Comparative studies on the 5-aminolaevulinic acid dehydratases from *Pisum sativum*, *Escherichia coli* and *Saccharomyces cerevisiae*

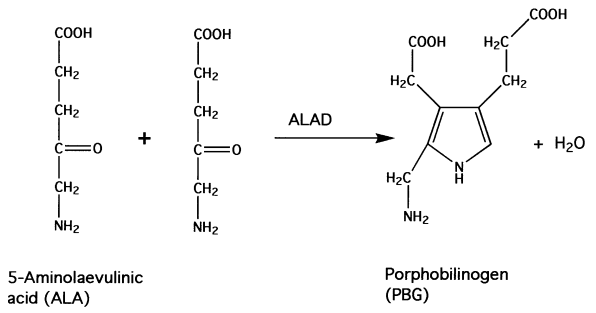
Natalie M. SENIOR*, Keith BROCKLEHURST†, Jon B. COOPER‡, Stephen P. WOOD‡, Peter ERSKINE‡, Peter M. SHOOLINGIN-JORDAN§, Paul G. THOMAS‖ and Martin J. WARREN*¶

*Department of Molecular Genetics, Institute of Ophthalmology, University College London, Bath Street, London EC1V 9EL, U.K., †Department of Biochemistry, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, U.K., ‡Department of Crystallography, Birkbeck College, Malet Street, London WC1E 7HX, U.K., §Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, U.K., and ‖Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire RG12 6EY, U.K.

5-Aminolaevulinic acid dehydratase (ALAD) is an essential enzyme in most organisms, catalysing an inaugural step in the tetrapyrrole biosynthetic pathway, the Knorr-type condensation reaction of two molecules of 5-aminolaevulinic acid (ALA) to form the monopyrrole porphobilinogen. ALADs can be conveniently separated into two main groups: those requiring Zn^{2+} for activity (typified here by the enzymes from *Escherichia coli* and *Saccharomyces cerevisiae*, yeast) and those requiring Mg^{2+} (represented here by the enzyme from *Pisum sativum*, pea). Here we describe a detailed comparison of these two metal-dependent systems. Kinetically influential ionizations were identified by using pH-dependent kinetics. Groups with pK_a values of approx. 7 and 10 (assigned to cysteine and lysine residues) were detected in the free enzyme and enzyme–substrate states of all three enzymes, and a further ionizable group with a pK_a of approx. 8.5 (assigned to histidine) was found to be additionally important to the yeast enzyme. The importance of these residues was confirmed by using protein modifying reagents. Shifts in the pK_a values of the pea and *E. coli* enzymes consequent on E–S complex formation suggest a change to a less hydrophobic microenvironment when substrate binds. Studies with inhibitors revealed that the three enzymes exhibit differential susceptibilities and, in the case of succinylacetone, this is reflected in K_i values that vary by three orders of magnitude. In addition, the crystallization of the yeast ALAD is described, raising the possibility of an X-ray-derived three-dimensional structure of this enzyme.

INTRODUCTION

5-Aminolaevulinic acid dehydratase (ALAD; also named porphobilinogen synthase; EC 4.2.1.24) catalyses an early step in the biosynthesis of all modified tetrapyrroles, performing an asymmetric Knorr-type condensation of two molecules of 5-aminolaevulinic acid (ALA) to form the monopyrrole porphobilinogen (PBG) (Scheme 1). The end products of the tetrapyrrole pathway, such as haem, chlorophyll and corrins, are intricately involved in many aspects of metabolism, from electron transport to photosynthesis. Modified tetrapyrroles are able to perform this range of functions owing to their differential oxidation and conjugation states, permitting the chelation of various metal ions that fine-tune the prosthetic groups to their function. Studies over the past 40 years have resulted in the characterization of the biochemical events leading to the synthesis of tetrapyrrole-derived compounds and today there are few steps for which the reaction mechanism and biosynthetic intermediates are unknown (reviewed in [1]).



Scheme 1 The Knorr-type condensation catalysed by ALAD

Two molecules of ALA are condensed to give PBG, the pyrrole building block of all haems, chlorophylls and corrins.

ALADs have been purified to homogeneity from a wide variety of sources, including bovine liver [2], human erythrocytes [3], *Rhodopseudomonas capsulatus*, *Rhodobacter sphaeroides*, [4,5], *Escherichia coli* [6,7] and spinach (*Spinacia oleracea*) [8]. Although the fundamental catalytic properties of all ALADs are similar, differences in enzyme primary structure, metal ion requirement and thiol sensitivity have been observed between the various purified enzymes. Metal dependency allows ALADs to be divided into two main categories, the Zn^{2+}-dependent and the Mg^{2+}-dependent dehydratases. The Zn^{2+}-dependent enzymes include the ALADs from mammalian sources, which have 'pH optima' of between pH 6.3 and 7.1 and have been shown to require Zn^{2+} for maximal catalytic activity [9,10]. The yeast and *E. coli* enzymes can also be included in this class, requiring Zn^{2+} for activity but with more alkaline pH optima than their animal counterparts: 9.8 for the yeast enzyme and 8.5 for the enzyme from *E. coli* [6,11]. It should be noted, however, that the

Abbreviations used: ALA, 5-aminolaevulinic acid; ALAD, 5-aminolaevulinic acid dehydratase; CHES, 2-(*N*-cyclohexylamino)ethanesulphonic acid; DEP, diethyl pyrocarbonate; IAM, iodoacetamide; NEM, *N*-ethylmaleimide; o-phe, 1,10-phenanthroline; PBG, porphobilinogen; PLP, pyridoxal phosphate.

