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Crystallization and preliminary crystallographic analysis of an NADH oxidase that functions in peroxide reduction in *Thermus aquaticus* YT-1

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NADH oxidase from *Thermus aquaticus* is a thermostable flavoenzyme that is similar in amino-acid sequence and other properties to the flavoenzyme component of the NADH peroxidase systems from *Salmonella typhimurium* and *Amphibacillus xylanus*. The enzyme has been isolated from *T. aquaticus* and crystallized using the hanging-drop method of vapour diffusion with sodium citrate as a precipitant at pH 8.5. The crystals belong to the hexagonal space group *P*622 with unit-cell dimensions $a = b = 89.9$, $c = 491.6$ Å, and diffract to 2.5 Å resolution.

1. Introduction

NADH oxidase from *Thermus aquaticus* is a dimeric flavoprotein with subunits of molecular mass 54 kDa and one FAD per subunit, and it couples the oxidation of NADH to the reduction of electron acceptors including oxygen, dyes and 5,5′-dithio-bis(2,2′-dinitrobenzoate) (Toomey & Mayhew, 1998). First isolated as an NADH oxidase that reduces oxygen to hydrogen peroxide (Cocco *et al.*, 1988), it is thought to function physiologically in a two-protein system that catalyses the reduction of peroxides, similar to that found in *Salmonella typhimurium* (Poole, 1996; Poole & Ellis, 1996) and *Amphibacillus xylanus* (Niimura *et al.*, 1995). The system in *S. typhimurium* consists of a flavoprotein (AhpF) that has two redox-active disulfide groups in addition to FAD, together with a small protein (AhpC) that similarly contains a redox-active disulfide. The two proteins together catalyse the reduction of alkyl hydroperoxides and hydrogen peroxide to water, with reducing equivalents from NADH being passed from reduced FAD to the redox-active disulfides in the flavoprotein and then, *via* thiol–disulfide interchange, to the redox-active disulfide in AhpC. The flavoprotein from *T. aquaticus* has been shown to substitute for *S. typhimurium* AhpF in this system (Toomey *et al.*, 1997), and a protein similar to AhpC has been identified in *T. aquaticus* (Toomey & Mayhew, 1998). There is evidence that similar peroxide-reducing systems occur widely (Chae *et al.*, 1994) and, as summarized by Toomey & Mayhew (1998), flavoenzymes similar to *S. typhimurium* AhpF and NADH oxidase from *T. aquaticus* and *A. xylanus* have been identified in a variety of bacteria. The sequenced fragments of the *T. aquaticus* enzyme show 69% sequence identity with NADH oxidase from *A. xylanus* (Fig. 1). It has been suggested that enzymes with NADH oxidase activity could be used in biosensors and other devices that require the catalytic determination of NADH or the regeneration of NAD^+ (McNeil *et al.*, 1989; Compagnone *et al.*, 1995).

It is clear that some features of the group of flavoenzymes represented by AhpF from *S. typhimurium* and the NADH oxidases from *A. xylanus* and *T. aquaticus* resemble those of other flavoenzymes containing redox-active disulfide groups, and similarities with thioredoxin reductase are particularly striking. Thus, the amino-acid sequence towards the C-terminus of the polypeptide chain of the AhpF group of enzymes is similar to the full sequence of thioredoxin reductase (32% identity with NADH oxidase from *A. xylanus*), and it contains motifs for flavin and NAD(P)(H) binding (Poole, 1997). However, the *S. typhimurium* enzyme has about 200 residues at the N-terminal end that do not occur in thioredoxin reductase. Poole (1997) has modelled the C-terminal half of the *S. typhimurium* enzyme using the three-dimensional structure of thioredoxin reductase as the template.

We have crystallized the *T. aquaticus* enzyme and initiated a three-dimensional structure determination using X-ray crystallography. This knowledge may help to elucidate the relationship between the structures and functions of flavoenzymes of this type, and to unravel the structural relationships between enzymes similar to AhpF on the one hand and thioredoxin reductase on the other. The study of this enzyme, which is stable for several hours at 353 K, may lead to a better understanding of the structural characteristics that distinguish thermally stable flavoenzymes that bind flavin non-covalently from their mesophilic homologues.

The crystal structure of an NADH oxidase from a different thermophilic bacterium,

Thermus thermophilus, has been reported (Hecht *et al.*, 1995). This enzyme also catalyses the reduction of oxygen to hydrogen peroxide, possibly using free flavin as the intermediate electron carrier, but it appears to use FMN as the prosthetic group rather than FAD. Its subunit size is only about half that of the *T. aquaticus* enzyme and the two enzymes differ in amino-acid sequence (Fig. 1). The determination of the three-dimensional structure of the *T. aquaticus* enzyme will allow a more detailed comparison of two different flavoenzymes which have NADH oxidase activity.

