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**Jane A. Irwin · Susan V. Lynch · Suzie Coughlan
Patrick J. Baker · Haflidi M. Gudmundsson
Gudni A. Alfredsson · David W. Rice · Paul C. Engel**

Alanine dehydrogenase from the psychrophilic bacterium strain PA-43: overexpression, molecular characterization, and sequence analysis

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Abstract The gene encoding alanine dehydrogenase (AlaDH; EC 1.4.1.1) from the marine psychrophilic bacterium strain PA-43 was cloned, sequenced, and overexpressed in *Escherichia coli*. The primary structure was deduced on the basis of the nucleotide sequence. The enzyme subunit contains 371 amino acid residues, and the sequence is 90% and 77% identical, respectively, to AlaDHs from *Shewanella* Ac10 and *Vibrio proteolyticus*. The half-life of PA-43 AlaDH at 52 °C is 9 min, and it is thus more thermolabile than the AlaDH from *Shewanella* Ac10 or *V. proteolyticus*. The enzyme showed strong specificity for NAD^+ and L-alanine as substrates. The apparent K_m for NAD^+ was temperature dependent (0.04 mM–0.23 mM from 15 °C to 55 °C). A comparison of the PA-43 deduced amino acid sequence to the solved three-dimensional structure of *Phormidium lapideum* AlaDH showed that there were likely to be fewer salt bridges in the PA-43 enzyme, which would increase enzyme flexibility and decrease thermostability. The hydrophobic surface character of the PA-43 enzyme was greater than that of *P. lapideum* AlaDH, by six residues. However, no particular modification or suite of modifications emerged as being clearly responsible for the psychrophilic character of PA-43 AlaDH.

Keywords Alanine dehydrogenase · *Shewanella* · Psychrophilic · Primary structure · Sequence homology

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J.A. Irwin · S.V. Lynch · S. Coughlan · P.C. Engel (✉)
Department of Biochemistry and Conway Institute
of Biomolecular and Biomedical Research,
University College Dublin, Belfield, Dublin 4, Ireland
E-mail: paul.engel@ucd.ie
Tel.: +353-1-7161547
Fax: +353-1-2837211

P.J. Baker · D.W. Rice
Department of Molecular Biology and Biotechnology,
The Krebs Institute, University of Sheffield,
Western Bank, Sheffield, S10 2TN, UK

H.M. Gudmundsson · G.A. Alfredsson
Microbiology Laboratory, Institute of Biology,
University of Iceland, Ármúli 1A, IS-108
Reykjavik, Iceland

Pressent address: J.A. Irwin
Department of Veterinary Physiology and Biochemistry,
University College Dublin, Belfield, Dublin 4, Ireland

Introduction

In the last few years, information has begun to emerge about the enzymes of psychrophilic or cold-adapted organisms. Most of the studies to date have been of enzymes from Antarctic and deep-sea bacteria and some polar fish and have largely dealt with secreted enzymes. Many intracellular enzymes have also been studied; for reviews, see Russell (2000) and Sheridan et al. (2000). Psychrophilic enzymes tend to share the following three characteristics: they have high catalytic efficiency (k_{cat}/K_m) at low temperatures, they display high conformational flexibility, and they are thermolabile (Feller et al. 1996). Structural comparisons to date have revealed only subtle differences between psychrophilic enzymes and mesophilic homologues. Furthermore, few if any structural characteristics have been found to be unique to psychrophilic enzymes. Some of the characteristics observed in psychrophilic enzymes appear to be opposite to those that make thermophilic enzymes more rigid. These include reduced numbers of salt bridges, hydrogen bonds, aromatic-aromatic interactions, proline and arginine residues, as well as larger loops and greater exposure of nonpolar groups to solvent (Scandurra et al. 2000). However, this is not a reliable generalization.

Among the enzymes that have been used as models to elucidate structure-function relationships is alanine dehydrogenase. All alanine dehydrogenases found so far (AlaDH; EC 1.4.1.1) are solely NAD^+ dependent and catalyse the reversible deamination of L-alanine to pyruvate. This enzyme is found only in cyanobacteria

and in a few bacterial species. AlaDH is important in assimilating L-alanine as an energy source through the tricarboxylic acid cycle in *Bacillus* sp. (Siranosian et al. 1993). In other species, e.g., *Rhizobium* sp., ammonia formed through N_2 fixation is fixed into alanine through AlaDH, thereby linking nitrogen assimilation and carbon catabolism (Poole and Allaway 2000). AlaDH also has a biotechnological application in the production of optically active amino acids (Galkin et al. 1997). Most of the AlaDHs purified to date, such as those of several *Bacillus* sp. (Ohshima and Soda 1990) and the cyanobacterium *Phormidium lapideum* (Sawa et al. 1994), have been reported to be homohexamers with a subunit relative molecular mass of about 40,000. The cyanobacterial *Plectonema boryanum* L-AlaDH, however, is reportedly octameric (Perepelitsa et al. 1995), and the soybean nodule bacteroid and *Streptomyces fradiae* enzymes are tetramers (Smith and Emerich 1993; Vancura et al. 1989). AlaDH does not show sequence similarity to members of the glutamate, phenylalanine, leucine, and valine dehydrogenase superfamily (Sedelnikova et al. 1998).

An X-ray crystallographic structure is available for the moderately thermophilic *P. lapideum* AlaDH (Baker et al. 1998) and has permitted homology modeling studies on two cold-adapted AlaDHs from two Antarctic bacterial strains: the gram-negative *Shewanella* Ac10 and the gram-positive bacterium *Carnobacterium* sp. strain St 2. These structures were compared to modeled structures of AlaDH from the mesophile *Vibrio proteolyticus*, as well as mesophilic and thermophilic *Bacillus* sp. The thermal instability of the cold-adapted AlaDHs was attributed to their low content of salt bridges, which declined in the order thermophilic AlaDH—mesophilic AlaDH—psychrotrophic AlaDH (Galkin et al. 1999).

