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Crystallization and preliminary analysis of a water-forming NADH oxidase from *Lactobacillus sanfranciscensis*

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Single crystals have been obtained of NADH oxidase (Nox), a flavoenzyme cloned from *Lactobacillus sanfranciscensis*. The enzyme catalyzes the oxidation of two equivalents of NAD(P)H and reduces one equivalent of oxygen to yield two equivalents of water, without releasing hydrogen peroxide after the reduction of the first equivalent of NAD(P)H. The enzyme crystallizes in space group $P2_12_12_1$, with unit-cell parameters a = 59.6, b = 92.6, c = 163.5 Å. The crystals diffract to 1.85 Å resolution using synchrotron radiation. Matthews coefficient calculations suggest the presence of two molecules per asymmetric unit (V_M = 2.3 Å^3 Da^{-1}, 45.5% solvent content), which has been confirmed by the molecular-replacement solution using a search molecule derived from NADH peroxidase (PDB code 1f8w).

1. Introduction

Lactobacillus sanfranciscensis is used in the production of sourdough bread and is thus an important member of the family of lactic acid bacteria. It is an aerotolerant anaerobe and an obligatory heterofermentative microorganism that obtains most of its energy from the fermentation of maltose (Gobbetti & Corsetti, 1997). Genomic sequencing and analysis of several lactic acid bacteria suggests that most do not synthesize hemes or cytochromes (Bolotin *et al.*, 2001; Klaenhammer *et al.*, 2002; Kleerebezem *et al.*, 2003; de Vos *et al.*, 2004; Siezen *et al.*, 2004). Consequently, they do not use an electron-transport chain, O_2 and oxidative phosphorylation for energy metabolism, but rather satisfy all their energy requirements with glycolysis. Fermentation helps to maintain an optimized intracellular NAD^+/NADH ratio that is essential for efficient glycolysis. It also yields lactic acid, which acidifies the media and reduces competition from other microorganisms (De Angelis *et al.*, 2001; De Angelis & Gobbetti, 2004). However, *L. sanfranciscensis* tolerates O_2 in order to maintain a symbiotic relationship with other aerobic microbes, most notably the yeast found in sourdough breads, and for the fermentation of other cereals.

Oxidative stress in many lactic acid bacteria and other facultative and strict anaerobic bacteria is managed in part by the expression of one or more flavin-dependent NAD(P)H oxidase(s) (Klenk *et al.*, 1997; Baker *et al.*, 2001; Bolotin *et al.*, 2001; De Angelis *et al.*, 2001; Kleerebezem *et al.*, 2003; De Angelis & Gobbetti, 2004; Rabus *et al.*, 2004). The enzymes catalyze reactions of the type illustrated in Fig. 1 and thus can be further classified as (i) H_2O_2-producing NADH oxidases (PrxR and some Nox), (ii) peroxidases that reduce H_2O_2 to H_2O (Npx, Prx and other peroxiredoxins) and (iii) water-producing NAD(P)H oxidases (Nox). In addition to the FAD cofactor (except for Prx and other peroxiredoxins, which do not contain flavins), each enzyme includes one or more redox-active cysteine residue(s). This catalytically essential residue alternates between the thiol/thiolate and the sulfenic acid states (Nox and Npx), the thiol/thiolate and the disulfide state (PrxR and Prx) or the disulfide and sulfenic acid state (Prx) (for recent reviews, see Claiborne *et al.*, 1999, 2001; Poole *et al.*, 2000; Hofmann *et al.*, 2002; Wood *et al.*, 2003; Argyrou & Blanchard, 2004; Takeda *et al.*, 2004). The NAD(P)H oxidases exhibit a strong preference for O_2 as the electron acceptor (Ahmed & Claiborne, 1989*a*,*b*, 1992*a*,*b*;

$$\begin{aligned}
&NADH + O_2 + H^+ \rightarrow NAD^+ + H_2O_2 && \text{(PrxR, some Nox)}\\
&NADH + H_2O_2 + H^+ \rightarrow NAD^+ + 2H_2O && \text{(Npx and Prx)}\\
&2NAD(P)H + O_2 + 2H^+ \rightarrow 2NAD(P)^+ + 2H_2O && \text{(Nox)}
\end{aligned}$$

Figure 1
Reactions catalyzed by lactic acid bacteria and other facultative or strict anarobic bacteria.

