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Crystallization of the dI component of transhydrogenase, a proton-translocating membrane protein

Sveta E. Sedelnikova,[a] Jackie Burke,[a] Patrick A. Buckley,[a] David W. Rice,[a] J. Baz Jackson,[b] Nick P. J. Cotton,[b] Rachel L. Grimley[b] and Patrick J. Baker[a]*

[a]The Krebs Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2TN, England, and [b]School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, England

Correspondence e-mail: p.baker@sheffield.ac.uk

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Nicotinamide nucleotide transhydrogenase couples the exchange of a hydride-ion equivalent between NAD(H) and NADP(H) to the translocation of protons across an energy-transducing membrane. Peripheral components of 380 and 200 residues bind NAD(H) (dI) and NADP(H) (dIII), respectively, while a third component forms a membrane-spanning region (dII). The NAD(H)-binding component dI of *Rhodospirillum rubrum* transhydrogenase has been crystallized in a form which diffracts to beyond 3.0 Å resolution and is in space group $P2$ or $P2_1$, with unit-cell parameters a = 69.3, b = 117.8, c = 106.6 Å, β = 107.2° and two dimers in the asymmetric unit. The sequence of the dI component is similar to that of alanine dehydrogenase. A full structure determination will lead to important information on the mode of action of this proton pump and will permit the comparison of the structure–function relationships of dI with those of alanine dehydrogenase.

1. Introduction

Nicotinamide nucleotide transhydrogenase (E.C. 1.6.1.1) is a proton pump. It couples the transfer of reducing equivalents between NAD(H) and NADP(H) to the translocation of protons across a membrane,

$$\mathrm{NADH} + \mathrm{NADP^+} + \mathrm{H^+_{out}} \longleftrightarrow \mathrm{NAD^+} + \mathrm{NADH} + \mathrm{H^+_{in}}.$$

The protein, found in the inner membrane of animal mitochondria and the cytoplasmic membrane of some bacteria, has a tripartite structure (Jackson *et al.*, 1998; Rydström *et al.*, 1998). The dI (approximately 380 amino-acid residues) and dIII (approximately 200 residues) components protrude from the membrane and carry the binding sites for NAD(H) and NADP(H), respectively. The dII component (approximately 400 amino-acid residues) is embedded in the membrane and thus provides at least part of the pathway for proton translocation. The structures of bovine and human dIII, the NADPH-binding portion of this enzyme, have recently been reported (Prasad *et al.*, 1999; White *et al.*, 2000). The structure of dIII is a variant of the classical Rossmann fold, with the dinucleotide in an inverted binding orientation to that normally seen, perhaps indicating how the nicotinamide ring of the bound $NADP^+$ may interact with that of the NADH bound to dI.

The three components of transhydrogenase are located, depending upon species, on either one, two or three polypeptides. In the transhydrogenase from *R. rubrum*, dI exists as a separate polypeptide of 384 residues and M_r = 40 240 which can be easily separated from the other components (Cunningham *et al.*, 1992). In other species, such as *Escherichia coli* and *Bos taurus*, the dI polypeptide is fused to all or part of the dII component, with a separate polypeptide constructing the remainder of dII and all of dIII (Clarke *et al.*, 1986; Yamaguchi *et al.*, 1988). Sequencing studies have shown the presence of a dinucleotide-binding fingerprint G*x*G*xx*G (Wierenga *et al.*, 1985) in the dI component, which suggests that part of the structure folds to form a prototypic dinucleotide-binding domain (Williams *et al.*, 1994; Yamaguchi & Hatefi, 1994; Fjellstrom *et al.*, 1995). Furthermore, dI possesses significant sequence similarity to L-alanine dehydrogenase (AlaDH; Cunningham *et al.*, 1992), a soluble protein found widely in bacteria (Ohshima & Soda, 1990; Vancura *et al.*, 1989) with known three-dimensional structure (Baker *et al.*, 1998), suggesting an evolutionary relationship between these two proteins.

The dI and dIII proteins of *R. rubrum* transhydrogenase can be expressed separately and purified as stable water-soluble proteins (Diggle *et al.*, 1995, 1996). Both the dI and dIII components are highly conserved across different species, with approximate sequence identities of 25 and 30%, respectively. The dI component appears to assemble as a dimer in solution (Diggle *et al.*, 1995). A mixture of dI and dIII carries out very rapid single-turnover reduction of the NAD^+ analogue $AcPdAD^+$ by

NADPH even in the absence of dII, showing that the apparatus for the hydride-transfer reaction is located entirely within dI and dIII (Venning *et al.*, 1997, 1998). The transfer of the hydride-ion equivalent between NAD(H) bound to dI and NADP(H) bound to dIII is direct and does not involve redox intermediates (Venning *et al.*, 1997). The structure determination of dI is essential for understanding the mechanism of action of the complete protein. A comparison with the structure of AlaDH will permit further exploration of the evolutionary relationships between the two proteins.