We have investigated AlaDH from a psychrophilic gram-negative microorganism, strain PA-43, originally isolated from a sea urchin found off the southwest coast of Iceland. This microorganism was placed by 16S rRNA gene sequencing among the genus *Shewanella* (Irwin et al. 2001). It grows optimally at 16–17 °C and fails to grow at temperatures above 21 °C. This places it close to, but just outside of, the definition of a psychrophile according to Morita (1975). By Morita's criteria, a psychrophile has an optimum growth temperature ≤ 15 °C and a maximum of ≤ 20 °C. In contrast, *Shewanella* Ac10, with an optimum growth temperature of 20 °C and a maximum growth temperature of 30 °C, qualifies as a psychrotroph (or psychrotolerant). AlaDH and malate dehydrogenase were purified from this bacterium (Irwin et al. 2001) and were subjected to preliminary characterization and partial amino acid sequencing. This indicated that PA-43 AlaDH was similar (about 90% identical) to the *Shewanella* Ac10 enzyme and that PA-43 MDH was most similar to MDH from *Escherichia coli* (also about 90% identical).

Strain PA-43 AlaDH appears to be less thermostable than the *Shewanella* Ac10 enzyme, but its amino acid sequence is very similar. We therefore considered that it might be a good candidate for sequencing and structural analysis, to see if subtle differences between closely related enzymes could be attributed to particular residues or interactions between residues. We have cloned and sequenced the gene encoding AlaDH from PA-43 and interpreted the sequence data in the light of the published three-dimensional structure of the *P. lapideum* enzyme. We have also overexpressed the enzyme in *E. coli* and carried out kinetic studies.

Materials and methods

Materials

Grade II NAD^+ (98%, free acid), grade II NADH (98%, disodium salt), M_r markers for SDS-PAGE, and Expand DNA polymerase were obtained from Roche Molecular Biochemicals. Q-Sepharose Fast Flow, Sephacryl S-300, and NAD^+ analogues were supplied by Sigma-Aldrich Chemical Co. Sepharose CL-6B was purchased from Amersham Pharmacia Biotech. Restriction enzymes, *Taq* polymerase, and Wizard minipreps were from Promega. Primers were obtained from Sigma-Genosys, UK, and the pCRT7 Topo TA cloning kit was from Invitrogen. *Escherichia coli* (DE3) cells were purchased from Novagen. The GeneClean kit was obtained from Anachem.

Methods

Growth of strain PA-43

PA-43 was cultured at 15 °C in a rich medium as described by Irwin et al. (2001) and was used as the source of AlaDH for purification of the wild-type enzyme. Bacteria were harvested by centrifugation and stored at –20 °C until required.

Isolation of PA-43 genomic DNA

Cell paste (1 g) was washed with TES buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, pH 8.0), and the cells were then lysed by treatment with 5 mg/ml lysozyme. The mixture was incubated for 2 h at 37 °C. Proteinase K and SDS were then added to the suspension for a final concentration of 0.5 mg/ml and 1% w/v, respectively, and the incubation was continued at 55 °C for a further 2 h. The DNA was purified by phenol-chloroform treatment, as described by Sambrook et al. (1989).

Design of primers for PCR

The N-terminal amino acid sequence for the AlaDH was determined and sequence of internal tryptic and chymotryptic peptides, comprising about 30% of the total sequence, was also obtained by N-terminal sequencing (Irwin et al. 2001)

Initially, a degenerate N-terminal DNA primer was designed using the N-terminal sequence, and a C-terminal primer was designed using a sequence common to both the *Shewanella* Ac10 and *Vibrio proteolyticus* AlaDH sequences, CKEVA (amino acids 352–356). The N-terminal primer, AlaDH1, contained a *Bam*H1 site (5′-AGAGGATCATGATHATHGGNATH-3′) and the C-terminal primer, AlaDH2, contained a *Sal*1 site (5′-GGCGGTCGACNACNACYTCYTTRCA-3′). These primers were used to amplify a 1.1-kb fragment containing 96% of the gene.

PCR reaction

PA-43 genomic DNA was partially digested with *Sal*1 prior to PCR to facilitate primer binding. The amplification was performed using the Expand polymerase system, containing *Taq* and *Pwo* polymerases. The reaction mixtures (100 μl) contained 1 μg genomic DNA, 300 nM primers, 200 μM dNTP, 10 μl manufacturer's Mg^{2+}-free reaction buffer, 0.5 mM $MgCl_2$, and 2.5 U enzyme. The amplification procedure consisted of denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 40 °C for 1 min, and 72 °C for 2 min. A 1.1-kb fragment was purified from a 1% agarose gel (Geneclean) and subcloned into the phage M13mp19 through the *Bam*H1 and *Sal*1 sites that were incorporated during the PCR reaction.

Cloning of the 1.1-kb fragment and further PCR

The recombinant phage vector containing the amplified PCR product was transfected into *E. coli* TG1 cells using standard techniques. Single-stranded DNA was isolated from the phage and sequenced to reveal 96% of the DNA sequence. The fragment was labeled with digoxygenin (DIG High-Prime Labeling and Detection Kit, Roche Molecular Biochemicals) and used to probe Southern blots of single and multiple restriction enzyme digests of PA-43 genomic DNA. However, this approach failed to yield reproducible results and, as a result, an alternative PCR approach was devised in which a C-terminal primer was designed based on the highly homologous sequences of the *Shewanella* Ac10 and *V. proteolyticus* alanine dehydrogenase genes (5′-TCATTTAAKCAAGYAGGCTTTTYGGTTCAGYGWAYTC-MAGRTTMAACGCTTC-3′). This primer included the conserved region and extended four bases beyond the termination codon for the *Shewanella* Ac10 alanine dehydrogenase gene. The N-terminal primer was identical to the N-terminal DNA sequence (5′-ATGATTGGTATCCCTACTGAAATCAAAAAC-CAT-3′).

PCR using these primers was carried out as described above, except that the $MgCl_2$ concentration was 1 mM and 1 U *Taq* polymerase was used. The product containing the full gene (1.1 kb) was excised from agarose gel, purified, and ligated into the pCR 2.1-TOPO vector, which provides a topoisomerase to facilitate the incorporation of a *Taq*-amplified product with 3′A overhangs. This was transfected into One Shot (TOP10F') cells and grown on Luria-Bertani (LB) agar plates containing X-gal and 50 μg/ml ampicillin at 37 °C. White colonies containing the insert were picked and grown in 5 ml LB medium at 37 °C, and plasmid DNA was purified using Wizard minipreps eluted with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE buffer).