¶ To whom correspondence should be addressed.

differences in 'pH optima' between the enzymes might be misleading because investigators have measured 'rates' of reaction at fixed concentrations of substrate; this produces a mixture of the effects of changes in enzyme K_m and V_{max}. The animal, yeast and *E. coli* ALADs have a homo-octameric structure and have thiol groups that are extremely sensitive to oxidation. The oxidation of the thiol groups has been shown to be accompanied by a decrease in catalytic activity and a stoichiometric loss of bound metal ions [12], thereby demonstrating that the cysteine residues are required for Zn^{2+} binding. It has been established that ALADs from this class contain both catalytic and non-catalytic Zn^{2+} [13]. Techniques such as EXAFS predict that the non-catalytic Zn^{2+} has a tetrahedral co-ordination of at least two and often four cysteine residues. The catalytic Zn^{2+} can be bound in either a tetrahedral or pentaco-ordinate fashion with cysteine, histidine and often water as ligands [13–15].

The Mg^{2+}-dependent class of dehydratases includes the plant ALADs, which have been reported to have alkaline pH optima of approx. 8.0–8.5 [16], but again these values were determined by measurement of an average rate of reaction as described above. They have an absolute dependence on Mg^{2+} as well as subtle differences in their primary structure, especially in the putative metal-binding domains. In addition, some of the plant enzymes seem to be homohexameric and to be less sensitive to oxidation than their animal counterparts; consequently a minor role has been postulated for their thiol groups [17]. This may be due to the fact that the cysteine residues are not involved in metal chelation in the Mg^{2+}-dependent enzymes and their oxidation therefore does not lead to the loss of metal ions.

In an attempt to show definitive differences and/or similarities in the ALADs, a detailed study of the properties of the ALADs from *E. coli*, yeast and pea was undertaken. Evidence is presented supporting the hypothesis that the variances in metal binding between the enzymes are a reflection of significant biochemical differences that affect substrate recognition and binding and can therefore be used in the design of specific inhibitors. It is also revealed that the yeast enzyme, previously assumed to be solely Zn^{2+}-binding, is similar to the *E. coli* ALAD in that Mg^{2+} can be substituted at the catalytic site to restore enzyme activity although there is no stimulation of activity. Finally, the crystallization of the yeast ALAD is reported, which will permit the determination of the structure of the enzyme by X-ray diffraction methods.

MATERIALS AND METHODS

Materials

Perchloric acid was purchased from Aldrich Chemicals, Chelex 100 resin from Bio-Rad, and most other chemicals including yeast genomic DNA were purchased from Sigma (Poole, Dorset, U.K.). Chromatographic material and PD-10 columns were purchased from Pharmacia Biotech.

Growth of recombinant *E. coli* cells

All strains and plasmids used in this study are given in Table 1. Recombinant *E. coli* ALAD was purified from CR261, a TB1 strain containing pCAR261 (pUC19:*hemB*) [21]. Recombinant pea ALAD was purified from *E. coli* RP523 cells transformed with pS35M. This plasmid encodes for the mature form of the pea enzyme, expressing residues 35–398, and was constructed by cloning a modified version of preALAD-P [22] into pKK233-2 (M. P. Timko, personal communication). All bacteria were grown in baffled flasks with 800 ml of Luria broth containing 50 μg/ml ampicillin for 18 h at 37 °C in an orbital shaker at 200 rev./min. Cells were harvested by centrifugation at 10000 ***g*** for 15 min at room temperature and stored at −20 °C as a paste in gel-filtration buffer [50 mM potassium phosphate (pH 7.0) for *E. coli* ALAD or 50 mM 2-(*N*-cyclohexylamino)ethanesulphonic acid (CHES) (pH 8.5) for pea ALAD, containing 5 mM $MgCl_2$, 100 μM $ZnCl_2$ and 10 mM 2-mercaptoethanol]. Buffer (5 ml) was added per 4 g wet weight of cells. *E. coli* JM109 cells carrying pUC18 with the yeast *HEM2* gene (pNS1) were grown similarly except that the cells were induced with 500 μM isopropyl β-D-thiogalactoside 2 h before harvest. Cells (JM109/pNS1) were harvested and resuspended as described for the pea ALAD.

Table 1 List of strains and plasmids used in this study

Strain/plasmid	Genotype/properties	Reference
TB1	JM83 *hsdR* (r_k^- m_k^+)	[18]
RP523	C600 *hemB*, haem permeable	[19]
JM109	pJM107 *rec*A1	[20]
pCAR261	*E. coli hemB* cloned into pUC19	[21]
CR261 (TB1/pCAR261)	Overexpresses *E. coli* ALAD	[21]
pS35M	pea ALAD cDNA cloned into pKK233-3	M. P. Timko, unpublished work
ALAD-P (RP523/pS35M)	Overexpresses mature pea ALAD	M. P. Timko, unpublished work
pNS1	yeast *HEM2* cloned into pUC18	This study
NS1 (JM109/pNS1)	Overexpresses yeast ALAD	This study

Expression cassette PCR to amplify, clone and express yeast *HEM2*

The sequence of the yeast *HEM2* gene had been previously described [23] and specific PCR primers were synthesized corresponding to the 5′ and 3′ ends of the open reading frame to enable amplification of the *HEM2* coding sequence. To facilitate cloning and expression of this gene, the primers also incorporated features of an expression cassette system [24]. These features included a G/C clamp, a ribosome-binding site, transcription signals and a restriction enzyme site. PCR was performed with genomic yeast DNA (100 ng) as template, 10 μM primers and 40 cycles with a hybridization temperature of 60 °C.

The product of the PCR was analysed by agarose gel electrophoresis, and the approx. 1 kb band was extracted from the preparative 1.5 % NuSieve agarose gel with a GeneClean kit (Bio 101). The isolated amplified product was restricted with *Eco*RI and *Hin*dIII, and the expression cassette was ligated into pUC18 by using T4 DNA ligase. Plasmids containing the insert were identified after transformation of JM109 cells by blue/white colour selection on Luria broth/ampicillin/isopropyl β-D-thiogalactoside plates. Clones containing the correct-sized plasmid insert and demonstrating enhanced enzyme activity were sequenced with the Pharmacia random-labelling technique in accordance with the manufacturer's instructions.

