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Crystallization of the alanine dehydrogenase from *Phormidium lapideum*

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Abstract

Amino-acid dehydrogenases catalyse the interconversion of their respective amino acids to the corresponding keto acid, with concomitant reduction of NAD or NADP. The enzymes phenylalanine, glutamate, leucine and valine dehydrogenase all share a similar three-dimensional subunit structure and a high degree of sequence similarity, indicating that they belong to an enzyme superfamily related by divergent evolution. In contrast, alanine dehydrogenase shows no sequence similarity with any of these enzymes despite catalysing a reaction with the same chemistry and thus it is predicted that it possesses a different three-dimensional structure. The alanine dehydrogenase from *Phormidium lapideum* has been crystallized in space group *R*32, cell dimensions $a = b = 123.1$ and $c = 184.8$ Å, with a monomer in the asymmetric unit. The structure determination of this enzyme will shed light on how nature has evolved two different systems to carry out the same reaction.

1. Introduction

Alanine dehydrogenase (AlaDH), E.C. 1.4.1.1, catalyses the reversible reductive amination of pyruvate to L-alanine (Yoshida & Freese, 1970), with the concomitant oxidation of NADH, according to the following reaction

$$\mathrm{NADH} + \mathrm{NH_4^+} + \text{pyruvate} + \mathrm{H^+} \rightleftharpoons \mathrm{NAD^+} + \mathrm{H_2O} + \text{L-alanine}.$$

This enzyme is found in vegetative cells and spores of various bacilli and in some other bacteria (Vancura *et al.*, 1989; Ohshima & Soda, 1990 and references therein). AlaDH has an important role in the carbon and nitrogen metabolism of micro-organisms (Freese *et al.*, 1964; McCowen & Phibs, 1974) and is a key factor in the assimilation of L-alanine as an energy source through the tricarboxylic acid cycle during sporulation (Ohshima & Soda, 1990). Alanine dehydrogenase has been isolated from a number of *Bacillus* species and its enzymological properties elucidated (Yoshida & Freeze, 1965; Ohshima & Soda, 1979; Porumb *et al.*, 1987). The enzyme has also been purified and characterized from both the N_2-fixing cyanobacterium *Anabaena cylindrica* (Rowell & Stewart, 1976) and the non-N_2-fixing cyanobacterium *Phormidium lapideum* (Sawa *et al.*, 1994). AlaDH exhibits an A-type (*pro-R*) stereospecificity for the coenzyme (Ohshima & Soda, 1990; Grimshaw & Cleland, 1981), which is the opposite to that seen in the other amino-acid dehydrogenases, phenylalanine dehydrogenase (PheDH), leucine dehydrogenase (LeuDH) and glutamate dehydrogenase (GluDH). This *pro-R* stereospecificity also occurs in lactate dehydrogenase, which also uses pyruvate as a substrate.

The majority of AlaDH's isolated and purified exist as homohexamers, with subunit molecular weights between 39 500 and 40 000 Da; AlaDH's in this class include those from all *Bacillus* species (Ohshima & Soda, 1990), *P. lapideum* (Sawa *et al.*, 1994), *M. tuberculosis* (Andersen *et al.*, 1992) and *Streptomyces phaeochromogenes* (Itoh & Morikawa, 1983). An octamer of subunit M_r of 48 kDa has been reported for the AlaDH from *Streptomyces aureofaciens* (Vancurova *et al.*, 1988) and tetramers with subunit M_r of 43 kDa for soybean nodules (Smith & Emerich, 1993) and M_r of 51 kDa for *Streptomyces fradiae* (Vancura *et al.*, 1989).

The amino-acid sequences of the alanine dehydrogenases from *B. sphaericus*, *B. stearothermophilus* (Kuroda *et al.*, 1990), *Mycobacterium tuberculosis* (Andersen *et al.*, 1992) and *Phormidium lapideum* (Y. Sawa, personal communication) have been determined and show a very high degree of homology with one another, with for example 52% identity between the AlaDH from *P. lapideum* and that of *B. stearothermophilus*. Significant similarity (29% identity) has also been observed between the sequences of the AlaDH's and the N-terminal NAD-binding domain of the proton-translocating transhydrogenase of both *E. coli* and bovine mitochondria, perhaps suggesting some common evolutionary ancestor for this domain (Delforge *et al.*, 1993).