Fluorescent automated DNA sequencing of the insert was carried out commercially by Cambridge Biosciences (now Cytomyx) Cambridge, UK.

Expression of recombinant AlaDH in E. coli BL21 (DE3)

The 1,116 bp long open reading frame (ORF) encoding AlaDH was amplified by PCR using genomic DNA purified from 5 ml of sonicated PA-43 cell paste as template. The following oligonucleotides were designed to amplify the gene: N-terminal ADH5′: 5′-GGCCGGATCC**ATGATTATTGGTATCCCTA C**-3′ and C-terminal ADH3′: 5′-CGGGTTTAGGTCGAC**TCATTTAATC-AAGCAGGCTT**-3′ (bold face denotes AlaDH specific sequence; underline denotes the *Bam*HI and *Sal*I sites, respectively). The primers were designed to insert the gene downstream of the IPTG inducible hybrid *trp*/*lac*UV5 promoter of the overexpression vector pTAC. The PCR reaction was performed in a mixture containing 200 ng of template, 15 pmol of each oligonucleotide primer, 5 μl polymerase buffer containing 2 mM $MgSO_4$, and 200 μM dNTP for a final volume of 50 μl. The reaction mixture was incubated at 95 °C for 5 min prior to the addition of 2.5 U *Pwo* polymerase. The amplification cycle was as follows: one step of 95 °C for 60 s; 30 cycles of 42 °C for 30 s, 72 °C for 30 s, and 95 °C for 30 s; and a final extension step of 72 °C for 5 min.

The amplified product was digested with *Bam*HI and *Sal*I and then ligated to the corresponding sites of the expression vector pTAC. The recombinant DNA was transformed into *E. coli* BL21 (DE3) cells. Several clones were screened for the insert, and one construct was chosen and sequenced to ensure that the open reading frame was in frame with the ribosome-binding site.

This in-frame clone housing the AlaDH/pTAC construct was grown overnight at 37 °C in 100 ml LB broth containing ampicillin (100 μg/ml). Ten ml of this overnight culture was used to inoculate 1-l volumes of LB broth containing ampicillin (100 mg/ml). Flasks were incubated at 37 °C under continuous shaking until the culture reached an OD_{595} of 0.6. The flasks were placed in a water bath at 42 °C for 20 min, to allow for expression of inducible heat shock proteins that assist in protein folding, prior to being transferred to another water bath at 25 °C for 10 min to allow culture recovery. Expression of AlaDH was induced in recovered cells by adding IPTG to a final concentration of 1 mM. The culture was then re-incubated at 29 °C for 20 h, AlaDH activity was measured, and a further 100 mg/ml of ampicillin was added (method communicated to us by Dr. Michael McPherson). Cells were harvested by centrifugation for 20 min at 20,200 *g* at 4 °C.

Enzyme and protein assay

The enzyme was routinely assayed spectrophotometrically at 340 nm and 25 °C (Uvikon 941 Plus, Kontron Instruments) in the direction of both reductive amination and oxidative deamination. For the forward reaction, two different assays were used. Substrate specificity in the direction of oxidative deamination was determined using the assay of Irwin et al. (2001), in which the reaction mixtures contained the following, in a volume of 1 ml: 50 mM sodium pyrophosphate, pH 8.8; 1 mM NAD^+; 40 mM L-alanine (or other amino acid); and enzyme. Other studies were carried out using the conditions given by Galkin et al. (1999) to permit direct comparison, and the assay mixture contained 0.1 M glycine/KOH buffer, pH 10; 1 mM NAD^+; 40 mM L-alanine; and enzyme. For assays in the direction of reductive amination, the reaction mixture contained 0.1 M Tris-HCl, pH 8.5; 0.2 mM NADH; 160 mM $(NH_4)_2SO_4$; 5 mM pyruvate; and enzyme. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol NADH/min in the oxidative deamination of L-alanine.

Protein concentration was determined by the Bio-Rad assay method (Bradford 1976) using bovine serum albumin as standard. Protein elution patterns from columns were monitored using the absorbance at 280 nm.

Purification of recombinant AlaDH

Wild-type AlaDH was purified from PA-43 as described by Irwin et al. (2001). Recombinant enzyme was purified from *E. coli* as follows. The cell pellet obtained from centrifugation of 2×1 l *E. coli* culture was resuspended in 50 mM phosphate buffer pH 7, containing 10 mM EDTA and 1 mM β-mercaptoethanol. The resuspended cells were disrupted by 2×30 s bursts of sonication on ice. The sample was centrifuged at 3,000 *g* for 30 min at 4 °C, and the cell-free supernatant was applied to a Procion Red HE-3B Sepharose column (50 ml) equilibrated with the same buffer. The column was then washed with three column volumes of buffer and eluted with one column volume of 1 mM NADH in the same buffer. Fractions containing enzyme activity were concentrated by ultrafiltration and stored in 30% glycerol at −20 °C.

The purity of the fractions from each chromatographic step was analysed by SDS-PAGE (Laemmli 1970) using the Mini-Protean II apparatus (Bio-Rad) on 10% polyacrylamide gels. This showed that the protein was purified to near homogeneity, with a faint contaminating band corresponding to the presence of malate dehydrogenase in some preparations.

Calculation of kinetic constants

Initial velocity experiments were performed by varying the concentration of one substrate (six different concentrations: NAD^+ 0.05–1 mM; L-alanine, 5–40 mM; NADH, 0.025–0.20 mM; pyruvate, 0.25–5 mM; NH_4Cl, 20–160 mM) at a fixed concentration of the other substrate(s) (1 mM NAD^+; 40 mM L-alanine; 0.20 mM NADH; 5 mM pyruvate; 160 mM NH_4Cl). Values of K_m and V_{max} were calculated with the program Enzpack 3.0 (Biosoft Ltd, Cambridge, UK) using the Wilkinson method (Wilkinson 1961).

