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The NADP(H)-binding component (dIII) of human heart transhydrogenase: crystallization and preliminary crystallographic analysis

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Transhydrogenase is a membrane protein which uses the energy of the proton motive force to drive the reduction of $NADP^+$ by NADH. The enzyme has three domains: dII spans the membrane, while dI and dIII protrude from the membrane and contain the binding sites for NAD(H) and NADP(H), respectively. DIII from human heart transhydrogenase has been expressed in *Escherichia coli*. The purified protein has been crystallized with bound $NADP^+$ using the hanging-drop vapour-diffusion method with ammonium sulfate as a precipitant. The crystals belong to the tetragonal space group $P4_122$ or $P4_322$, with unit-cell parameters $a = b = 58.1$, $c = 251.0$ Å. A 2.1 Å resolution native data set has been collected with an $R_{\rm merge}$ of 6.8%.

1. Introduction

Nicotinamide nucleotide transhydrogenase is found in the inner membranes of mitochondria and in the cytoplasmic membranes of many bacteria. It catalyses the transfer of a hydride ion equivalent from the 4A position of NADH to the 4B position of $NADP^+$. Hydride transfer is coupled to the transfer of a proton across the membrane (1),

$$\mathrm{NADH} + \mathrm{NADH}^+ + n\mathrm{H}^+_{\rm out} \longleftrightarrow \mathrm{NAD}^+ + \mathrm{NADPH} + n\mathrm{H}^+_{\rm in}, \quad (1)$$

where n is probably equal to 1 (Bizouarn *et al.*, 1996). The physiological role of transhydrogenase is not clearly understood, but it is thought to function in the production of NADPH for biosynthesis and the maintenance of reduced glutathione levels (Hoek & Rydström, 1988). A further role in the fine control of flux through the TCA cycle is suggested in animal cells (Sazanov & Jackson, 1994).

Transhydrogenase sequences have been identified in over 20 species and although some shuffling has occurred at the genetic level, all the enzymes appear to have the same basic tridomain structure. The membrane-associated dII component consists of ~400 residues, which are predicted to form between 10 and 14 transmembrane helices (Meuller, 1999). The dI and dIII components are both predominantly hydrophilic and protrude from the membrane into the bacterial cytoplasm or the mitochondrial matrix (Yamaguchi *et al.*, 1988). NAD^+ and NADH bind to dI (~400 residues), while $NADP^+$ and NADPH bind to dIII (~200 residues).

In recent years, dI and dIII proteins from a number of species have been cloned and expressed in *E. coli* (Diggle, Cotton *et al.*, 1995; Diggle, Hutton *et al.*, 1995; Yamaguchi & Hatefi, 1995, 1997; Diggle *et al.*, 1996; Fjellström *et al.*, 1997; Peake, Venning & Jackson, 1999). The dI proteins are isolated as dimers which bind NADH with high affinity and NAD^+ with lower affinity. The balance of available evidence suggests that the dIII proteins are isolated as monomers. Recombinant dIII proteins are associated with tightly bound NADP(H). The human dIII protein comprises residues 837–1086 of the complete transhydrogenase protein (M_r = 22 260 Da).

The amino-acid sequence of dI is similar to that of alanine dehydrogenase, the crystal structure of which has recently been solved (Baker *et al.*, 1998). Sequence comparison with the alanine dehydrogenase structure suggests that dI might form two subdomains (dI.1 and dI.2), each having the characteristic Rossmann fold of nucleotide-binding proteins (Quirk, Smith *et al.*, 1999). NAD^+ and NADH are thought to bind to dI.2; the function of dI.1 is unknown. An NMR structure of the dIII protein from the photosynthetic bacterium *Rhodospirillum rubrum* suggests that this also forms a Rossmann fold (Jeeves *et al.*, 1999; Quirk, Jeeves *et al.*, 1999).

The mechanism by which transhydrogenase couples hydride transfer to the translocation of protons across the membrane is thought to involve Δp-driven changes in the binding energy of NADP(H) to dIII. Conformational changes resulting from protonation/deprotonation lead to the stabilization of NADPH relative to $NADP^+$ (Venning & Jackson, 1999;

Table 1
Data-collection statistics.

Numbers in parentheses are for data in the outer shell only (2.21–2.10 Å).

No. of measured reflections	178595 (12091)
No. of independent reflections	25584 (3236)
Resolution (Å)	2.1
$R_{\rm merge}$†	6.8 (25.5)
Completeness (%)	97.5 (88.1)
Completeness [$>3I/\sigma(I)$] (%)	82 (44.5)
$I/\sigma(I)$	6.1 (2.9)

† $R_{\rm merge} = \sum_j |\langle I\rangle - I_j| / \sum \langle I\rangle$, where I_j is the intensity of the jth reflection and $\langle I\rangle$ is the average intensity.

Peake, Venning, Cotton *et al.*, 1999). This causes a shift in the on-enzyme equilibrium at the hydride-transfer step favouring the physiological 'forward' reaction.

