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Crystallization of L-aspartate oxidase, the first enzyme in the bacterial *de novo* biosynthesis of NAD

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The flavoenzyme L-aspartate oxidase from *Escherichia coli* was crystallized using the hanging-drop vapour-diffusion technique with PEG 4000 as precipitant. The crystals belong to space group $P3_121$ (or $P3_221$) with unit-cell parameters $a = b = 84.9$, $c = 159.9$ Å. A solvent content of 42% corresponds to a monomer (60 kDa) in the asymmetric unit. A complete 2.8 Å resolution data set was collected using a rotating-anode X-ray generator.

1. Introduction

Several bacteria are able to carry out the *de novo* biosynthesis of NAD starting from L-aspartate and dihydroxyacetone phosphate. These two metabolites are first converted to quinolinic acid by the enzymes L-aspartate oxidase (E.C. 1.4.3.16; LASPO) and quinolinate synthase. Quinolinate is then used as a building block to complete the synthesis of NAD. Unlike prokaryotes, most eukaryotes are unable to employ L-aspartate and dihydroxyacetone phosphate to synthesize quinolinate, which is normally produced *via* the degradation of tryptophan (Foster & Moat, 1980; Tritz, 1987). In recent years, there has been a growing interest in the enzymes involved in NAD biosynthesis, which have been described as a potential target for drugs against pathogenic bacteria such as *Mycobacterium tuberculosis* (Foster & Moat, 1980; Eads *et al.*, 1997) and *Haemophilus influenzae* (Denicola-Seoane & Anderson, 1990). In particular, the crystal structures of quinolinic acid phosphoribosyltransferase (Eads *et al.*, 1997) and NAD synthetase (Rizzi *et al.*, 1996), two enzymes involved in the conversion of quinolinic acid to NAD, have recently been solved.

LASPO catalyses the oxidation of L-aspartate to iminoaspartate (Fig. 1; Nasu *et al.*, 1982). The gene coding for LASPO from *E. coli* has been cloned and the protein overexpressed, allowing the production, purification and biochemical characterization of the enzyme (Seifert *et al.*, 1990; Mortarino *et al.*, 1996; Tedeschi *et al.*, 1996). LASPO is a flavoprotein consisting of 540 amino acids (molecular weight 60354 Da) and contains a non-covalently bound flavin–adenine dinucleotide (FAD) molecule which is essential for catalysis. The protein is in equilibrium between a monomeric and dimeric form, with the monomer being prevalent at pH values below 5 and the dimer being favoured by pH values higher than 7. The two enzyme forms are fully active at pH 8.0 (Tedeschi *et al.*, 1999).

Analysis of the primary sequence (Seifert *et al.*, 1990) has revealed a clear similarity (30% sequence identity) between LASPO and the flavin-containing subunits of succinate dehydrogenase and fumarate reductase, two membrane-bound multi-subunit enzymes which are part of the respiratory chain (see Ackrell *et al.*, 1991 and references therein). The structural similarity finds a counterpart in several functional properties which LASPO shares with the members of the fumarate reductase/succinate dehydrogenase class of oxidoreductases (Tedeschi *et al.*, 1996, 1997). Particularly, in addition to oxidase activity, LASPO displays the ability to use fumarate as electron acceptor, so that the enzyme can function as a L-aspartate/fumarate oxidoreductase (Fig. 1). This feature may be of physiological significance, since it provides facultative aerobic bacteria such as *E. coli* with the ability to carry out NAD biosynthesis in both aerobic and anaerobic conditions (Tedeschi *et al.*, 1996). In line with this characteristic, and despite its name, LASPO displays only part of the properties which typically characterize the oxidase class of flavin-dependent enzymes (Massey, 1995). For instance, the turnover number with oxygen (156 min^{-1}) is comparable with that with other electron acceptors such as fumarate (turnover number 333 min^{-1}) or menadione (67 min^{-1}). Moreover, the enzyme can stabilize the blue semiquinone form of FAD, a feature which is unusual for an oxidase (Ghisla & Massey, 1989). These observations have led to the suggestion that LASPO can be viewed as a particularly soluble fumarate reductase, capable of reducing fumarate but unable to oxidize succinate (Tedeschi *et al.*, 1997).