2. Methods and results

NADH oxidase was purified from *T. aquaticus* as described elsewhere (Toomey & Mayhew, 1998). Hexagonal shaped crystals were obtained using ammonium sulfate as precipitant at pH 4.5 but these diffracted to less than 10 Å resolution. Further crystallization trials, using a sparse-matrix screen similar to that of Jancarik & Kim (1991), yielded crystals at pH 8.5 with a similar morphology but which showed diffraction to 2.5 Å resolution. Crystals were obtained by the hanging-drop method (McPherson, 1982) at 293 K. The drops were produced by adding 3 ml protein solution (20 mg ml^{-1}, 100 m*M* Tris–HCl, pH 7.5) to 3 ml precipitant solution (1.4 *M* sodium citrate, 70 m*M* Tris–HCl, pH 8.5).

The crystals were initially characterized using data collected on an Enraf–Nonius FR 591 rotating-anode generator using Cu $K\alpha$ radiation with a MAR imaging-plate system. The data were indexed with the program *XDS* (Kabsch, 1988). Subsequent data collection was carried out using synchrotron radiation from the EMBL beamline BW7B at the DORIS storage ring, DESY, Hamburg on a 345 mm MAR Research imaging-plate scanner. Crystals of approximate dimensions 0.25 × 0.25 × 0.3 mm were used for data collection at 298 K. The crystals showed diffraction to 2.5 Å resolution, but they were found to be radiation sensitive.

A crystal-to-detector distance of 550 mm, corresponding to 2.8 Å maximum resolution, was necessary to resolve the reflections on one axis (Fig. 2). A 98% complete data set was collected to 2.8 Å from two crystals which were found to belong to the hexagonal space group *P*622 with unit-cell dimensions $a = b = 89.9$, $c = 491.6$ Å. A total of 139822 observations were merged into 30953 unique reflections using the *HKL* suite of programs (Otwinowski & Minor, 1997), giving an overall $R_{\rm merge}$ of 7.7% [$R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)_i \rangle| / \sum_h \sum_i I(h)_i$] with $I/\sigma(I) > 6$ in the last resolution bin. The completeness of the highest resolution shell was 95%. Higher symmetry than *P*622 was ruled out by the lack of general absences. Attempts to collect data from crystals at liquid-nitrogen temperature have so far failed, owing to increases in crystal mosaicity on freezing which prevented spot resolution on the long axis.

The solvent content is estimated, according to the method of Matthews (1968), at 54% (two molecules per asymmetric unit) or 77% (one molecule per asymmetric unit). No non-crystallographic symmetry was observed in the native Patterson map or using the self-rotation function in *AMoRe* (Collaborative Computational Project, Number 4, 1994). An attempt to solve the structure by molecular replacement using the program *AMoRe* with the structure of thioredoxin reductase (coordinate file 1TDE in the Brookhaven Protein Data Bank; Waksman *et al.*, 1994) as a search model was unsuccessful. A search for suitable heavy-atom derivatives is currently under way.

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T. aquaticus      1 MLLDADIKAQLAQYLQLLENDIVLTVSAGDDNVSRDMLAL 40
A. xylanus          ML-DKDIKQQLEQYLALLENDIVIKVSVGDDKVSKDTLEL
T. thermophilus     MEATLPVLVAKTAALKRRSIRRYRKDPVPEGLLREILEAA
                                  (a)

A. xylanus      185 MTIEDILGHLG 195      275 EYNIDVMKGLR 285
T. aquaticus        MSLEDILAIXK             EYNVDIMNSQR

 320 DIGVPGEKEYK 330      458 IGEIVIDKKGQTSVPGIFAAGDCTDIPYK 486
     XLGVPGEEEFK              FGEIIVDKRGATNIEGVFAAGDCTDSAYK
                                  (b)
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Figure 1
Comparison of partial amino-acid sequences from *T. aquaticus* NADH oxidase with those of the *A. xylanus* and *T. thermophilus* enzymes (Toomey & Mayhew, 1998). Amino acids identical to those of *T. aquaticus* NADH oxidase are shown in bold. (*a*) Comparison of the N-terminal sequences. (*b*) Alignment of four tryptic fragments from the *T. aquaticus* enzyme with *A. xylanus* NADH oxidase.

Figure 2
Diffraction image (2.8 Å resolution at outer edge).

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