Mallett & Claiborne, 1998; Mallett *et al.*, 1999). Moreover, they only rarely release H_2O_2 as the product, in contrast to most flavin-dependent oxidases (Mallett & Claiborne, 1998; Fraaije & Mattevi, 2000; Massey, 2000; Ward *et al.*, 2001; Kengen *et al.*, 2003). For example, during aerobic NADH turnover by *L. sanfranciscensis* Nox, less than 0.5% of the reducing equivalents can be detected as H_2O_2, suggesting that hydrogen peroxide is not released from the active site (Riebel *et al.*, 2002, 2003). In contrast, the Npx and Prx enzymes do not react with O_2, but rather use H_2O_2 or alkylperoxides as the electron acceptor (Poole & Claiborne, 1986, 1988; Ahmed & Claiborne, 1992*a*; Stehle *et al.*, 1993; Parsonage & Claiborne, 1995; Crane *et al.*, 2000; Poole *et al.*, 2000). Most Nox and Npx enzymes exhibit a strong preference for NADH, whereas Nox from *L. sanfranciscensis* will use either reduced nicotinamide adenine dinucleotide.

All the enzymes may facilitate regeneration of oxidized pyridine nucleotides for glycolysis and help protect the organism against oxidative stress. For example, the NADH oxidases from *Streptococcus pyogenes* and *L. delbrueckii* have been shown to contribute significantly to aerobic metabolism under conditions of high O_2 stress (Gibson *et al.*, 2000; Marty-Teysset *et al.*, 2000). Although no crystal structures have been reported of an H_2O-producing NADH oxidase, several have been characterized and described in the literature (Koike *et al.*, 1985; Ross & Claiborne, 1991, 1992; Peterson *et al.*, 1993; Bult *et al.*, 1996; Matsumoto *et al.*, 1996). *BLASTP* (v.2.2.8) searches with the protein sequence of *L. sanfranciscensis* Nox return other water-producing enzymes with sequence identities ranging from 59% for the enzyme from *L. plantarum* WCFS1 (NP_786664.1) to 39% for the crystallographically defined Npx from *Enterococcus faecalis* (Stehle *et al.*, 1991; Crane *et al.*, 2000). In each case, the most highly conserved portions from the sequence comparisons are associated with either the redox-active cysteine (Cys42 in Npx), the FAD-binding or the NAD(P)H-binding regions. The Nox from *L. sanfranciscensis* (ATCC 27651) is a homodimer comprised of approximately 50 kDa subunits, similar to other NADH oxidases. In contrast, Npx is a homotetramer with *D*2 symmetry relating the four subunits. In order to describe the structural basis of the substrate preference and lack of H_2O_2 generation by *L. sanfranciscensis* Nox, we have initiated a crystallographic analysis of the enzyme. Here, we report the crystallization conditions and initial X-ray diffraction analysis of the H_2O-forming NADH oxidase from *L. sanfranciscensis*.

2. Materials and methods

2.1. Protein expression and purification

The cloning, expression and preparation of *L. sanfranciscensis* Nox was essentially as previously reported, with slight modifications (Riebel *et al.*, 2002, 2003). Briefly, starter cultures of *Escherichia coli* strain JM101 containing the *nox2* gene from *L. sanfranciscensis* in the pKK223-3 vector were grown in 5 ml LB media augmented with 100 µg ml^{-1} ampicillin (LB/amp) at 310 K to an OD_{600} of 1.0. 1 l cultures of LB/amp medium were inoculated with the starter cultures and grown at 303 K in baffled 2.8 l Fernbach shake flasks with 200 rev min^{-1} shaking. When the culture reached an OD_{600} of 0.7, protein expression was induced by the addition of 1.0 m*M* IPTG for 3 h. Additional ampicillin was added to 200 µg ml^{-1} at the induction time and 1.5 h later. Cultures were harvested by centrifugation at 277 K and the resulting cell pellet was frozen at 193 K.

