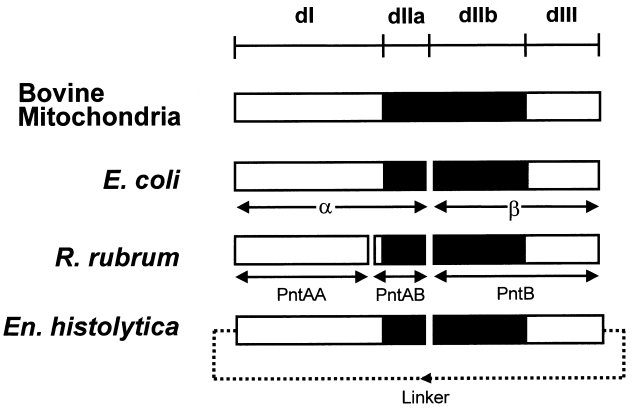

Fig. 1. Subunit organisation of transhydrogenases. See text.

After dialysis of the purified protein (87 μM in buffer containing 4 μM $NADP^+$, see Section 2) against 1000 volumes of 20 mM Tris–HCl, pH 8.0 and 2 mM dithiothreitol, to remove excess and weakly associated nucleotide, it remained fully active (see below). Subsequent analysis (see [20]) indicated that the dialysed material retained 0.54 mol $NADP^+$ per mol protein; there was no bound NADPH, NAD^+ or NADH. Under equivalent conditions, the isolated dIII component of *R. rubrum* [20], *E. coli* [21] and human [27] transhydrogenases also retain tightly bound nucleotide.

The catalytic properties of the purified, **tethered** *eh*dIII–dI protein were generally very similar to those of the complex formed from a mixture of the **isolated** dI and dIII components of *R. rubrum* [20] or *E. coli* [21] transhydrogenase, or of various hybrids of the bacterial and mammalian enzymes [27,28]. Thus, purified *eh*dIII–dI catalysed both 'reverse transhydrogenation' (the reduction of the NAD^+ analogue, $AcPdAD^+$, by NADPH, Fig. 2), and 'forward transhydrogenation' (the reduction of thio-$NADP^+$ by NADH, data not shown), only at very low rates. However, the protein catalysed 'cyclic transhydrogenation' (the reduction of $AcPdAD^+$ by NADH in the presence of $NADP^+$, Fig. 2) at a very high rate. The rapid rate of this reaction, which involves the alternate reduction of $NADP^+$ by NADH, and the oxidation of NADPH by $AcPdAD^+$ [29], shows that (a) NAD(H) and AcPdAD(H) binding and release, and (b) the two hydride transfer reactions on the protein ($NADH \rightarrow NADP^+$ and $NADPH \rightarrow AcPdAD^+$), are both relatively fast. Cyclic transhydrogenation proceeds rapidly when $NADP^+$ and NADPH remain bound to the protein [29]. It is therefore concluded that the low rates of forward and reverse transhydrogenation by *eh*dIII–dI (similar to the reactions catalysed by *R. rubrum* dI:dIII complexes) are a consequence of slow release of product NADPH and $NADP^+$, respectively. Note that the substrate inhibition of the cyclic reaction, observed at high concentrations of NADH (Fig. 2), is probably a consequence of competition between NADH and $AcPdAD^+$ for the same binding site [30].

The pH dependence of reverse transhydrogenation by *eh*dIII–dI resembles that described in detail for the dI:dIII complex of *R. rubrum* transhydrogenase (Fig. 3); the increase in rate at low pH is attributed to acceleration of $NADP^+$ release due to protonation of dIII (J.D. Venning and D. Rodrigues, personal communication). The pH dependence of the cyclic reaction (Fig. 3), thought to be dominated by the protonation state of dI, reveals a $pK_a \approx 7.2$, intermediate between those of the *R. rubrum* [31] and *E. coli* [21,28] dI:dIII complexes.

4. Discussion

The results presented above show that the tethered dIII–dI component of the dIIb–dIII–dI–dIIa protein from *En. histolytica* has transhydrogenase activity. The relative rates of the reverse and cyclic reactions catalysed by *eh*dIII–dI prove that the hydride transfer steps on the protein are fast, but that the rates of $NADP^+$ and NADPH release from their binding sites are slow. For the well-characterised transhydrogenases from bacteria and animal mitochondria, it is established that interactions with the membrane-spanning dII accelerate $NADP^+$ and NADPH release from dIII, and that this is important in the coupling to proton translocation [1]. Because the characteristics of the proteins are so similar, it is likely that the same