The availability of large amounts of homogeneous recombinant enzyme has allowed us to undertake the crystallization of *E. coli*

Table 1
LASPO X-ray diffraction data-collection statistics.

Resolution (Å)	Completeness (%)	Independent reflections	I/σ	R_{merge}† (%)
99.0–8.85	91.7	533	17.3	2.4
8.85–6.26	96.1	972	12.5	5.1
6.26–5.11	98.4	1275	8.3	7.6
5.11–4.43	98.6	1496	10.4	6.5
4.43–3.96	99.2	1694	8.8	7.8
3.96–3.62	98.4	1849	7.3	9.9
3.62–3.35	97.2	1974	5.5	13.3
3.35–3.13	97.3	2117	3.9	19.4
3.13–2.95	96.3	2221	3.1	21.7
2.95–2.80	97.3	2372	2.0	29.2
Overall	97.5	16503	6.7	9.8

† $R_{merge} = \sum_h \sum_i |I_{ih} - \langle I_h \rangle| / \sum_h \sum_i I_{ih}$ where $\langle I_h \rangle$ is the mean intensity of the i observations of reflection h.

LASPO as a first step towards the structure determination. This study is carried out in the framework of two projects presently being developed in our laboratory, focused on the catalytic properties of flavin-dependent oxidases (Mattevi *et al.*, 1996, 1997) and on the structural biology of enzymes involved in the NAD biosynthesis (Rizzi *et al.*, 1996). Knowledge of the LASPO three-dimensional structure will shed light on the dual nature of the enzyme, which can function either as an oxidase or an oxidoreductase. Moreover, it is expected that the X-ray analysis of LASPO will be of interest for gaining insight into the properties of the fumarate reductase/succinate dehydrogenase class of proteins.

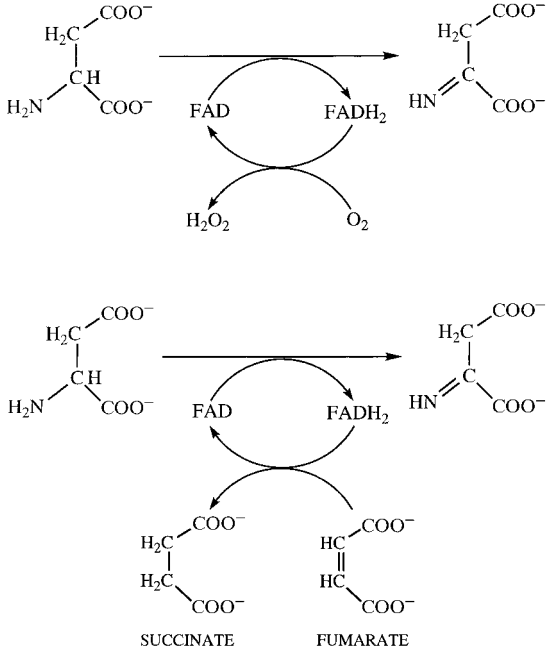

Figure 1
Scheme of the reactions catalysed by LASPO: oxidation of aspartate and reduction of oxygen to hydrogen peroxide (top) and oxidation of aspartate coupled to the reduction of fumarate to succinate (bottom).

2. Materials and methods

2.1. Purification

The enzyme was purified following the protocol developed by Mortarino *et al.* (1996). All purification steps were carried out at 278 K. 35 g cell paste was suspended in 200 ml 50 m*M* phosphate buffer pH 8.0, 1 m*M* PMSF, 1 m*M* EDTA, 10 m*M* succinate, 20 µ*M* FAD and sonicated for 5 min on ice. After centrifugation for 35 min at 10000*g*, 1 ml of protamine sulfate was added per milligram of protein, and the suspension was centrifuged for 30 min at 28000*g*. The supernatant was collected and ammonium sulfate was added to 50% final concentration, followed by a centrifugation step at 16000*g*. The precipitate was dialysed overnight in 50 m*M* phosphate buffer pH 8.0, 10 m*M* succinate, 1 m*M* EDTA, 1 m*M* PMSF, 20% glycerol, 20 µ*M* FAD and loaded on a DEAE–Sepharose CL-6B column equilibrated in the same buffer. The enzyme was eluted with a gradient of 50–300 m*M* phosphate buffer. The active fractions were concentrated by precipitation with 60% ammonium sulfate, dialysed against 50 m*M* HEPES pH 8.0, 20% glycerol, 1 m*M* EDTA, 10 m*M* succinate and applied to a Sephacryl S-200 HR equilibrated with the same solution. The active fractions were collected, concentrated by ammonium sulfate and dialysed in 20 m*M* HEPES pH 8.0, 0.02% NaN_3. Finally, the protein was then concentrated to 100–130 mg ml^{-1} using a Centricon 30 (Amicon). For storage, a cocktail of protease inhibitors (Complete from Boehringer Mannheim GmbH, Mannheim, Germany) was added to the protein solution at the concentration suggested by the producer.