Results

Cloning and sequence analysis of the gene encoding AlaDH

The PCR product corresponding to the gene encoding AlaDH was cloned in the pCRT7 Topo TA vector and sequenced. The gene and protein sequences obtained are shown in Fig. 1. The PCR fragment obtained was 1,120 bp long and encoded a protein of 371 amino acids, a stop codon, and four extra bases at the 3′ end. The G + C composition of the alanine dehydrogenase gene was 43%. The deduced primary structure gave a perfect match to partial sequences for the N-terminus and tryptic and chymotryptic fragments (Irwin et al. 2001). The sequence was lodged to GenBank under the accession number AY081059.

Figure 2 displays amino acid alignments of the AlaDHs from strain PA-43, *Shewanella* Ac10, *Vibrio proteolyticus*, and *Phormidium lapideum*. The enzyme displayed closest identity to the *Shewanella* enzyme (90.5%), followed by 77% to *V. proteolyticus* AlaDH and 52% to *P. lapideum* AlaDH. Many of the differences between the sequences of the PA-43 AlaDH and the *V. proteolyticus* and *Shewanella* Ac10 enzymes were conservative substitutions, e.g., leucine for isoleucine, arginine for lysine, or isoleucine for valine. A BLAST search (http://www.ncbi.nlm.nih.gov/blast/) was carried out to determine the similarity of the PA-43 AlaDH to other AlaDHs present in the GenBank database. The enzyme showed the following percentage identities to AlaDHs from the following microorganisms: *Vibrio cholerae* (73%); *Mezorhizobium loti* (63%); *Sinorhizobium meliloti* and *Rhizobium leguminosarium* (62%); *Streptomyces coelicolor*, *Staphylococcus aureus* and *Bacillus sphaericus*, (54%); *Enterobacter aerogenes* and *Bacillus subtilis* (53%); *Mycobacterium tuberculosis* and *Deinococcus radiodurans* (52%); *Synechocystis* sp. 6803 and *Carnobacterium* sp. St2 (50%); *Helicobacter pylori* (37%). The high identity to *Vibrio* sp. is not surprising, as they belong to the same group in the γ-subdivision of the class Proteobacteria as *Shewanella*.

Purification of AlaDH

To overexpress the enzyme in *E. coli*, a blunt-ended PCR product was initially cloned into the pCRT7 TOPO TA cloning vector, and the overexpressed protein had a polyhistidine tag fused to the N-terminal end to allow for purification by metal chelate affinity chromatography. However, while the protein was overexpressed, it formed insoluble aggregates in the pellet fraction. Attempts to solubilize these fractions using detergent were successful, but this soluble protein had no alanine dehydrogenase activity. As an alternative, the gene was

Fig. 1 Nucleotide and amino acid sequences of PA-43 AlaDH

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   1 - ATGATTATTGGTATCCCTACTGAAATCAAAAACCATGAATACCGTGTAGGTATGGTTCCT - 60
     - M  I  I  G  I  P  T  E  I  K  N  H  E  Y  R  V  G  M  V  P
  61 - TCAAGCGTTCGCGAATTAACGATTAAAGGCCATGAAGTATTTGTTCAATCTAATGCAGGT - 120
     - S  S  V  R  E  L  T  I  K  G  H  E  V  F  V  Q  S  N  A  G
 121 - ATGGGCATTGGTTTTACTAACCAAGATTATTTAAATGTTGGTGCCGCTATTTTAGAAACT - 180
     - M  G  I  G  F  T  N  Q  D  Y  L  N  V  G  A  A  I  L  E  T
 181 - GCGGAAGAAGTGTTTGCACAATCAGACATGATTGTGAAAGTAAAAGAACCACAAGCTGTT - 240
     - A  E  E  V  F  A  Q  S  D  M  I  V  K  V  K  E  P  Q  A  V
 241 - GAACGTGCAATGCTACGTGAAGACCAAATCTTATTCACGTATTTACACCTTGCACCTGAC - 300
     - E  R  A  M  L  R  E  D  Q  I  L  F  T  Y  L  H  L  A  P  D
 301 - ATGCCACAGACTGAAGACTTGCTAAAAAGTAAGGCTGTTTGTATCGCTTATGAAACCGTA - 360
     - M  P  Q  T  E  D  L  L  K  S  K  A  V  C  I  A  Y  E  T  V
 361 - ACTGATGACCGTGGAGGCCTGCCATTACTCGCTCCAATGTCAGAAGTTGCTGGCCGTATG - 420
     - T  D  D  R  G  G  L  P  L  L  A  P  M  S  E  V  A  G  R  M
 421 - TCTATCCAAGCAGGTGCTCGCGCACTGGAAAAATCATTGGGTGGACGTGGTATGTTACTT - 480
     - S  I  Q  A  G  A  R  A  L  E  K  S  L  G  G  R  G  M  L  L
 481 - GGTGGCGTACCCGGTGTTGAACCCGCTAAAGTTGTCATCATTGGCGGCGGTATGGTTGGT - 540
     - G  G  V  P  G  V  E  P  A  K  V  V  I  I  G  G  G  M  V  G
 541 - ACTAATGCAGCGCAGATGGCAGTAGGCATGGGCGCTGACGTTGTTGTACTTGATCGCAGC - 600
     - T  N  A  A  Q  M  A  V  G  M  G  A  D  V  V  V  L  D  R  S
 601 - ATTGATGCATTACGTCGTCTTAATGTGCAATTTGGCTCACCTGTAAAAGCAATTTATTCA - 660
     - I  D  A  L  R  R  L  N  V  Q  F  G  S  P  V  K  A  I  Y  S
 661 - ACTGCTGATGCTATCGAACGTCATGTGATAGAAGCTGATTTAGTCATTGGTGGTGTACTG - 720
     - T  A  D  A  I  E  R  H  V  I  E  A  D  L  V  I  G  G  V  L
 721 - GTTCCTGGTGCTGCAGCACCTAAGCTTGTTACTCGCGATATGATTACTCGTATGAAGCCA - 780
     - V  P  G  A  A  A  P  K  L  V  T  R  D  M  I  T  R  M  K  P
 781 - GGCAGTGCCATTGTTGATGTTGCAATTGACCAAGGTGGTTGTGTTGAAACGTCTTATGCA - 840
     - G  S  A  I  V  D  V  A  I  D  Q  G  G  C  V  E  T  S  Y  A
 841 - ACAACCCACCAAGACCCAACTTATATCATCGATGATGTGGTTCATTACTGTGTTGCAAAC - 900
     - T  T  H  Q  D  P  T  Y  I  I  D  D  V  V  H  Y  C  V  A  N
 901 - ATGCCAGGCGCGGTTGCTCGCACCTCTACATTTGCACTTAATAATGCAACGCTTCCATAC - 960
     - M  P  G  A  V  A  R  T  S  T  F  A  L  N  N  A  T  L  P  Y
 961 - ATTATTAAGCTTGCGAACTTGGGTTATAAGTCTGCATTACTTCAAGATAAACACTTACTA - 1020
     - I  I  K  L  A  N  L  G  Y  K  S  A  L  L  Q  D  K  H  L  L
1021 - AATGGCCTAAATGCTATTCATGGAAAGCTAGTTTGTAAGGAAGTAGCTGAAGCGTTTAAC - 1080
     - N  G  L  N  A  I  H  G  K  L  V  C  K  E  V  A  E  A  F  N
1081 - CTGGAGTACACTGAACCAAAAAGCCTGCTTGATTAA - 1116
     - L  E  Y  T  E  P  K  S  L  L  D  *
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Fig. 2 Sequence alignment of strain PA-43 AlaDH with homologous AlaDHs. The sequences were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank) and aligned using the program Clustal W (version 1.8). The accession numbers used were *Shewanella* Ac10, AAC23578; *Vibrio proteolyticus*, AAC23579; *Phormidium lapideum*, BAA24455. Shewanella denotes *Shewanella* Ac10, Vib denotes *V. proteolyticus*, and P. lap. is *Phormidium lapideum*. Identical residues are shown as *asterisks*, conserved residues by a *colon* or a *period*

