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Crystallization and preliminary X-ray analysis of human nicotinamide mononucleotide adenylyltransferase (NMNAT)

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Nicotinamide mononucleotide adenylyltransferase catalyses the final step in the synthesis of nicotinamide-adenine dinucleotide (NAD^+) by transferring the adenylyl moiety of ATP to nicotinamide mononucleotide (NMN) with the release of pyrophosphate. The human enzyme was crystallized in the presence of NAD^+. Crystals grew in the orthorhombic space group $C222_1$, with unit-cell parameters a = 140.3, b = 235.5, c = 89.3 Å, and diffract to a maximum resolution of 3.0 Å. Packing considerations suggest a trimer or higher multimer to be present in the asymmetric unit of the crystal. Two archaeal homologues have been described to form hexamers.

1. Introduction

Human nicotinamide mononucleotide adenylyltransferase (NMNAT; E.C. 2.7.7.1) is an oligomeric enzyme consisting of identical 33 kDa subunits. It catalyses the final step in the biosynthesis of nicotinamide-adenine dinucleotide, the major coenzyme in cellular redox reactions (Dahmen *et al.*, 1967; Magni *et al.*, 1999),

$$\mathrm{ATP} + \mathrm{NMN} \rightarrow \mathrm{NAD}^+ + \mathrm{PP_i}.$$

NAD^+ also appears to be an important constituent of several intracellular signalling pathways (Ziegler, 2000).

NMNAT is located within the nucleus (Balducci *et al.*, 1992; Hogeboom & Schneider, 1952; Ziegler, 2000) and may be associated with poly(ADP-ribosyl) polymerase (PARP1) (Ruggieri *et al.*, 1990; Uhr & Smulson, 1982). PARP1 takes part in DNA base-excision repair (D'Amours *et al.*, 1999; Lindahl & Wood, 1999; Oei *et al.*, 1997) and may consume a considerable amount of cellular NAD^+ under conditions of genotoxic stress (Das & Berger, 1986; Sims *et al.*, 1983). Therefore, NMNAT is expected to play an important role in the restoration of the cellular NAD^+ pool following episodes of increased DNA damage.

NMNAT has been proposed as a chemotherapeutical drug target since its activity is down-regulated in tumour cells (Emanuelli *et al.*, 1995; Hughes *et al.*, 1983; Jayaram *et al.*, 1986, 1999). Human NMNAT has been purified from placenta (Emanuelli *et al.*, 1992) and also as recombinant protein expressed in *Escherichia coli* (Emanuelli *et al.*, 2001; Schweiger *et al.*, 2001). It can be easily assayed by monitoring the reduction of the reaction product, NAD^+, by a dehydrogenase such as alcohol dehydrogenase (Balducci *et al.*, 1995).

Two structures of archaeal NMNATs are known, one from *Methanococcus jannaschii* (D'Angelo *et al.*, 2000; PDB code 1f9a) and one from *Methanobacterium thermoautotrophicum* (Christendat *et al.*, 2000; Saridakis *et al.*, 2001; PDB code 1ej2). Both structures show an $\alpha\beta$ topology, with two trimers forming a hexamer. Whereas the amino-acid sequences of the two bacterial enzymes are very closely related, their difference from the human sequence is much larger, necessitating several insertions, one of them 58 amino-acids long, in a sequence alignment with *ClustalW* (Thompson *et al.*, 1994; Fig. 1). The *ClustalW* scores of pairwise alignments are Mja–Mth, 58; Mja–hum, 11; Mth–hum, 15.

2. Material and methods

2.1. Cloning, expression and purification

The cDNA of NMNAT was cloned and the recombinant protein was purified as described previously (Schweiger *et al.*, 2001). Briefly, the PCR product was cloned into the vector pQE30 (Qiagen) and transfected into *E. coli* (JM109). The cells were grown in TY medium containing ampicillin; the overexpression of the gene was induced by IPTG. Cells were lysed in a French pressure cell and NMNAT, containing a N-terminal His_6 tag, was purified *via* Ni-NTA chromatography according to the manufacturer's protocol (Qiagen).

2.2. Crystallization

NMNAT was crystallized by the hanging-drop vapour-diffusion method, mixing 1 µl of

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Mja  -------MRGFIIGRFQPFHKGHLEVIKKIAEEVD---------EIIIGIGSAQKSHTLE 44
Mth  -------MRGLLVGRMQPFHRGHLQVIKSILEEVD---------ELIICIGSAQLSHSIR 44
hum  ENSEKTEVVLLACGSFNPITNMHLRLFELAKDYMNGTGRYTVVKGIISPVGDAYKKKGLI 60
            :  :  * ::*: . **.:::   : ::          :*  :*.*  .: :

Mja  NPFT---------------------------------------------------------- 48
Mth  DPFT---------------------------------------------------------- 48
hum  PAYHRVIMAELATKNSKWVEVDTWESLQKEWKETLKVLRHHQEKLEASDCDHQQNSPTLE 120
      .:

Mja  --AGERILMITQSLKDYDL---TYYPIP----------IKDIEFNSIWVS-YVESLTPPF 92
Mth  --AGERVMMLTKALSENGIPASRYYIIP----------VQDIECNALWVG-HIKMLTPPF 95
hum  RPGRKRKWTETQDSSQKKSLEPKTKAVPKVKLLCGADLLESFAVPNLWKSEDITQIVANY 180
       . :*    *:  .:          :*          ::.:    :* .  :  :.. :

Mja  DIVYS---GNPLVRVLFE-----ERGYEVKRPEMFNRKEYSGTEIRRRMLNGEKWEHLVP 144
Mth  DRVYS---GNPLVQRLFS-----EDGYEVTAPPLFYRDRYSGTEVRRRMLDDGDWRSLLP 147
hum  GLICVTRAGNDAQKFIYESDVLWKHRSNIHVVNEWIANDISSTKIRRALRRGQSIRYLVP 240
     . :     **   : ::.     :   ::     :  .  *.*::** :  . . . *:*

Mja  KAVVDVIKEIKGVERLRK-------LAQTDK------- 168
Mth  ESVVEVIDEINGVERIKH-------LAKKEVSELGGIS 178
hum  DLVQEYIEKHNLYSSESEDRNAGVILAPLQRNTAEAKT 278
     . * : *.: :  .   .       **  :
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Figure 1
ClustalW multiple sequence alignment of NMNAT from *Methanococcus jannaschii* (Mja), *Methanobacterium thermoautotrophicum* (Mth) and human (hum).

protein solution (20 mg ml^{-1}) with the same volume of reservoir solution containing 1.9 *M* Na/KH_2PO_4, 0.1 *M* Tris–HCl pH 7.5 and 5% 2-propanol. The protein solution contained 70 m*M* $(NH_4)_2SO_4$, 100 m*M* imidazole, 300 m*M* NaCl, 1 m*M* $MgCl_2$, 2.5 m*M* DTT and 1 m*M* NAD^+. Crystals grew within 4 d to dimensions of 1.0 × 0.7 × 0.2 mm at 293 K.

2.3. Data collection and processing

Prior to diffraction experiments, crystals were soaked in reservoir solution containing an additional 20% ethylene glycol and flash-frozen in liquid nitrogen. A diffraction data set was collected at DESY Hamburg beamline BW7B at 100 K. 110 frames of $\Delta\varphi = 1^\circ$ at a distance of 510 mm were collected to a maximal resolution of 3.0 Å and processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

NMNAT can be crystallized in a variety of high-salt conditions. Starting from the Hampton Research Crystallization Screen (Jancarik & Kim, 1991), crystals could be observed in a number of high-salt conditions, including those with Li_2SO_4, $(NH_4)_2SO_4$, Na/KH_2PO_4 and even NaCl. Refinement of the conditions using different combinations of salt and buffer were judged by the diffraction quality of the respective crystals and resulted in the optimal crystallization condition described above. Crystals often grew into each other which could be overcome by the addition of 3–6%(*v*/*v*) 2-propanol.

Figure 2
Typical crystal of NMNAT (approximate dimensions 600 × 350 × 200 µm).

Although crystals grew to a relatively large size and had a clean morphology (Fig. 2), diffraction quality was often poor. Bragg reflections were smeared out, often only in one region of the diffraction pattern. In addition, diffraction was often anisotropic with respect to resolution and mosaicity. Usually, diffraction quality was better when guiding the beam through the smallest crystal dimension compared to the largest dimension which contained more layers.

A variety of conditions were tested to improve diffraction quality. Equilibration of the crystal over a high or low salt-content reservoir, soaking in different cryoprotectants (ethylene glycol, glycerol, PEG, glucose, saccharides) and/or with solutions of high or low salt content, as well as varying the temperature and the protein concentration did not yield reproducible results. In fact, the type of crystal specimen was more important for diffraction quality. Higher diffraction quality without a loss in resolution was observed with crystals grown evenly in three dimensions, even if they reached only moderate size (up to approximately 0.3 × 0.3 × 0.3 mm).

Table 1
Data-collection statistics.

Values in parentheses refer to the outer resolution shell.

Resolution (Å)	30–3.00 (3.03–3.00)
No. of observations	370484
Unique reflections	28373 (852)
Data completeness (%)	94.8 (85.7)
Average $I/\sigma(I)$	12.9 (1.4)
$R_{\rm merge}$† (%)	9.1 (64.1)
$R_{\rm r.i.m.}$† (%)	10.3 (73.7)
$R_{\rm p.i.m.}$† (%)	4.6 (38.2)
Space group	$C222_1$
Unit-cell parameters (Å)	
a	140.3
b	235.5
c	89.3

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i - \langle I\rangle|/\sum\langle I\rangle$, $R_{\rm r.i.m.} = \sum_{hkl}[N/(N-1)]^{1/2}\sum_i |I_i - \langle I\rangle|/\sum\langle I\rangle$ and $R_{\rm p.i.m.} = \sum_{hkl}[1/(N-1)]^{1/2}\sum_i |I_i - \langle I\rangle|/\sum\langle I\rangle$, where I_i is the intensity of the observation of reflection hkl, $\langle I\rangle$ is the average intensity of a reflection and N is the redundancy.