The protein was purified by a two-step procedure at 277 K unless otherwise noted. Approximately 31 g of frozen cells were thawed and suspended in 30 ml 100 m*M* 1-methylpiperazine buffer pH 5.0 plus 1 m*M* EDTA, 5 m*M* DTT and 20 m*M* spermine. The cell slurry was sonicated at approximately 277–283 K for six 2 min pulses and centrifuged at 16 000 rev min^{-1}. Acid precipitation of a range of impurities was accomplished by dialyzing the cell-free extract overnight at 303 K with three changes of 1.5 l 20 m*M* 1-methylpiperazine pH 5.0 plus 1 m*M* EDTA and 5 m*M* DTT. After centrifugation, the enzyme was then purified by anion-exchange chromatography. The supernatant was loaded onto a Hiprep 16/10 Source 30Q column (Amersham Pharmacia, Piscataway, NJ, USA) and washed with ten column volumes of 20 m*M* 1-methylpiperazine pH 5.0 plus 5 m*M* DTT. The enzyme was then eluted with a linear gradient from 0 to 1 *M* NaCl in the same buffer at a flow rate of 5 ml min^{-1}. SDS–PAGE analysis suggested that the protein was >95% pure.

L. sanfranciscensis Nox activity was typically assayed with 0.2 m*M* NADH at 303 K in 0.1 *M* triethanolamine (TEA) pH 7.5. The reaction was followed at 340 nm and the activity was calculated using a 6220 M^{-1} cm^{-1} extinction coefficient for NADH. Protein concentration was determined by the Bradford method (Pierce Chemical, Rockford, IL, USA). The specific activity of *L. sanfranciscensis* Nox was typically 221 U mg^{-1}.

2.2. Crystallization and X-ray data collection

Initial crystallization conditions for NADH oxidase were determined using sparse-matrix screens from Hampton Research (Laguna Nigel, CA, USA) and Nextal Biotechnologies (Montreal, Quebec, Canada). Typically, 2 µl protein (10 mg ml^{-1} in 20 m*M* 1-methylpiperazine pH 5.0, 0.15 *M* sodium chloride and 5 m*M* dithiothreitol) was mixed with an equal volume of reservoir solution on a silanized cover slip. Equilibration took place at room temperature by vapor diffusion in 24-well VDX plates (Hampton Research). Crystals appeared after approximately 5 d with reservoir solution containing 100 m*M* HEPES buffer pH 7.0–7.7, 4–10%(*v*/*v*) 2-propanol and 18–24%(*w*/*v*) polyethylene glycol 4000. Optimization of the initial conditions involved mixing 2 µl protein solution with 2 µl reservoir solution and 1 µl freshly prepared 100 m*M* dithiothreitol and screening pH and precipitant concentration. Crystals used for data collection were obtained by transferring seed crystals appearing in drops equilibrated over 100 m*M* HEPES pH 7.5, 24%(*w*/*v*) polyethylene glycol 4000 and 6%(*v*/*v*) 2-propanol by streak-seeding with a cat whisker into a pre-equilibrated drop containing 2 µl 10 mg ml^{-1} protein solution, 2 µl reservoir solution [100 m*M* HEPES pH 7.0, 18%(*w*/*v*) polyethylene glycol 4000 and 8%(*v*/*v*) 2-propanol] and 1 µl 100 m*M* dithiothreitol. Crystals from the streak-seeding experiment appeared after approximately 4 d and reached maximum dimensions of 0.2 × 0.1 × 0.1 mm within two weeks.