Purification of *E. coli* ALAD

E. coli ALAD was purified by the method of Spencer and Jordan [6]. Fractions containing purified enzyme after Sephacryl S-300 gel-filtration chromatography were analysed by SDS/PAGE [10 % (w/v) gel]. Electrophoresis was performed at 40 mA for 30 min [25]. Active and pure fractions were pooled, filter-sterilized through a 0.22 μm filter and maintained at 4 °C.

Purification of yeast ALAD

The purification strategy developed for the recombinant yeast ALAD was based on that used for the *E. coli* enzyme. The cell paste from 2.4 litres of NS1 (15 ml) was thawed and the cells were disrupted by sonication in an ice bath with an MSE Soniprep Ultrasonic Disintegrator four times for 1 min each at an amplitude of 10 μm. The sample was cooled for 3 min between sonications. The resulting lysate was centrifuged at 10000 ***g*** for 10 min at 4 °C and the cell debris pellet discarded. The supernatant was heated to 50 °C for 5 min and the resulting precipitate was collected by centrifugation and discarded. The clarified solution was made 33 % (w/v) with respect to ammonium sulphate and was left at room temperature for 10 min. The precipitate was removed by centrifugation at 10000 ***g*** for 10 min at 4 °C. The supernatant was made 40 % (w/v) with respect to ammonium sulphate and, after being left at room temperature for 10 min, the precipitate was collected by centrifugation at 10000 ***g*** for 10 min at 4 °C. The resulting pellet was carefully resuspended in 3 ml of 25 mM CHES buffer, pH 9.0, containing 5 mM $MgCl_2$, 100 μM $ZnCl_2$ and 10 mM 2-mercaptoethanol, and was applied to a Sephacryl S-300 gel filtration column (100 cm × 3 cm) that had been pre-equilibrated with 2 litres of the same buffer. The sample was eluted overnight at 4 °C from the column in 2 litres of buffer and was collected in a series of 8 ml fractions. Active fractions were determined as described above. After gel filtration the active fractions were applied to a MonoQ column attached to a Biologic system and eluted in a gradient of 0–400 mM NaCl in 50 mM Tris/HCl, pH 8.0, containing 100 μM $ZnCl_2$, 5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. Pure samples were filter-sterilized through a 0.22 μm filter and maintained at 4 °C.

Purification of pea ALAD

Bacterial cells containing the recombinant pea ALAD (12 g in 15 ml of buffer) were disrupted by sonication as described for the yeast enzyme. The disrupted cells were centrifuged at 10000 ***g*** for 15 min at 4 °C to remove the cell debris. The supernatant was made 33 % (w/v) with respect to ammonium sulphate and the precipitated protein removed by centrifugation at 10000 ***g*** for 10 min at 4 °C. The supernatant was made 40 % (w/v) with respect to ammonium sulphate and the precipitated protein removed by centrifugation at 10000 ***g*** for 10 min at 4 °C. The supernatant was made 50 % (w/v) with respect to ammonium sulphate and the pellet was collected after centrifugation at 10000 ***g*** for 10 min at 4 °C. This pellet was resuspended in a minimum volume (approx. 3 ml) of 25 mM CHES buffer, pH 9.0, containing 5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The resuspended protein was applied to a column of Sephacryl S-300 (100 cm × 3 cm) that had been pre-equilibrated in the same buffer. The column was eluted with a further 2 litres of the same buffer and 8 ml fractions were collected. The purest fractions, as determined by SDS/PAGE, were applied to a Sephacel DEAE ion-exchange column (20 cm × 2 cm) pre-equilibrated in 50 mM Tris/HCl buffer, pH 8.0, containing 5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The enzyme was eluted at 4 °C in a gradient of 0–400 mM NaCl dissolved in the original Tris/HCl buffer (500 ml total volume). The fractions judged to contain most of the ALAD were saturated with ammonium sulphate to a final concentration of 33 % (w/v) and applied to a phenyl-agarose column (20 cm × 2 cm) that had been pre-equilibrated in the same buffer [50 mM Tris, pH 8.0, containing 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 33 % (w/v) ammonium sulphate]. The column was washed with 33 % (w/v) ammonium sulphate and the enzyme eluted in a gradient of 33–0 % (w/v) ammonium sulphate in Tris/HCl buffer (total volume 250 ml). Active fractions were assayed as before and the purity of fractions was judged by SDS/PAGE [10 % (w/v) gel] after overnight dialysis against salt-free CHES buffer to remove salt from the enzyme samples. The final step in the purification was column chromatography on Cibacron Blue resin (Sigma), based on a modification of a procedure described by M. P. Timko (personal communication). Active and pure fractions were pooled, filter-sterilized through a 0.22 μm filter and maintained at 4 °C.

Enzyme assay

ALAD activity was determined by the amount of PBG formed in the following assay. Enzyme (2–10 μg) was preincubated for 5 min at 37 °C in CHES buffer (25 mM, pH 9.0, containing 5 mM $MgCl_2$, 100 μM $ZnCl_2$ and 10 mM 2-mercaptoethanol). The reaction was started by the addition of ALA (5 mM final concentration) to give a final volume of 500 μl and proceeded for 5 min at 37 °C. The incubation was terminated by the addition of 0.1 M mercuric chloride (500 μl) in 10 % trichloroacetic acid. The mixture was centrifuged at 10000 ***g*** for 5 min at room temperature and 500 μl of the clear supernatant removed and reacted with an equal volume of Ehrlich's reagent [26]. The colour was left to develop for 15 min and PBG was detected spectrophotometrically by absorbance of the pyrrole at 555 nm with the use of a molar absorption coefficient for PBG of $6.02 \times 10^4\ M^{-1} \cdot cm^{-1}$.