2.2. Crystallization

Crystallization conditions were screened using the hanging-drop vapour-diffusion technique. The hanging drops were formed by mixing equal volumes of the protein solution, which consisted of 20 mg ml^{-1} enzyme in 20 m*M* HEPES (pH 8.0), and the reservoir. Various precipitants such as low and high molecular weight polyethylene glycols, 3-methyl 2,4-pentanediol, ammonium sulfate and sodium chloride were tested within a pH range of 5.0–9.0. These initial screening experiments revealed that crystals of the enzyme could be obtained in about two weeks using as precipitant 20%(*w*/*v*) PEG 4000 in 100 m*M* Tris–HCl (pH 8.3) and 0.02% NaN_3 at 293 K. Further experiments indicated that the size and reproducibility of the crystals could be improved by the addition of 100 m*M* sodium acetate and the use of high protein concentrations, coupled with a decrease in the PEG 4000 concentration in the reservoir solution. Thus, in the optimal crystallization conditions, a protein solution containing 100–130 mg ml^{-1} protein in 20 m*M* HEPES buffer (pH 8.0) was equilibrated at 293 K against a reservoir containing 100 m*M* Tris–HCl (pH 8.3), 100 m*M* sodium acetate, 0.02% NaN_3 and 12–15%(*w*/*v*) PEG 4000. The crystals had a tetrahedral shape and reached a maximum size of 0.3 × 0.3 × 0.6 mm.

2.3. Data collection

For data collection, crystals were soaked for a few seconds in a solution containing 25% PEG 4000, 30%(*v*/*v*) glycerol, 100 m*M* sodium acetate and 100 m*M* Tris–HCl buffer (pH 8.3). The crystals were then mounted in a rayon loop (Hampton Research) supported by a brass mounting pin and quickly transferred to a liquid N_2 stream maintained at 100 K. A complete 2.8 Å data set was collected using a rotating-anode X-ray generator operated at 160 mA and 60 kV and equipped with a graphite monochromator. An R-AXIS II imaging-plate system was used as detector. The data were integrated with *MOSFLM* (A. G. W. Leslie) and scaled using the program *SCALA* from the *CCP4* suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Crystals of recombinant *E. coli* LASPO reproducibly grow at pH 8.3 using PEG 4000

as precipitant. The crystals are amenable to the employment of cryocrystallographic techniques, so that diffraction data can be collected at 100 K. A data set was measured using a rotating-anode X-ray generator. A total of 62360 measured intensities were merged into a set of 16503 independent reflections with an $R_{\rm merge}$ of 9.8% and a completeness of 97.5% to 2.8 Å resolution. In the highest resolution shell (2.95–2.80 Å), the $R_{\rm merge}$ and the completeness are 29.2 and 97.3%, respectively (Table 1). The space group was determined according to the $R_{\rm merge}$ value and systematic extinctions. Crystals belong to the $P3_121$ (or $P3_221$) space group with unit-cell dimensions $a = b = 84.9$, $c = 159.9$ Å. Assuming one protein molecule per asymmetric unit results in a V_m parameter (Matthews, 1968) of 1.8 Å^3 Da^{-1} and a solvent content of 42%. At the pH of crystallization (8.3), the enzyme should predominantly exist as an active dimer. This feature is fully compatible with the crystal symmetry, since the two subunits of the oligomer could be related by a crystallographic twofold axis.

For structure determination, the multiple isomorphous replacement method will be employed. A search for heavy-atom derivatives is currently in progress.

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