Crystals for X-ray diffraction data collection were harvested with a nylon loop and transferred to mother liquor supplemented with 15%(*w*/*v*) polyethylene glycol 400 and allowed to soak for approximately 30 s. The crystals were flash-frozen by rapid submersion into liquid N_2. All diffraction data were collected from crystals held at approximately 100 K on beamline 22-ID operated by the South East Regional Collaborative Access Team (SER-CAT) at the Advanced Photon Source (APS), Argonne National Laboratory using a MAR CCD 225 detector. Each image was collected with a 1 s exposure time, a 0.5° oscillation range and a 180 mm crystal-to-detector distance. The data were integrated with

Table 1
Data-collection statistics for *L. sanfranciscensis* NADH oxidase.

Data were collected with a MAR CCD 225 detector. Values for the highest resolution shell of data are given in parentheses.

X-ray source	SER-CAT beamline 22-ID, APS
Beamline	22-ID
Wavelength (Å)	0.9997
Detector	MAR CCD 225
Resolution range (Å)	46.4–1.85 (1.92–1.85)
Mosaic spread (°)	∼0.7
Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	59.6
b (Å)	92.6
c (Å)	163.5
Total reflections	675265
Unique reflections	76768
Multiplicity	8.8 (5.4)
Completeness (%)	98.1(86.3)
$I/\sigma(I)$†	30.7 (2.6)
R_{sym}‡ (%)	9.9 (42.1)

† $I/\sigma(I)$ is the root-mean-square value of the intensity measurements divided by their estimated standard deviation. ‡ $R_{sym}(I)$ gives the average agreement between the independently measured intensities such as $\sum_h \sum_i |I_i - I|/\sum_h \sum_i$, where I is the mean intensity of the i observations of reflection h.

*HKL*2000 and merged with *SCALEPACK* (Otwinowski & Minor, 1997). Molecular replacement was carried out with the *MOLREP* program (Vagin & Teplyakov, 1997) from the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). A search model was prepared using a monomer of NADH peroxidase (PDB code 1f8w; Crane *et al.*, 2000) with all non-identical residues to *L. sanfranciscensis* Nox mutated to alanine and searching for two molecules in the asymmetric unit with data between 46 and 3 Å resolution. Rigid-body refinement and simulated annealing of the *MOLREP* solution was performed with *CNS* (Brünger *et al.*, 1998).

3. Results and discussion

The first crystals of *L. sanfranciscensis* Nox appeared as a cluster of small yellow rods and plates that were birefringent under polarized light. Refinement of pH and precipitant concentration yielded larger reproducible crystals, but frequently failed to yield single crystals. Moreover, the single crystals obtained from these conditions typically only diffracted to approximately 3.5 Å resolution. The addition of dithiothreitol and streak-seeding yielded single crystals that were suitable for data collection at high resolution (Fig. 2). The statistics for the X-ray diffraction data collection are reported in Table 1. The data sets were obtained from 180° of φ rotation and are strong [overall $I/\sigma(I) = 30.7$], of good quality (R_{sym} = 9.9%) and redundant (overall 8.8-fold multiplicity). The space group is $P2_12_12_1$ and Matthews coefficient calculations suggest the presence of two molecules in the asymmetric unit (V_M = 2.3 Å^3 Da^{-1}, 45.5% solvent content; Matthews, 1968; Kantardjieff & Rupp, 2003).

The R303M mutant isoform of NADH peroxidase (Npx) from *E. faecalis* (PDB code 1f8w; Crane *et al.*, 2000) has 39% sequence identity to *L. sanfranciscensis* Nox. A monomeric molecular-replacement search model consisted of 447 residues of the 452 residues in a full-length monomer of *L. sanfranciscensis* Nox. All non-identical residues to Nox were truncated to alanine, unless they were glycine residues in Npx. All *B* factors were set to 20.0 Å^2. The top molecular-replacement solution obtained from *MOLREP* yielded a correlation coefficient of 0.4 for two molecules in the asymmetric unit. The top result was approximately two times greater than the next best solution. The solution was subjected to rigid-body refinement and simulated annealing to 3.0 Å resolution, which improved the *R* factors to R_{cryst} = 0.43 and R_{free} = 0.48. The resulting electron-density maps were clearly interpretable. Moreover, greater than 3σ positive difference features were apparent for the active-site FAD and the side chains of the residues missing those atoms in the search model. Refinement of the model to the high-resolution limit is currently in progress.