Study of pH-dependent kinetics

A range of buffers varying by 0.2 pH unit were prepared within the pH range 6.0–10.2. Mes buffer was used for pH values between 6.0 and 7.0, Hepes between pH 7.0 and 8.4, and CHES between pH 8.4 and 10.2. All buffers contained 5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. A range of substrate concentrations between 0.02 and 0.4 mM ALA for *E. coli* ALAD, 0.05 and 1 mM for the yeast enzyme and 0.5 and 4 mM for the pea enzyme was prepared in the buffer solutions. Enzyme (2 μg) was assayed as described above. The results obtained were checked for adherence to Michaelis–Menten kinetics by constructing plots of initial rate (v) against substrate concentration [S], [S]/v against [S] (Hanes–Woolf) and finally a direct linear plot of rate (v) and substrate concentration [S] (Eisenthal–Cornish-Bowden), with the results plotted in parameter space rather than the conventional observation space.

These results were analysed with SIGMAPLOT 4.1 (Jandel Scientific) and a Tandon MCS 486/33 computer to generate values of K_m and k_{cat} at each pH. An error structure of constant relative error was assumed and weighting factors were inversely proportional to v^2. Values of the characterizing parameters of the pH-dependent kinetics (macroscopic pK_a values and pH-independent values of the rate constants, $\tilde{k}_{cat}/\tilde{K}_m$ and $\tilde{k}_{cat}$) were obtained by using SKETCHER [27,28], a multitasking application program written in ANSI C running under RISCOS on an Acorn Archimedes. Chemical modification reactions targeted at the side chains of cysteine, lysine and histidine residues were performed as follows: enzyme (2 μg) was suspended in a total volume of 450 μl of 50 mM Tris buffer, pH 7.5, containing a range of concentrations of reagent; at intervals between 1 min and 2 h enzyme samples were removed, ALA was added to a final concentration of 5 mM and enzyme activity was assayed as before. This method was used with iodoacetamide (IAM), *N*-ethylmaleimide (NEM) and pyridoxal phosphate (PLP), which were prepared in aqueous solution. Diethyl pyro-

carbonate (DEP) stock solutions were prepared in ethanol and inactivation was performed in 50 mM potassium phosphate buffer, pH 6.2. At intervals, aliquots were removed, treated with 20 mM final concentration of imidazole and assayed in the usual way.

Analysis of metal binding by *E. coli*, yeast and pea ALADs

The metal dependence of the enzymes was determined by the method of Mitchell and Jaffe [7]. Enzymes were incubated in 50 mM potassium phosphate buffer, pH 7.5, at 37 °C for 10 min in the presence of 10 mM 1,10-phenanthroline (o-phe) for the *E. coli* enzyme and 0.5 mM o-phe for the yeast enzyme. The minimum concentration of o-phe was used to remove the bound metal. Solutions of o-phe were prepared fresh in ethanol. After o-phe treatment, enzymes were incubated with a range of Mg^{2+} and Zn^{2+} concentrations for a further 10 min at 37 °C and assayed for activity in the usual way.

Metal binding stoichiometries were determined as follows: purified enzymes were applied to a PD-10 gel filtration desalting column, eluted in 50 mM CHES (pH 9.0)/10 mM 2-mercaptoethanol, prepared in thoroughly degassed analytical quality water (BDH) and loaded into dialysis tubing. After overnight dialysis in the same buffer, enzyme samples were assayed with the standard method. The metal content of the enzymes was confirmed by atomic absorption spectroscopy against standard solutions of Mg^{2+} and Zn^{2+}.

Atomic absorption spectroscopy

Samples for Zn^{2+} and Mg^{2+} determination were injected into an Instrumentation Laboratory 157 atomic-absorption spectrometer after treatment as described above. Concentrations of metal ions were determined with reference to a previously constructed standard curve and samples were diluted to bring them into the linear range of 5–30 μM for Zn^{2+} and 5–40 μM for Mg^{2+}.

Investigation of putative ALAD inhibitors

Inhibitors were identified by microtitre screening of potential target molecules which had been selected using similarity searching. Thus a range of compounds with some structural similarity to ALA were selected and screened for inhibitor action as described below. Microtitre screening followed the standard enzyme assay system, except that enzymes were preincubated with inhibitor (2 p.p.m.) before the addition of ALA. 2-Mercaptoethanol was omitted from thoroughly degassed buffers, permitting the addition of Ehrlich's reagent directly to the reaction mixture without a precipitation step. Dilutions were performed with Titertek automatic pipetting instruments and plates were read at 550 nm with a Dynatech 1000.

After the screening, potential inhibitors were studied in greater detail. *E. coli* enzyme (2 μg), yeast enzyme (2 μg) or pea enzyme (100 μg) was incubated with a range of inhibitor concentrations between 10 nM and 10 mM for 30 min at room temperature in a buffer of 50 mM CHES, pH 9.0, containing 50 μM $MgCl_2$ and 10 mM 2-mercaptoethanol in a final volume of 500 μl. ALA was added to a concentration of 5 mM and the enzyme was assayed with the standard method. K_i values for competitive inhibitors were determined by repeating the experiment at two selected inhibitor concentrations that gave approx. 50 % and 75 % inhibition under the conditions used. The enzymes were incubated with inhibitor concentrations in the presence of a range of ALA concentrations of between 0.02 and 0.4 mM for *E. coli* ALAD, 0.05 and 1 mM for the yeast enzyme, and 0.5 and 4 mM for pea ALAD. The formation of PBG was measured as described above.

Dilution experiments were used to differentiate between covalent and non-covalent interactions of enzyme with inhibitors. In these experiments, the enzymes were incubated with a concentration of inhibitor known to cause approx. 50 % inhibition. The inhibitor–enzyme complex was then diluted 100-fold and the activity was monitored at time points between 5 min and 4 h.