Table 1 Summary of purification procedure for recombinant AlaDH from strain PA-43 expressed in *Escherichia coli*. The activity at each step was measured using 1 mM NAD^+ and 40 mM L-alanine in 0.1 M glycine/NaOH buffer, pH 10

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
E. coli supernatant	13.2	1890	754	0.40	100	1
Procion Red HE3B chromatography	28.3	56.4	1,398	24.8	185	62

cloned into pTAC, a plasmid that did not express any tags, as described above. This expression gave rise to soluble and active enzyme, which suggested that the His-tag was responsible for inhibiting enzyme activity. A purification table for recombinant alanine dehydrogenase is shown in Table 1. AlaDH was purified in a one-step process using Procion Red HE-3B eluted with 1 mM NADH. The increase in yield after purification is likely to be due to the removal of inhibitors in the crude extract. In some preparations, the yield of recombinant enzyme was as low as 1% of the total protein, comparable with that found in the wild-type microorganism, but in other preparations the yield was 3%. The purified enzyme showed the same mobility on SDS-PAGE as the wild-type enzyme and had AlaDH activity when run on a native PAGE gel and activity stained using the method of Irwin et al. (2001).

Effect of temperature

Strain PA-43 AlaDH was found to be quite thermolabile. The half-lives of the wild-type enzyme at 50 °C, 56 °C, and 60 °C were reported previously as 42 min, 2.25 min, and 15 s, respectively (Irwin et al. 2001). The half-life of both the recombinant and wild-type enzymes at 52 °C was 9 min, strongly suggesting that the AlaDHs prepared by the two methods were identical in terms of folding. If one compares the temperatures at which different AlaDHs lost 50% of their original activity over 30 min, the value obtained for the PA-43 enzyme was 50–51 °C, compared to 63 °C for *V. proteolyticus* AlaDH and 50 °C for *Shewanella* Ac10 AlaDH (Galkin et al. 1999). The Arrhenius activation energy was calculated from an Arrhenius plot, using the data for k_{cat} given in Table 2, as 41.5 kJ/mol.

Substrate and coenzyme specificity of AlaDH

PA-43 AlaDH showed a strong preference for L-alanine as substrate. This property is shared by the AlaDHs of *P. lapideum*, *Streptomyces* sp., and *Anabaena variabilis*, but not by those of *Pseudomonas* and *Rhodobacter* (Sawa et al. 1994) or by that of *Bacillus* sp. DSM730, which can use L-serine at 10% of the rate observed with

Table 2 Biocomputing parameters for alanine dehydrogenases from PA-43, *Shewanella* Ac10, *Vibrio proteolyticus*, and *Phormidium lapideum*

	PA-43	*Shewanella*	*V. proteolyticus*	*P. lapideum*
Total number of amino acids	371	371	374	361
Molecular weight	39,595.8	39,527.4	39,802.9	38,557.4
Theoretical pI	5.35	5.70	5.56	5.45
Molar extinction coefficient (280 nm) $M^{-1}cm^{-1}$	14,320	13,040	13,040	22,900
Aliphatic index	104.88	106.44	101.47	106.90
Grand average of hydropathy	0.177	0.228	0.178	0.184
Instability index[a]	34.17	30.38	21.87	31.67

[a] A protein whose instability index is <40 is predicted to be stable, whereas a value >40 predicts that the protein may be unstable (Guruprasad et al. 1990).The calculated molar extinction coefficient is estimated on the assumption that all the Cys residues are half cystines. The aliphatic index is calculated using the method of Ikai (1980), and the grand average of hydropathy is calculated according to the method of Kyte and Doolittle (1982)

L-alanine (Nagata et al. 1989). AlaDH from *Mycobacterium smegmatis* was found to function also as a glycine dehydrogenase (Usha et al. 2002). Crystallographic data from *P. lapideum* AlaDH show that pyruvate binds deep in the cleft between the enzyme's two domains and that the methyl group of pyruvate packs close to the hydrophobic residues Tyr 93, Met 132, and Leu 129 (Baker et al. 1998). It is likely that the tight packing of these three amino acid residues in the amino acid binding site may not allow amino acids with larger side chains to fit into the cleft in the right orientation, if at all. These three residues are invariant in *P. lapideum*, PA-43, AlaDHs from various *Bacillus* sp., and *M. smegmatis*, and thus the differences in specificity are possibly due to subtle differences in their spacing. However, no protein sequence is available in GenBank for *Bacillus* DSM 730, *Pseudomonas*, or *Rhodobacter* AlaDHs; therefore, at present it is impossible to verify that these residues are the only residues conferring amino acid substrate specificity on AlaDH. A definitive explanation awaits the determination of a crystal structure of a binary complex between AlaDH and L-Ala.