2. Protein purification and crystallization

Wild-type *R. rubrum* dI was cloned and expressed in *E. coli* as described previously (Diggle *et al.*, 1995). For preparation of dI labelled with selenomethionine, *E. coli* strain DL41 was transformed with pCD1 and cells were grown on controlled minimal media supplemented with selenomethionine. The cells of either the selenomethionine or wild-type protein were harvested by centrifugation and stored at 253 K. For purification, the cell paste was defrosted and suspended in buffer *A* (40 m*M* Tris–HCl pH 8.0, 2 m*M* EDTA). The cells were disrupted by ultrasonication for 3 × 20 s at 16 µm amplitude. Debris was removed by centrifugation at 42 000*g* for 10 min. The supernatant fraction was applied to a 30 ml column of DEAE–Sepharose Fast Flow and the proteins were eluted with a 200 ml gradient of NaCl from 0 to 0.22 *M* in buffer *A* at a flow rate of 3 ml min^{-1}. Collected fractions were analysed for protein concentration by the method of Bradford (1976) using the Bio-Rad Dye Reagent. The dI component elutes as the main protein peak. Fractions with the highest protein concentration were combined, concentrated to a volume of 1–2 ml using a Viva-Spin concentrator and applied to a 1.6 × 60 cm Hi-Load Superdex-200 gel-filtration column (Pharmacia) equilibrated with buffer *B* (50 m*M* sodium phosphate pH 7.5, 15 m*M* ammonium sulfate) at a flow rate of 1 ml min^{-1}. The dI protein was eluted from this column as a protein of apparent molecular mass $\simeq$ 95 kDa, indicating its dimeric state in solution under these buffer conditions. The yield of protein was 6–14 mg from 800 ml culture, with a purity estimated by SDS–PAGE of ∼95%. For crystallization using the hanging-drop method, dI was concentrated to 18–22 mg ml^{-1} on a Viva-Spin concentrator and 10 µl samples in 10 m*M* sodium phosphate buffer pH 7.5, 30 m*M* ammonium sulfate, 10 m*M* NAD$^+$ were mixed with equal volumes of reservoir solution, 200 m*M* sodium phosphate buffer pH 7.5, 30 m*M* ammonium sulfate and 10–20% methyl ethyl PEG 2000. Crystals of plate-like morphology grew within two weeks to maximum dimensions of 0.5 × 0.3 × 0.1 mm.

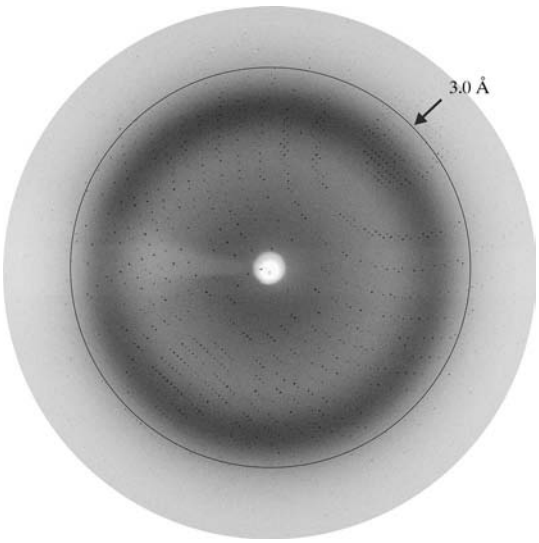

Figure 1
A diffraction image of a test crystal of the dI component of *R. rubrum* transhydrogenase exposed at the CCLRC SRS Daresbury synchrotron at station PX 7.2, showing diffraction to beyond 3.0 Å resolution.

3. Space-group determination

For data collection, a single crystal was stabilized in 15% PEG 400, 10 m*M* NAD$^+$, 25% methyl ethyl PEG 2000, 200 m*M* sodium phosphate buffer pH 7.5 and 30 m*M* ammonium sulfate prior to flash-freezing at 100 K. The crystals diffracted to beyond 3.0 Å on a synchrotron source (Fig. 1) and preliminary data were collected from a native crystal to 4.2 Å resolution using a MAR 345 detector with double-mirror focused Cu $K\alpha$ X-rays produced by a Rigaku RU-200 rotating-anode generator. 180 1° rotation images were processed and merged using *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997). The autoindexing algorithm of *DENZO* suggested that the space group was either $P2$ or $P2_1$, with unit-cell parameters a = 69.3, b = 117.8, c = 106.6 Å, β = 107.2°. The data were 98.7% complete (97.3% in the 4.3–4.2 Å shell), were merged to an overall R factor of 0.11 (0.22 in the 4.3–4.2 Å shell) and had an overall $I/\sigma(I)$ of 13.8 (6.9 in the highest resolution shell). Although there appears to be a $2n$ condition on the $0k0$ reflections, possibly indicating that the space group is $P2_1$, higher resolution data will be required to verify this. The value of V_m for four chains in the asymmetric unit is 2.58 Å^3 Da^{-1}, which is within the range given by Matthews (1977) and suggests that two dimers of dI are present in the asymmetric unit. A self-rotation function using data in the range 10–4.2 Å and an integration radius of 20 Å shows the presence of a twofold axis with peak height 87% of the origin lying along a^*, possibly indicating the direction of one of the non-crystallographic twofold axes. Attempts to solve the structure are under way and once completed should lead to important information on the mode of action of this proton pump.

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