Schiff base trapping

Enzymes (20 μg) in a volume of 20 μl were incubated with a single crystal of NaB^3H_4 in the presence of CHES buffer (approx. 250 mM), pH 8.0, and ALA (5 mM). After mixing, unlabelled $NaBH_4$ (5 μmol) was added and two further aliquots of this solution were added over the next 30 min. Reactions were subjected to SDS/PAGE [10 % (w/v) gel] and after staining, brief destaining and Autofluor (National Diagnostics) treatment, autoradiographs were taken.

Crystallization

The yeast enzyme was crystallized by the vapour-diffusion method [29]. The enzyme was concentrated to 5 mg/ml and the protein was used in hanging-drop trials to ascertain the optimum conditions for crystallization.

RESULTS AND DISCUSSION

Cloning and expression of yeast ALAD

Recent findings have shown that the *E. coli* ALAD is a Zn^{2+}-dependent enzyme that is stimulated by Mg^{2+} [6,7], whereas the pea ALAD is solely a Mg^{2+}-dependent enzyme (M. P. Timko,

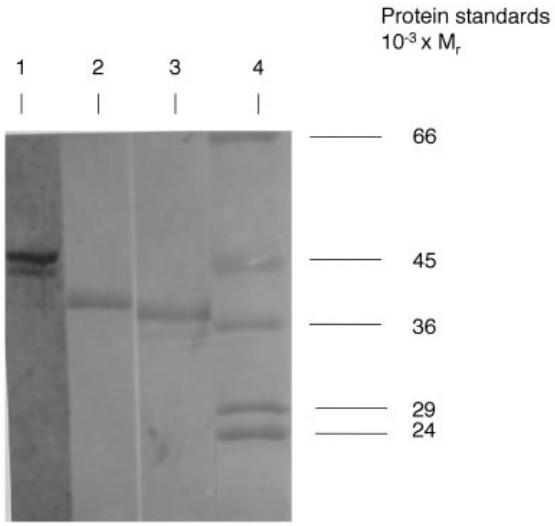


Figure 1 Three purified recombinant ALADs

Lane 1, 10 μg of the recombinant pea ALAD; lane 2, 10 μg of purified recombinant yeast ALAD; lane 3, 10 μg of recombinant *E. coli* ALAD; lane 4, molecular-mass markers as shown. Both the *E. coli* and yeast proteins ran as single bands with M_r values of approx. 35 000 and approx. 37 000 respectively, whereas the pea enzyme migrated as a doublet with an M_r of approx. 43 000.

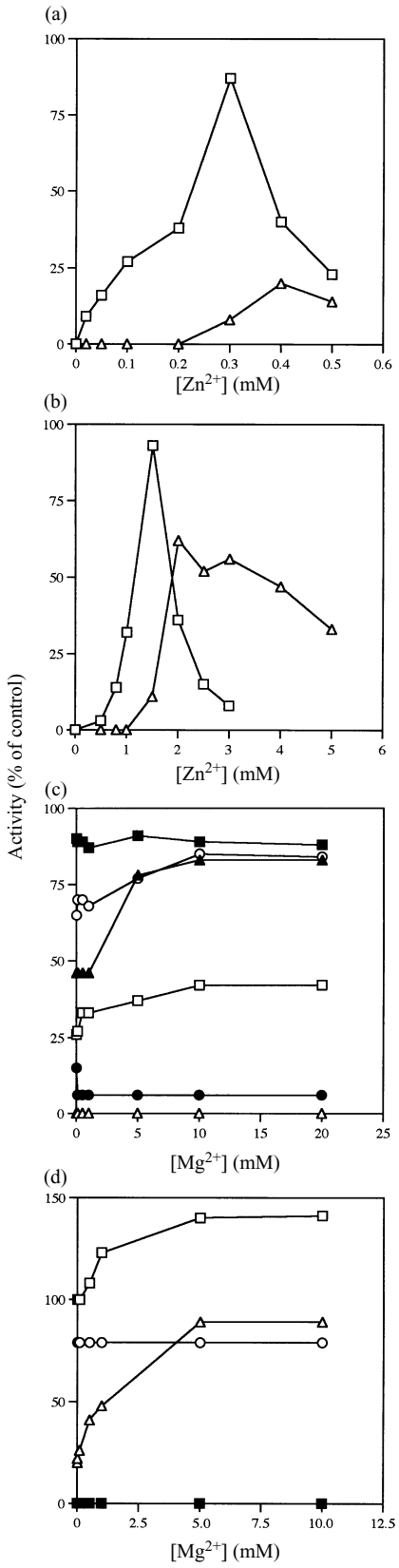


Figure 2 Effect of Zn^{2+} and Mg^{2+} on the activity of the yeast and *E. coli* ALADs

The two enzymes were treated with the minimum concentration of o-phe required to chelate all bound metals. For the *E. coli* enzyme this was determined as 10 mM, whereas a concentration of 0.5 mM was required for the yeast protein. Metal ions (either Mg^{2+} or Zn^{2+} at the concentrations shown) were subsequently added back to the enzymes under reducing conditions at the pH indicated and the activity was monitored. Activity is expressed as percentage of control, where the control represents enzyme that was not treated with o-phe. The control samples were assayed in the presence of a full Zn^{2+} complement. (**a**) The effect of exogenous Zn^{2+} on the activity of apo-(yeast ALAD). The activity was monitored at pH 6.0 (△) and 8.0 (□) to gauge the ability of the yeast enzyme to occupy the β metal-binding site with Zn^{2+}. (**b**) The effect of exogenous Zn^{2+} on the activity of apo *E. coli* ALAD, at pH 6.0 (△) and 8.0 (□). The profile can be contrasted with (**a**). (**c**) The effect of Mg^{2+} on the activity of yeast ALAD in the presence of fixed concentrations of Zn^{2+}: △, 0 mM Zn^{2+}; □, 0.02 mM Zn^{2+}, pH 8.0; ▲, 0.05 mM Zn^{2+}, pH 8.0; ○, 0.1 mM Zn^{2+}, pH 8.0; ■, 0.3 mM Zn^{2+}, pH 8.0; ●, 0.3 mM Zn^{2+}, pH 6.0. (**d**) The effect of Mg^{2+} on the activity of *E. coli* ALAD in the presence of fixed concentrations of Zn^{2+}: ■, 0 mM Zn^{2+}; □, 1.5 mM Zn^{2+}, pH 8.0; △, 1.0 mM Zn^{2+}, pH 8.0; ○, 1.5 mM Zn^{2+}, pH 6.0.

personal communication). The yeast enzyme was cloned and overexpressed to allow a further comparison of the properties of an ALAD that was thought to be solely Zn^{2+}-dependent [11].