The following L-amino acids were found to be inert as substrates, showing no activity even at enzyme concentrations 10-fold greater than those normally used for AlaDH assay: arginine, proline, threonine, methionine, cysteine, aspartate, asparagine, tyrosine, phenylalanine, leucine, isoleucine, valine, glycine, serine, glutamate, glutamine, norvaline, norleucine, and β-chloro-L-alanine. D-alanine is not a substrate. L-2-amino-*n*-butyric acid showed very slight activity, $<1\%$ of that with L-alanine. The enzyme was also assayed in the direction of reductive amination. 2-Oxoglutarate was inert as a substrate, and 5 mM 2-oxobutyrate gave 3% of the activity observed with 5 mM pyruvate. Absolute specificity for pyruvate in the direction of reductive amination has not been observed with any AlaDH (Sawa et al. 1994).

AlaDH used NAD^+ preferentially as coenzyme. The rate with 1 mM $NADP^+$ was 5% of that obtained with 1 mM NAD^+. No activity was obtained when the enzyme was assayed with 0.2 mM NADPH. The activities with hypoxanthine nicotinamide nucleotide (deamino-NAD^+), 3-acetylpyridine adenine nucleotide, and N^6-etheno-NAD^+ (all 1 mM) were respectively 90%, 212%, and 17% of the activity with NAD^+.

Inhibition and activation by effectors

The effect of various compounds on AlaDH activity was examined. Enzyme activity was unaffected by 1 mM ATP, AMP, and GTP, suggesting that activity is not modulated by purine mononucleotides. FAD (0.5 mM) had no effect. Cibacron Blue F3GA, a ligand commonly used in dehydrogenase purification, inhibited AlaDH, abolishing 70% of the activity when added to a concentration of 100 μM in the assay mixture. DTNB, which modifies thiol groups, also inhibits (7% inhibition with 0.01 mM and 40% inhibition with 0.1 mM DTNB), which suggests that thiol groups may be directly or indirectly involved in enzyme catalysis or in the maintenance of enzyme structure. β-Mercaptoethanol exerted a slight activatory effect on the enzyme (107% of control at 1 mM). Of the L-amino acids tested, only L-cysteine inhibited the enzyme when it was assayed in the direction of oxidative deamination (40% inhibition in the presence of 10 mM L-cysteine). The following L-amino acids at a concentration of 10 mM did not inhibit the reaction (in the presence of 40 mM Ala): threonine, methionine, aspartate, tyrosine, phenylalanine, leucine, isoleucine, valine, serine, glutamate, and glycine. No inhibition was observed in the presence of 10 mM EDTA, which suggests that the enzyme does not require metal ions for activity, or 1 mM $MgCl_2$. Low levels of inhibition were observed when the enzyme was assayed in the presence of 1 mM $MnCl_2$ (10%) or 1 mM $CaCl_2$ (15%).

Kinetic parameters

The dependence of the enzyme's kinetic parameters on temperature is shown in Table 3. It should be noted first of all that where they were compared, the same values were obtained with recombinant enzyme as with enzyme purified from the original organism. It is notable that the

Table 3 Kinetic parameters: AlaDH activity in the direction of oxidative deamination was measured in 0.1 M glycine/NaOH buffer, pH 10, as described in Methods

Substrate	Temperature (°C)	K_m (mM)[a]	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}.mM^{-1}$)
Oxidative deamination				
NAD^+	10	0.04 ± 0.01	29 ± 2	725
	15	0.04 ± 0.01	41 ± 2	1,025
	25	0.05 ± 0.01	63 ± 3	1,260
	35	0.07 ± 0.01	128 ± 4	1,829
	45	0.10 ± 0.01	207 ± 6	2,070
	50	0.16 ± 0.02	211 ± 6	1,319
	55	0.23 ± 0.03	163 ± 9	710
L-alanine	10	10.1 ± 0.2	45 ± 2	4.4
	15	10.6 ± 0.7	67 ± 4	6.3
	25	9.7 ± 0.6	75 ± 4	7.7
	45	10.8 ± 0.3	228 ± 10	21.1
	55	23.0 ± 0.6	215 ± 20	9.3
Reductive amination				
NADH	5	0.04 ± 0.01	1008 ± 74	25,200
	25	0.08 ± 0.02	2209 ± 265	27,612
	40	0.08 ± 0.01	2871 ± 91	35,887
Pyruvate	5	0.39 ± 0.04	888 ± 24	2,277
	25	0.80 ± 0.12	1963 ± 102	2,453
	40	0.80 ± 0.16	2562 ± 192	3,203
NH_4^+	5	28 ± 3	977 ± 68	35
	25	25 ± 1	1755 ± 77	70
	40	30 ± 2	2472 ± 94	82

[a] The values for K_m and k_{cat} are given ± SEM

K_m for NAD^+ at 25 °C (0.05 mM) is considerably lower than the corresponding constants for the enzymes from *V. proteolyticus* (0.21 mM) and *Shewanella* Ac 10 (0.24 mM).

One of the obvious features of the temperature effect is that below 50 °C the k_{cat} values drop steadily, about 7-fold between 50 °C and 10 °C for the two-substrate reaction and about 3-fold between 40 °C and 5 °C for the three-substrate reaction. On the other hand, over the same temperature ranges there are also substantial decreases in the apparent K_m values for pyruvate, NADH, and NAD^+, tending to mitigate the effect of the diminished k_{cat}. The apparent K_ms for alanine and ammonia are virtually constant over these temperatures. The net effect of these trends is that at sub-saturating substrate concentrations, the observed rate is only moderately affected by decreased temperature, in keeping with the need for catalytic efficiency under physiological conditions.