We have crystallized the dIII component of transhydrogenase from human heart complexed with $NADP^+$. An understanding of the high-resolution structure of this protein is crucial to a proper understanding of the mechanism of action of the intact enzyme.

2. Materials and methods

2.1. Expression and purification

As described previously (Peake, Venning & Jackson, 1999), the DNA coding for *Homo sapiens* dIII was amplified from human heart cDNA (Clontech) by PCR and cloned into the vector pET11c (Novagen). The construct (pSJP2) was transformed into the expression host *E. coli* BL21(DE3) and protein expression was induced by the addition of isopropyl β-D-thiogalactoside (IPTG). The bacteria were harvested by centrifugation and stored at 253 K. The dIII protein was purified from the soluble fraction of the disrupted cells by column chromatography on QA-Trisacryl (IBF Biotechnics), Q-Sepharose High Performance (Pharmacia Biotech) and Butyl Toyopearl 650S (Tosch Corporation), using the buffer conditions employed with *R. rubrum* dIII (Diggle *et al.*, 1996; Bizouarn *et al.*, 1997).

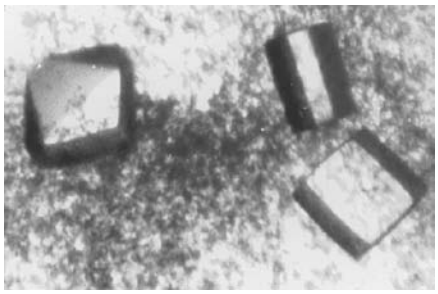

Figure 1
Crystals of *H. sapiens* transhydrogenase dIII. Their approximate dimensions are 0.1 × 0.1 × 0.04 mm.

2.2. Crystallization

All of the recombinant dIII proteins isolated to date have been found to be associated with tightly bound NADP(H). Since homogeneity is always desirable in crystallization experiments, we ensured that the nucleotide bound to the dIII protein was fully oxidized (since this is the most stable form). This was achieved as described for *R. rubrum* dIII (Jeeves *et al.*, 1999).

For crystallization trials, the purified protein was concentrated using Vivaspin 15 concentrators (molecular weight cutoff = 5000 Da; Vivascience) and the buffer exchanged for 10 m*M* Tris–HCl pH 8.0, 5 µ*M* $NADP^+$, 1 m*M* dithiothreitol (DTT). The protein concentration was estimated using the microtannin assay (Mejbaum-Katzenellenbogen & Drobryszycka, 1959) with bovine serum albumin as a standard. Crystallization was performed using the hanging-drop vapour-diffusion method. The first crystals were obtained using Crystal Screen I and Crystal Screen II from Hampton Research. A number of wells yielded crystals. However, the most promising were formed with 1.6 *M* $(NH_4)_2SO_4$, 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.5, 10% dioxane. Following optimization, diffraction-quality crystals grew under conditions where the reservoir contained 500 µl 1.6 *M* $(NH_4)_2SO_4$, 0.1 *M* 3-(*N*-morpholino)propanesulfonic acid (MOPS) pH 7.0, 8% dioxane and the drop was a mixture of 2 µl protein solution (30 mg ml^{-1}) and 6 µl reservoir solution at 291 K. The crystals were square plates with sharp edges (Fig. 1) and appeared after about 24 h. They grew to maximum dimensions of 0.1 × 0.1 × 0.04 mm within a few days.z

3. X-ray data collection and preliminary analysis

Diffraction data were collected using synchrotron radiation (λ = 0.93 Å) at station ID14-4 at ESRF, Grenoble, France at 100 K using a ADSC Quantum 4 CCD detector. The crystals were frozen directly in the Cryostream after soaking in 1.8 *M* $(NH_4)_2SO_4$, 0.1 *M* MOPS pH 7.0, 8% dioxane and 20% glycerol. Under cryo-conditions, the crystals had low mosaicity (<0.20°) and were stable under normal exposure. Crystals diffracted to at least 1.6 Å resolution, but this required exposures of more than 5 s per 0.5° oscillation and resulted in rapid crystal decay. A native data set was collected to 2.1 Å resolution (98% completeness) using 1 s exposures and 0.5° oscillations. Full data-reduction statistics are presented in Table 1.[1] The program *MOSFLM*6.0 (Leslie, 1992) was used for indexing and integration. Diffraction data were scaled and manipulated using the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994).

The crystals are tetragonal, with space group $P4_122$ or $P4_322$ as indicated by systematic absences. The unit-cell parameters of the crystals are $a = b = 58.1$, $c = 251.0$ Å. Assuming there to be two molecules of dIII in the asymmetric unit, the Matthews coefficient (V_m) is calculated to be 2.3 Å^3 Da^{-1}.

We are currently in the process of determining the dIII structure by the MAD technique using a selenomethionyl derivative of the human dIII protein.

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[1] Supplementary data, in the form of a more complete table of statistics, are available from the IUCr electronic archive (Reference: ad0101). Services for accessing these data are described at the back of the journal.

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