The cloning and sequencing of the *HEM2* gene encoding ALAD from *Saccharomyces cerevisiae* has previously been described [23]. Overexpression had been attained by cloning the *HEM2* gene into a multicopy plasmid, YEp24, which produced a 20-fold elevation of enzyme activity when transformed into yeast cells. The protein could be purified to near-homogeneity from recombinant yeast cells although the quantity of protein obtained was quite low (less than 200 μg per litre of culture). The purification did, however, allow some properties of the purified protein to be investigated, including basic kinetic parameters, metal requirement and molecular mass [11]. To produce the quantities of protein required in this study, the yeast gene was cloned into a vector system enabling overexpression in *E. coli*. Thus the yeast *HEM2* was incorporated into a prokaryotic expression cassette by PCR [24] and cloned into pUC18. The resulting plasmid, pNS1, gave rise to strain NS1 when transformed into *E. coli* JM109, and was seen to overexpress a protein band of the expected size when cell extracts were analysed by SDS/PAGE (results not shown). The recombinant yeast ALAD was purified from this strain by differential ammonium sulphate precipitation, Sephacryl S-300 gel filtration and high-resolution ion-exchange chromatography with a MonoQ HR10/10 column as described in the Materials and methods section. Approx. 10 mg of purified protein was obtained from 1 litre of JM109/pNS1, which is significantly higher than the overexpression system previously reported [11]. The purified protein had a M_r of 37000 when analysed by SDS/PAGE (Figure 1), a native M_r of 240000 as determined by gel filtration (results not shown) and a specific activity of 3.6 μmol PBG/h per mg. The specific activity is lower than that reported [11] but the reasons for the discrepancy in this value are unclear.

Yeast ALAD metal binding

The *E. coli* ALAD has been reported to bind two Zn^{2+} ions per subunit under reducing conditions [6,7]. The two ions bind at separate sites, termed α and β [30]. For catalytic activity the α site has to be occupied by Zn^{2+} but this site can also be replaced by Mg^{2+} if Zn^{2+} is present at the β site [30]. Furthermore, there is evidence for a third metal-binding site specifically for Mg^{2+}, termed Mg_c [15]. In contrast, the pea ALAD is exclusively Mg^{2+}-dependent ([22], and M. P. Timko, personal communication) and the enzyme is inactive in the presence of Zn^{2+}. Previous work on the yeast ALAD had shown it to be Zn^{2+}-dependent [11] but the stoichiometry of metal binding and the effect of Mg^{2+} had not been investigated. The latter points were addressed with protein

Table 2 Stoichiometry of metal binding

The activity of yeast and *E. coli* ALAD that had been treated with o-phe to generate apo-protein (see the Materials and methods section) was compared (a) directly, (b) after the addition of optimal zinc and (c) in the presence of suboptimal zinc and excess magnesium. The optimal and suboptimal concentrations of zinc used in these experiments are reported in Figure 2. Protein and metal concentrations were determined after removal of exogenous, unbound, metal by gel filtration.

Enzyme sample	Mol of metal per mol of enzyme	Specific activity (μmol of PBG/h per mg of protein)	Enzyme K_m (mM)
E. coli ALAD			
(a) o-phe-treated	0 $Zn + 0$ Mg^{2+}	0	–
(b) Optimal Zn^{2+}	15.35 ± 0.59 Zn^{2+}	34.08 ± 0.50	0.10
(c) Suboptimal $Zn^{2+} + Mg^{2+}$	7.72 ± 0.71 $Zn^{2+} + 8.72 \pm 0.84$ Mg^{2+}	47.84 ± 0.50	0.10
Yeast ALAD			
(a) o-phe-treated	0 $Zn^{2+} + 0$ Mg^{2+}	0	–
(b) Optimal Zn	16.06 ± 0.22 Zn^{2+}	3.59 ± 0.10	0.25
(c) Suboptimal Zn + Mg	7.33 ± 0.48 $Zn^{2+} + 7.95 \pm 0.35$ Mg^{2+}	3.09 ± 0.10	0.25

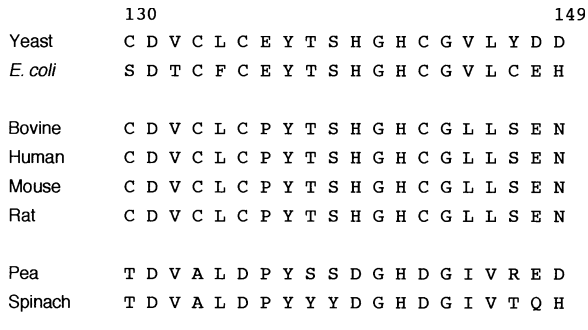


Figure 3 Alignment of a putative metal-binding domain

A pileup of the metal-binding sites, located between amino acid residues 130 and 149 (numbers refer to the yeast ALAD sequence). The metal-binding site in the yeast enzyme has a greater similarity to the mammalian metal binding sites than that found in *E. coli* ALAD.