Structural properties of AlaDH

Table 2 shows biocomputing parameters calculated from the sequence of PA-43 AlaDH, which were compared to those of three other AlaDHs. The subunit relative molecular weight is 39,596, giving a value for the hexamer of 237,576. This compares with the M_r value of 260,000 obtained by gel filtration (Irwin et al. 2001). The pI calculated using Protparam was 5.35, almost identical to the pI value of 5.3 obtained by isoelectric focusing, which is the lowest value for the four enzymes surveyed. The aliphatic index did not differ greatly among the four enzymes, and only the *Shewanella* Ac10 enzyme showed any significant difference in the grand average of hydropathy.

Discussion

We report the sequencing, overexpression, and molecular characterization of AlaDH from the psychrophilic microorganism strain PA-43. The enzyme showed high sequence identity to the enzymes from *Vibrio proteolyticus* and *Shewanella* Ac10, but it was more thermolabile than either of these enzymes. The apparent K_m for NAD^+ changed depending on the reaction temperature (Table 3), being lowest at or below 15 °C and increasing above 35 °C. This behavior is consistent with the designation of this enzyme as psychrophilic, and the temperature of 15 °C is close to that required for optimum growth, 16.5 °C (Irwin et al. 2001). The activation energy of 41.5 kJ/mol was also relatively low and would be advantageous to the catalysis of the reaction at low to moderate temperatures.

Several features conferring optimal cold activity and thermolability on psychrophilic enzymes have now been identified, using multiple alignments, crystal structures, and homology modeling. These data can now be used to suggest possible general rules for protein-engineering experiments aimed at producing enzymes catalytically active at low temperatures (Gianese et al. 2001).

Arginine residue content is considered to be important in maintaining protein stability, as arginine residues form more stable bonds and give rise to more favorable interactions at protein-protein and protein-solvent interfaces than do lysine residues (Galkin et al. 1999). In the case of the alanine dehydrogenases discussed above,

Table 4 Amino acid content of alanine dehydrogenases[a]

	PA-43	*Shewanella*	*V. proteolyticus*	*P. lapideum*
Glycine	34 (9.2%)	35 (9.4%)	33 (8.8%)	34 (9.4%)
Arginine	15	15	13	16
Arg/(Arg + Lys)	0.469	0.469	0.394	0.571
Proline	17 (4.6%)	16 (4.3%)	17 (4.5%)	21 (5.8%)
Hydrophobic residues (F, I, L, V)	106 (28.6%)	107 (28.8%)	106 (28.3%)	111(30.7%)

[a] Values calculated above are for one subunit of the hexameric protein. They were obtained using the Protparam tool from the Expasy computer facilities (http://www.expasy.ch)

a comparison of the Arg content and the ratio of Arg to total basic residues (Table 4) does not reveal any clear relationship between decreases in Arg content and thermostability. The enzymes from strain PA-43 and *Shewanella* Ac10 have two more Arg residues than the mesophile *V. proteolyticus*. However, the arginine residues at positions 243 and 244 in *P. lapideum* are replaced in the other three enzymes by alanine (positions 244 and 245), which may contribute to flexibility. Arg 346 is replaced with Lys at the corresponding position 349 in the other three enzymes, in a region between a β-strand and an α-helix.

The sequence changes in PA-43 AlaDH compared to the sequence in *P. lapideum* were plotted onto the *P. lapideum* structure, and the residues involved in making salt bridges were examined. Of the 18 salt bridges present in the *P. lapideum* structure, sequence changes in the equivalent residues in PA-43 would suggest that only 8–10 of these are present in PA-43 AlaDH. This implies that a decrease in salt bridge numbers may be a factor in increasing enzyme flexibility and decreasing thermostability. However, a precise knowledge of salt bridges in the PA-43 enzyme awaits structural resolution of the enzyme.

Proline and glycine residues are thought to affect backbone flexibility, thereby modulating the entropy of protein unfolding (Matthews et al. 1987). The three closely related AlaDHs have comparable percentage contents of Pro, but the value is increased in *P. lapideum*. One possible important substitution may be the substitution of Pro in the hinge region of *P. lapideum* (position 122) with Asn in *V. proteolyticus* and with Asp in *Shewanella* Ac10 and PA-43. The Pro residue at position 78 in *P. lapideum* is replaced at the corresponding position 79 with Ala and, similarly, at position 305 with Ala at position 306 in the other three AlaDHs. Both of these substitutions are found at the beginning of α-helices. Position 59 in both *P. lapideum* and *V. proteolyticus* AlaDH is occupied by a Pro residue, which is replaced with Glu in PA-43 and with Ala in *Shewanella* Ac10 AlaDH. The replacement of this residue, which is surface accessible and located at the beginning of helix 3, may also increase enzyme flexibility.

The differences in percentage Gly content among the four enzymes do not appear to correlate strongly with their thermostability profiles. Alanine residues at position 238 and 237 in the *V. proteolyticus* and *P. lapideum* enzymes, respectively, are replaced with glycines in the *Shewanella* and PA-43 enzymes. These residues are located at the end of a strand, and this change may also correlate with an increase in structural flexibility.

The accessible surface area of the *P. lapideum* AlaDH hexamer was calculated using the program AREAIMOL (CCP4 1994). Only those residues whose accessible fraction was greater than 0.25 were considered as lying on the surface. Of the 129 residues thus identified in *P. lapideum* AlaDH, 79 were hydrophilic in character and 50 were hydrophobic. Nineteen of the hydrophobic surface residues in *P. lapideum* AlaDH are hydrophilic in the PA-43 enzyme, and 25 of the hydrophilic surface residues in *P. lapideum* are hydrophobic in PA-43 AlaDH. There is thus a net increase in hydrophobic surface character in the psychrophilic enzyme of six residues. This trend is also seen in *Shewanella* Ac10 AlaDH, but in this case the net change was only two residues. It must be emphasized that this method can only indicate trends, and a more thorough investigation of the surface character of these psychrophilic AlaDHs will require a full structural determination.

The aliphatic index, which is the relative volume of a protein occupied by aliphatic side chains (Ikai 1980), does not vary significantly for all four enzymes (Table 2). This value has been found to be significantly higher in proteins from thermophilic bacteria, and it would be expected to be higher in the *P. lapideum* enzyme, which has an apparent optimum temperature for activity of 60–70 °C (Sawa et al. 1994). The grand average of hydropathy (Kyte and Doolittle 1982) is similar for the PA-43, *V. proteolyticus,* and *P. lapideum* enzymes, but is increased from approximately 0.18–0.228 for the *Shewanella* AlaDH. The calculated instability index (Table 2) was greatest for PA-43 AlaDH, correlating with its relatively low thermostability.