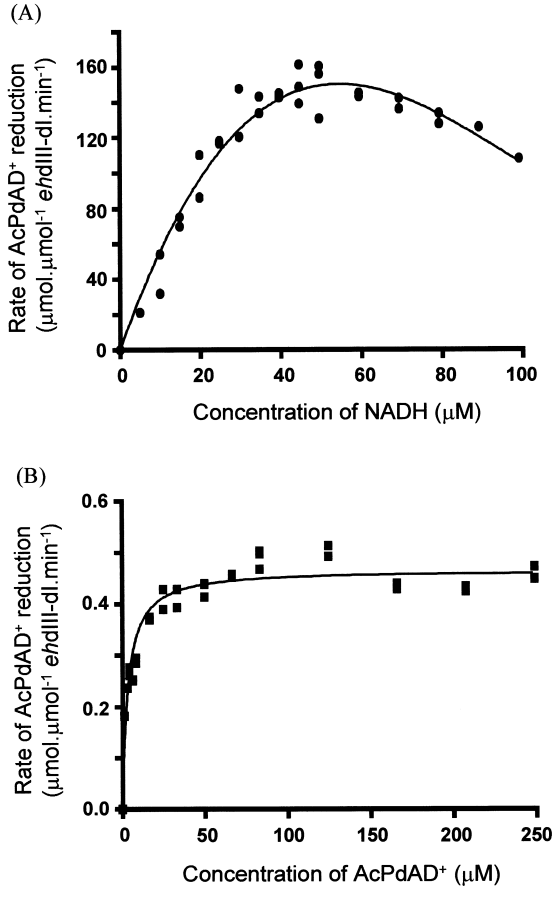

Fig. 2. Transhydrogenation reactions catalysed by *eh*dIII–dI. For cyclic transhydrogenase activity (A) the concentrations of *eh*dIII–dI, $NADP^+$ and $AcPdAD^+$ were 1, 21 and 219 μM, respectively. The concentration of NADH was varied as indicated. For reverse transhydrogenase activity (B) the protein concentration was 2 μM and the NADPH concentration was 25 μM. The concentration of $AcPdAD^+$ was varied as indicated. See Section 2.

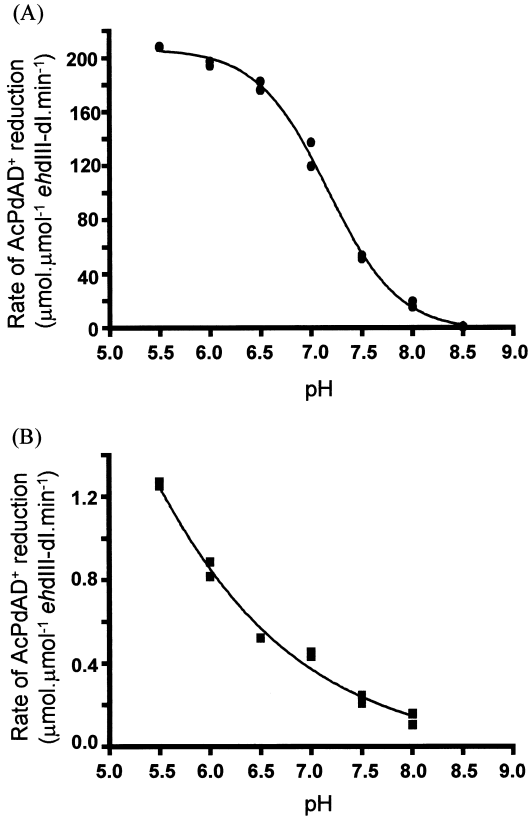

Fig. 3. The pH dependence of transhydrogenation catalysed by the *eh*dIII–dI protein. Cyclic transhydrogenation (A) was carried out in the presence of 630 nM *eh*dIII–dI, 21 μM $NADP^+$, 57 μM NADH and 193 μM $AcPdAD^+$. For reverse transhydrogenation (B), 1.26 μM protein was present, with 51 μM NADPH and 193 μM $AcPdAD^+$. See Section 2.

situation applies to the *En. histolytica* enzyme. Thus, the dIIb–dIII–dI–dIIa proteins of the protozoan parasites are very probably proton-translocating transhydrogenases, as predicted [10,11].

This conclusion re-focuses on the question as to the function of transhydrogenase in this group of organisms. The primary issue is to identify the protein(s) that operate in series with transhydrogenase in the expected chemiosmotic proton circuit. *En. histolytica* grows by fermentation [32]. It lacks some of the enzymes of the TCA cycle [32,33], and is reported not to have cytochromes [32,34]. However, the dIIb–dIII–dI–dIIa transhydrogenase is thought to be located in mitosomes (or cryptons) [14,15], which are mitochondrially derived organelles, present only in small numbers in the cell, and it is doubtful if earlier analyses would have been sensitive enough to detect small quantities of redox components in such a minor cellular component. Thus, it remains entirely possible that the mitosomes do possess a proton-pumping electron transport chain (not necessarily with O_2 as the terminal electron acceptor), that would drive transhydrogenation. The existence of F-type ATPase has not been reported in *En. histolytica*, although there is evidence for a V-type ATPase [35], the location of which is unknown. The relatively small amount of membrane associated with the mitosome make it very unlikely that transhydrogenase will play a significant role in the central metabolic pathways of the organism (contrast [32]). Thus, unless it is unusually enriched within the organelle membrane,

the enzyme will not have the catalytic capacity to shift large amounts of reducing power between cytoplasmic NAD(H) and NADP(H). More likely, the transhydrogenase, with its catalytic site facing the 'matrix' of the mitosome (as in its mitochondrial progenitor), will be responsible for controlling the redox state of the nicotinamide nucleotide pools within the organelle, but for what purpose, remains to be established. A biochemical examination of the mitosome is awaited to further an understanding of this problem.

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