purified from NS1 (JM109/pNS1; Table 1). The yeast ALAD was treated with o-phe, a Zn^{2+} chelator, under conditions enabling metal chelation [7]. The activity of the enzyme in increasing concentrations of exogenous Zn^{2+} was then determined and the results are shown graphically in Figure 2(a); for comparison a similar set of experiments performed on the *E. coli* enzyme is shown in Figure 2(b). Zn^{2+} was added to the enzyme at pH 6.0 and 8.0 as it is known that at pH 6.0 in the *E. coli* ALAD the β site is preferentially occupied, whereas at pH 8.0 the α site is preferentially occupied [30]. Two observations can be made from these experiments. First, a lower concentration of o-phe is required to remove the Zn^{2+} from the yeast enzyme: 0.1 mM compared with 10 mM for *E. coli* ALAD. This would suggest that either the metal is more tightly bound in the *E. coli* enzyme or that the metal is more accessible in the yeast ALAD. A lower concentration of Zn^{2+} is also required to reactivate the apo (metal-free) yeast ALAD, 0.3 mM as opposed to 1.5 mM, but this is probably a reflection of the higher endogenous o-phe concentration present in the incubation of the *E. coli* enzyme. Secondly, at pH 6.0, the activity of the yeast enzyme after Zn^{2+} had been added was much lower than that observed with the *E. coli* enzyme. This suggests that the occupation of the β site in the yeast enzyme is lower than that in the *E. coli* enzyme, reflecting a subtle structural difference.

The stoichiometry of Zn^{2+} binding was investigated by taking protein that had been purified in the presence of Zn^{2+}, removing the exogenous unbound metal ions by gel filtration as described in the Materials and methods section, and determining the concentration of protein and Zn^{2+} independently. The results, shown in Table 2, demonstrate that the yeast enzyme is associated with approx. two Zn^{2+} ions per protein subunit. The stoichiometry of metal binding in the yeast enzyme is very similar to that observed with the *E. coli* ALAD.

The effect of Mg^{2+} on the activity of yeast ALAD was also investigated. The enzyme, which had been treated with o-phe, was assayed for activity in the presence of fixed quantities of Zn^{2+} and variable amounts of Mg^{2+}. The results of these experiments are shown in Figure 2(c), and a comparison with the *E. coli* enzyme is shown in Figure 2(d). In the absence of exogenous and endogenous Zn^{2+}, both the *E. coli* and yeast ALAD display no enzymic activity. If 0.3 mM Zn^{2+} (optimal) is added to the apo-(yeast ALAD), addition of Mg^{2+} has no effect on activity. However, if the enzyme is assayed in the presence of suboptimal concentrations of Zn^{2+}, i.e. concentrations below 0.3 mM, addition of Mg^{2+} increases the activity of the enzyme so that it is restored to a near-maximal level. Collectively these results suggest that the yeast enzyme has two metal-binding sites, one of which has to be bound with Zn^{2+}. The second site can also be filled with Zn^{2+} but it can be replaced by Mg^{2+}. A similar observation has been made with the effect of Mg^{2+} on the *E. coli* ALAD [6,7]. However, a major difference between the yeast and *E. coli* enzymes is that if the *E. coli* enzyme is assayed in the presence of Mg^{2+} and the optimal concentration of Zn^{2+} (1.5 mM), further stimulation of enzyme activity is observed, suggesting that the *E. coli* enzyme has a third, stimulatory, metal-binding site for Mg^{2+} (Mg_c), in agreement with Jaffe [15]. In both cases, Mg^{2+} exerts its effect only at pH 8, the optimal pH for occupancy of the α site; no binding is observed at pH 6.0, the pH associated with occupancy of the β site. Mg^{2+} did not affect the K_m of the enzymes (Table 2).

The stoichiometry of Mg^{2+} binding to the yeast ALAD was also investigated as described for Zn^{2+} binding above. The results of this are shown in Table 2 and suggest that 8 mol of Mg^{2+} bind per mol of octamer, i.e. one Mg^{2+} per subunit. Again, this supports the theory that Mg^{2+} is able to occupy only one site (α) on the yeast ALAD subunit.

An examination of the primary structure of the yeast ALAD reveals that the enzyme clearly contains the putative Zn^{2+}-