The structural analysis of *L. sanfranciscensis* Nox will help to establish the structural basis for the nearly stoichiometric production of H_2O, the almost complete lack of H_2O_2 detected after reduction of the first equivalent of NAD(P)H and the apparent promiscuity for reduced nicotinamide adenine dinucleotide substrates. Indeed, *L. sanfranciscensis* Nox exhibits nearly identical K_m values for NADH and NADPH (6.7 and 6.1 μM, respectively), whereas the NADH oxidases from *Borrelia burgdorferi* or *L. brevis* only accept NADH (Riebel *et al.*, 2002; Hummel & Riebel, 2003; Riebel *et al.*, 2003). Moreover, comparisons to the other homologs may also reveal features that differentiate the various family members. For example, the structures of the NADH peroxidase and the biochemical analyses of the NADH oxidase from *E. faecalis* (Ross & Claiborne, 1992; Stehle *et al.*, 1993) reveal that a highly conserved redox-active cysteine residue plays an essential role in the catalytic cycle. *L. sanfranciscensis* Nox contains the analogous Cys42 residue that is proposed to cycle between a thiol/thiolate anion and a sulfenic acid (Cys-SOH). This residue is proposed to be largely responsible for altering the enzyme reaction coordinate to yield H_2O rather than H_2O_2 (Mande *et al.*, 1995; Parsonage & Claiborne, 1995; Yeh *et al.*, 1996; Mallett & Claiborne, 1998; Claiborne *et al.*, 2001). The X-ray diffraction data are of sufficient quality and resolution to support a refined crystal structure of *L. sanfranciscensis* Nox, which is in progress.

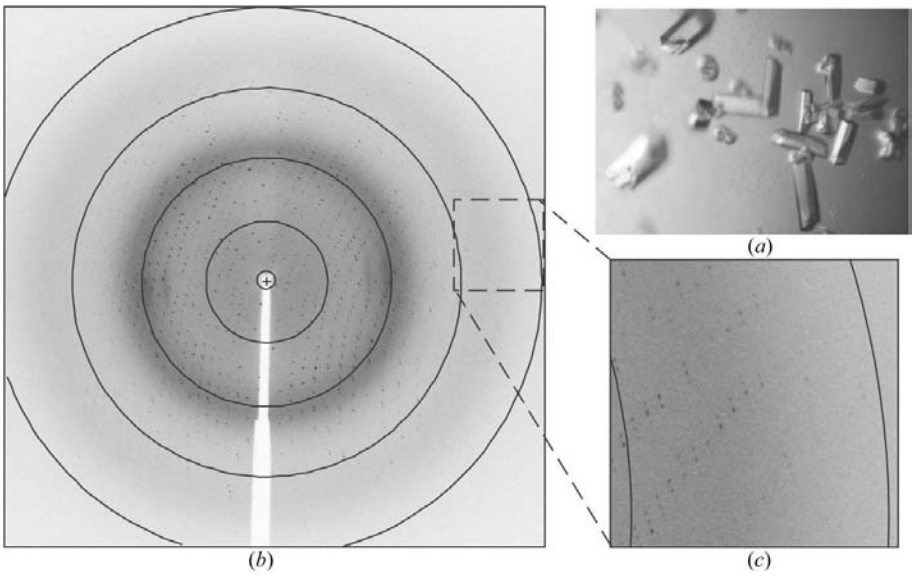

Figure 2
(*a*) Crystals of *L. sanfranciscensis* NADH oxidase with dimensions of approximately 0.2 × 0.1 × 0.1 mm photographed under polarized light. (*b*) The X-ray diffraction pattern obtained with 1 s exposure and 0.5° oscillation range about the vertical axis; arcs indicate 7.1, 3.6, 2.4 and 1.8 Å resolution. (*c*) An expanded and contrast-adjusted view of the diffraction pattern between 2.4 and 1.8 Å perpendicular to the φ rotation axis.

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