Four other amino-acid dehydrogenases, glutamate, leucine, phenylalanine and valine dehydrogenase (GluDH, LeuDH, PheDH and ValDH, respectively) have been sequenced and these sequences show a high degree of similarity with each other (Britton *et al.*, 1993). The three-dimensional structures of GluDH (Baker *et al.*, 1992; Stillman *et al.*, 1993) and LeuDH (Baker *et al.*, 1995) have been determined. These two enzymes share a very similar subunit structure, as predicted on the basis of sequence similarity (Britton *et al.*, 1993). The crucial residues involved in the recognition of the nicotinamide ring of the dinucleotide cofactor, and those which bind the substrate backbone and determine the catalysis are conserved, whereas there are important sequence changes in the substrate binding pocket which mediate the substrate specificity. Equivalent residues can be identified in both PheDH and ValDH and these four enzymes form an enzyme superfamily catalysing equivalent reactions on their respective substrates using the same molecular framework and reaction mechanism. The only sequence similarity between AlaDH and any of these enzymes is the presence of the ubiquitous dinucleotide-binding fingerprint, G*X*G*XX*G/A, which suggests that, like them and, indeed, many other dehydrogenases, AlaDH contains a dinucleotide-binding domain. However, as no other sequence similarity can be detected, the rest of its structure is likely to be different from that of the other amino-acid dehydrogenases. The structure determination of AlaDH will

thereby show how nature has evolved different protocols to catalyse essentially the same chemical process.

2. Protein purification and crystallization

P. lapideum AlaDH was overproduced in *E. coli* strain MV1174 carrying the pMAD1 plasmid, which encodes the gene for *P. lapideum* AlaDH. The cells were grown at 310 K in 5 l of Luria broth (1.0% tryptone, 1.0% NaCl and 0.5% yeast extracts) containing 50 μg ml^{-1} sodium ampicillin and induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG). The cells were harvested by centrifugation. For purification, cells (13 g of wet weight) were suspended in buffer *A* (40 m*M* Tris–HCl, pH 8.5, 2 m*M* EDTA) and disrupted by two passes through a French Press at 20 Kps. Debris was spun down by centrifugation at 30 000*g* for 10 min. The supernatant fraction (crude extract) contained 900 mg of total protein, as estimated by the Bio-Rad protein assay procedure with BSA as standard. 8% of the total protein was determined to be alanine dehydrogenase, using the assay described previously (Sawa *et al.*, 1994). The crude extract was loaded on a 20 ml DEAE–Sepharose Fast Flow column equilibrated with buffer *A*. To elute the protein, 300 ml of a linear gradient from 0 to 0.25 *M* NaCl in buffer *A* was applied. AlaDH activity was eluted from the column as a rather broad peak between 0.08 and 0.12 *M* NaCl. Fractions with AlaDH activity were collected (66 ml) and 22 ml of 4 *M* $(NH_4)_2SO_4$ was added, to give a final concentration of ammonium sulfate of 1 *M*. The resulting precipitate, which was 90% pure AlaDH as estimated by polyacrylamide gel electrophoresis and the enzymic assay, was collected by centrifugation (30 000*g*, 10 min).

For crystallization trials, samples of AlaDH were dialysed against 0.1 *M* MES, pH 6.7 and concentrated to 25–30 mg ml^{-1} using Centricon-30 or Microcon-30 microconcentrators (Amicon). The hanging-drop method was used with wells containing 6–10% PEG 20000 in 0.1 *M* MES, pH 6.7. 5 μl drops of protein solution were mixed with an equivalent volume of the precipitant and prism-like crystals grew in 2 weeks with maximum dimensions 0.5 × 0.5 × 0.5 mm.

3. Space-group determination

Preliminary data were collected from a single crystal at room temperature using a twin SDMS area-detector system (Xuong *et al.*, 1985; Hamlin, 1985) and graphite-monochromated Cu $K\alpha$ X-rays produced using a Rigaku RU 200 rotating-anode generator, with an 0.3 × 3 mm filament running at 55 kV 90 mA. A total of 26 315 measurements of 8037 independent reflections was made, with an R_{sym} of 0.035 and 85% completeness over the resolution range 20–3.1 Å, with mean $I/\sigma(I)$ = 3.9 in the highest resolution shell. The data were processed using the supplied software (Howard *et al.*, 1985). The auto-indexing algorithm suggested that the space group was *R*32, and inspection of the images showed a $-h + k + l = 3n$ condition on the data, confirming the space-group assignment. The cell parameters were refined to $a = b = 123.1$ and $c = 183.8$ Å, with a cell volume of 3.38×10^6 Å^3. Space group *R*32 demands some multiple of 18 polypeptide chains in the unit cell. The value of V_m for one chain in the asymmetric unit, with an M_r of 39 700 Da, is 3.38 Å^3 Da^{-1} this is within the range given by Matthews (1977), strongly suggesting that the biological hexamers of AlaDH must be constructed in 32 symmetry. We are currently searching for suitable heavy-atom derivatives.

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