Data collection and processing (Table 1) revealed that crystals grow in the orthorhombic space group $C222_1$, with unit-cell parameters a = 140.3, b = 235.5, c = 89.3 Å. With a completeness of the data set of 94% the $R_{\rm merge}$ is 9.1%. The redundancy-independent merging R factor ($R_{\rm r.i.m.}$) is 10.3% and the precision-indicating merging R factor ($R_{\rm p.i.m.}$) is 4.6% (Weiss, 2001).

The oligomeric state of human NMNAT remains unclear at present. Crystal-packing considerations (Matthews, 1968) in principle permit three, four, five or six monomers to be present in the asymmetric unit, yielding 66, 55, 44 or 33% solvent content, respectively. By analogy with the crystals of the two archaeal NMNATs (see above) we might expect to find a trimer or hexamer of human NMNAT in the asymmetric unit. The self-rotation function, calculated with the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994), indeed supports the presence of a threefold or sixfold rotational symmetry, as peaks are found at κ = 120 and 60° along the *c* axis. However, owing to the presence of smaller peaks at $\kappa = 90^\circ$ along the *b* axis, a tetrameric arrangement cannot be ruled out completely (Fig. 3). Peaks at $\kappa = 72^\circ$ (indicating fivefold symmetry) were not found.

The human enzyme as well as the NMNAT from *Methanobacterium thermoautotrophicum* crystallizes with NAD^+ as substrate. The structure of *Methanococcus*

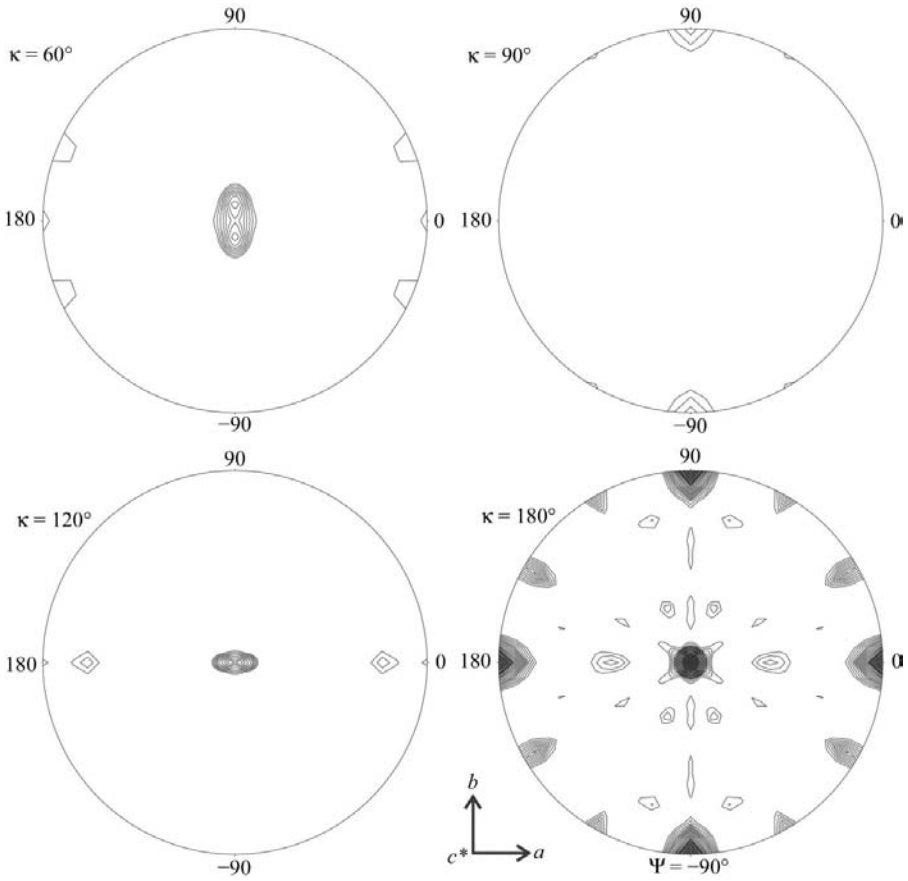

Figure 3
Self-rotation function. View along the *c* axis, *a*, *b* and Ψ as indicated, generated with the program *POLARRFN* in the resolution range 20–3.5 Å; integration radius 25 Å. Strong peaks at $\kappa = 180^{\circ}$ are consistent with the orthorhombic crystal symmetries.

jannaschii NMNAT was solved in complex with ATP and is very similar to the other archaeal structure. It thus appears unlikely, but cannot be ruled out, that a bound ligand influences the state of oligomerization of human NMNAT.

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