Gianese et al. (2001) identified significant residue exchanges in the transition from mesophile to psychrophile. One of these was the substitution of Val with Ile, usually in buried regions of the protein. This substitution is observed in several places in the AlaDH alignment. Valine in *P. lapideum* AlaDH is replaced with Ile at positions 57, 71, 141 (142), and 320 (321) in the other three enzymes. Val residues in *V. proteolyticus* AlaDH

are replaced with Ile at position 173, position 322 in *Shewanella* and PA-43 AlaDH, and position 250 in *Shewanella* only. However, the opposite substitution, i.e., Ile to Val, is also seen at several locations in the AlaDH structure. For example, Ile 35 and 135 are replaced by Val at the corresponding position in the other three AlaDHs, and Ile 195 in *P. lapideum* (196 in *V. proteolyticus*) is replaced by Val in *Shewanella* Ac10 and PA-43. This interchange suggests that Val-to-Ile exchange is unlikely to be crucial in determining thermostability.

It is therefore clear from this study that no single structural modification can adequately explain the psychrophilic character of AlaDH from PA-43, although salt bridges, and perhaps increases in surface hydrophobicity, may play important roles. The crystallization and structural determination of this enzyme are ongoing, and the determination of the crystal structure should shed more light on the molecular basis of its psychrophilic properties.

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References

Baker PJ, Sawa Y, Shibata H, Sedelnikova SE, Rice DW (1998) Analysis of the structure and substrate binding of *Phormidium lapideum* alanine dehydrogenase. Nat Struct Biol 5:561–567

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

[CCP4] Collaborative Computational Project, Number 4 (1994) The CCP4 Suite: Programs for protein crystallography. Acta Crystallogr D 50:760–763

Feller G, Narinx E, Arpigny JL, Aittaleb M, Baise E, Genicot S, Gerday C (1996) Enzymes from psychrophilic organisms. FEMS Microbiol Rev 18:189–191

Galkin A, Kulakova L, Yoshimura T, Soda K, Esaki N (1997) Synthesis of optically-active amino acids from the corresponding α-keto acids with *Escherichia coli* cells expressing heterologous genes. Appl Environ Microbiol 63:4651–4656

Galkin A, Kulakova L, Ashida H, Sawa Y, Esaki N (1999) Cold-adapted alanine dehydrogenases from two Antarctic bacterial strains; gene cloning, protein characterization, and comparison with mesophilic and thermophilic counterparts. Appl Environ Microbiol 65:4014–4020

Gianese G, Argos P, Pascarella S (2001) Structural adaptation of enzymes to low temperatures. Protein Eng 14:141–148

Guruprasad K, Reddy BV, Pandit MW (1990) Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting *in vivo* stability of a protein from its primary sequence. Protein Eng 4:155–161

Ikai A (1980) Thermostability and aliphatic index of globular proteins. J Biochem 88:1895–1898

Irwin JA, Gudmundsson HM, Marteinsson VT, Hreggvidsson GO, Lanzetti AJ, Alfredsson GA, Engel PC (2001) Characterization of alanine and malate dehydrogenases from a marine psychrophile strain PA-43. Extremophiles 5:199–211

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Matthews BW, Nicholson H, Becktel WJ (1987) Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. Proc Natl Acad Sci 84:6663–6667

Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 39: 144–167

Nagata S, Misono H, Nagasaki S, Esaki E, Tanaka H, Soda K (1989) Thermostable alanine dehydrogenase of *Bacillus* sp. DSM730: gene cloning, purification, and characterization. Biochimie 71:559–563

Ohshima T, Soda K (1990) Biochemistry and biotechnology of amino acid dehydrogenases. Adv Biochem Eng Biotechnol 42:187–209

Perepelitsa SI, Koltukova NV, Mendzhul MI (1995) Alanine dehydrogenase of the cyanobacterium *Plectonema boryanum* in the early period of cyanophage LPP-3 development. WMJ 67:47–52

Poole P, Allaway D (2000) Carbon and nitrogen metabolism in *Rhizobium*. Adv Microb Physiol 43:117–163

Russell NJ (2000) Toward a molecular understanding of cold activity of enzymes from psychrophiles. Extremophiles 4:83–90

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor NY

Sawa S, Tani M, Murata K, Shibata H, Ochiai H (1994) Purification and characterization of alanine dehydrogenase from a cyanobacterium, *Phormidium lapideum*. J Biochem 116:995–1000

Scandurra R, Consalvi V, Chiaraluce R, Politi L, Engel PC (2000) Protein stability in extremophilic archaea. Front Biosci 5:D787–D795

Sedelnikova S, Rice DW, Shibata H, Sawa Y, Baker PJ (1998) Crystallization of the alanine dehydrogenase from *Phormidium lapideum*. Acta Crystallogr D 54:407–408

Sheridan PP, Panasik N, Coombs JM, Brenchley JE (2000) Approaches for deciphering the structural basis of low temperature activity. Biochim Biophys Acta 1543:417–433

Siranosian KJ, Ireton K, Grossman AD (1993) Alanine dehydrogenase (ald) is required for normal sporulation in *Bacillus subtilis*. J Bacteriol 175:6789–6796

Smith MT, Emerich DW (1993) Alanine dehydrogenase from soybean nodule bacteroids: purification and properties. Arch Biochem Biophys 304:379–385

Usha V, Jayaraman R, Toro JC, Hoffner SE, Das KS (2002) Glycine and alanine dehydrogenase activities are catalyzed by the same protein in *Mycobacterium smegmatis*: upregulation of both activities under microaerophilic adaptation. Can J Microbiol 48:7–13

Vancura A, Vancurova I, Volc J, Jones SK, Flieger M, Basarova G, Behal V (1989) Alanine dehydrogenase from *Streptomyces fradiae*. Purification and properties. Eur J Biochem 179:221–227

Wilkinson GN (1961) Statistical estimations in enzyme kinetics. Biochem J 80:324–332