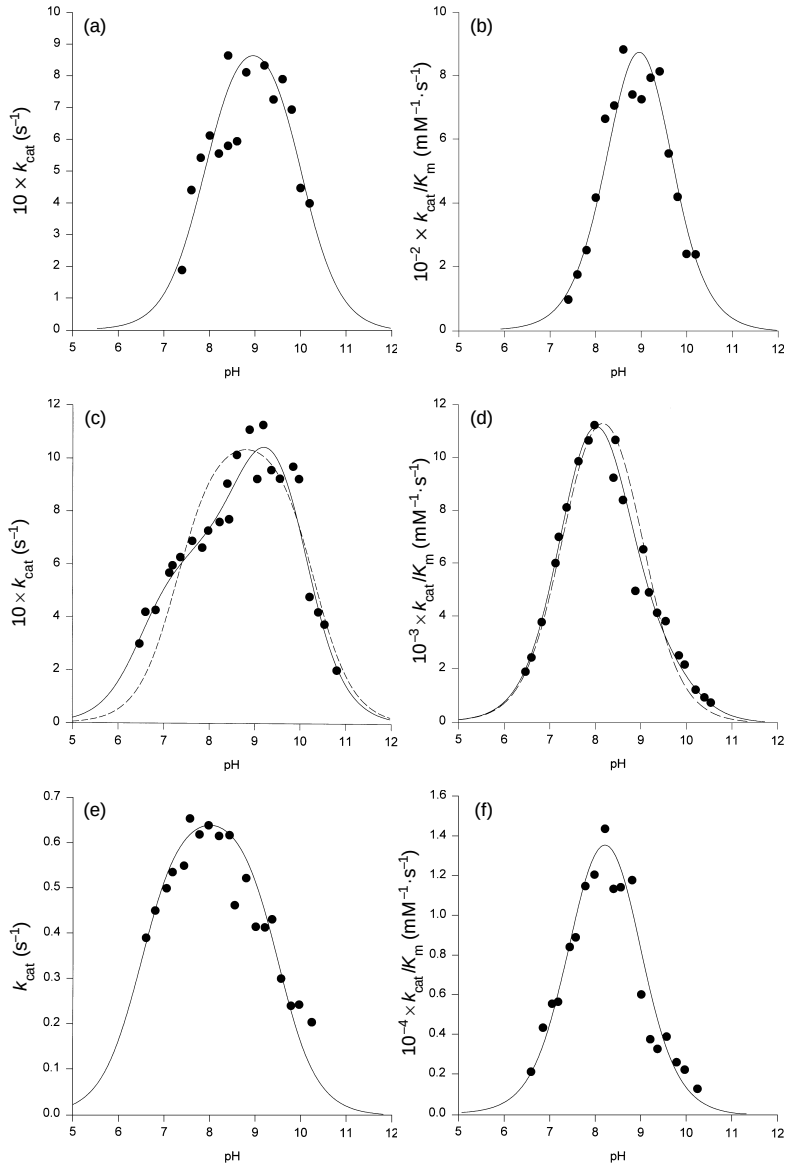


Figure 4 Effect of pH on k_{cat} and k_{cat}/K_m

The data points in the enzymes' active pH ranges (6.0–10.2 for *E. coli*, 6.4–10.8 for yeast and 7.4–10.2 for pea ALAD) were determined by measuring v (PBG formation) as described in the Materials and methods section. Measurements were taken such that $[S]_0 K_m$ ($K_m \approx 0.1$ mM for *E. coli*, $K_m \approx 0.25$ mM for yeast and $K_m \approx 1.0$ mM for pea over the active pH range). Values of k_{cat} and K_m were obtained from these results (see the Materials and methods section) and these were used to generate the points shown in (**a**–**f**). The continuous lines in (**a**), (**b**), (**e**) and (**f**) and the broken lines in (**c**) and (**d**) are theoretical for a reaction in one active protonic state; the continuous lines in (**c**) and (**d**) are theoretical for a reaction in two active protonic states; the characterizing parameters for the lines in (**a**–**f**) are given in Table 3. (**a**) Effect of pH on the turnover (k_{cat}) of the pea ALAD. (**b**) Effect of pH on the ratio k_{cat}/K_m of the pea ALAD. (**c**) Effect of pH on the turnover (k_{cat}) of the yeast ALAD. (**d**) Effect of pH on the ratio k_{cat}/K_m of the yeast ALAD. (**e**) Effect of pH on the turnover (k_{cat}) of the *E. coli* ALAD. (**f**) Effect of pH on the ratio k_{cat}/K_m of the *E. coli* ALAD.

binding arrangement of cysteine and histidine residues as found in human and bovine enzymes between residues 130 and 149 (Figure 3). However, the yeast sequence throughout also has a few changes from the mammalian enzymes, substituting instead residues that are found in the *E. coli* and plant ALAD sequences. The yeast primary structure contains no obvious Mg^{2+}-binding site so whether the yeast enzyme is able to bind Mg^{2+} as a result of these changes remains to be seen. If this is so, the earlier assumption that the ability of the *E. coli* enzyme to bind Mg^{2+} is a result of the change of a cysteine to a serine residue at position 229 in the *E. coli* ALAD [30] might prove to be over-simplistic, as the yeast enzyme has a cysteine residue at this equivalent position (position 234 in the yeast sequence).

pH-dependent kinetics

The status of the proton as the least sterically demanding pertubant of protein structure and the importance of the protonation state of specific side chains in enzyme function combine to make properly designed and carefully interpreted pH-dependent kinetic studies one of the most useful approaches to probing catalytic function [31,32]. The principal experimental

Table 3 Characteristics of the pH-dependence of k_{cat} and k_{cat}/K_m for the reactions catalysed by ALAD from pea, yeast and *E. coli*

Enzyme source	Free enzyme pK_a values		k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	Enzyme–substrate complex pK_a values		k_{cat} (s^{-1})	pH–rate constant profiles
	pK_1	pK_2		pK_1	pK_2		
Pea	8.4	9.5	1.37×10^{-2}	7.9	10.0	1.02	Figures 4(a) and 4(b)
Yeast	7.3*	9.0*	14.5	7.3†	10.3†	1.1†	Figures 4(c) and 4(d)
E. coli	7.5	8.9	19.0	6.5	9.5	0.68	Figures 4(e) and 4(f)

* The fit to the pH–k_{cat}/K_m results seems to be improved by using a three-pK_a rate equation (Figure 4d, continuous line): pK_1 = 7.3; pK_2 = 8.6; pK_3 = 10.0; k_{cat1}/K_{m1} = 15.5 $M^{-1} \cdot s^{-1}$; k_{cat2}/K_{m2} = 3.0 $M^{-1} \cdot s^{-1}$.

† The fit to the pH–k_{cat} results is clearly improved by using a three-pK_a rate equation (Figure 4c, continuous line): pK_1 = 6.5; pK_2 = 8.7; pK_3 = 10.1; k_{cat1} = 0.65 s^{-1}; k_{cat2} = 1.3 s^{-1}.

Table 4 The effect of protein-modifying reagents on the activity of the three ALADs

The effect of amino acid-modifying reagents on the activity of the three ALADs was monitored to support the assignment of amino acids deduced from the observed pK_a values given in Table 3. The concentrations of the reagents that gave rise to 50% inactivation of each enzyme are given.

Protein-modifying reagent	Likely site of modification	Pea ALAD K_m (mM)	*E. coli* ALAD K_m (mM)	Yeast ALAD K_m (mM)
DEP	Histidine	0.2	0.02	2.0
IAM	Cysteine	3.0	0.9	6.0
NEM	Cysteine	5.0	7.5	7.5
PLP	Lysine	0.1	0.6	0.1

objectives of such studies are the identification and determination of the characteristics (pK_a values approximating to those of individual ionizing groups and rate constants characteristic of the reaction of specific ionic forms of the reactants) of the kinetically influential ionizations.

The pH-dependences of k_{cat}/K_m and k_{cat} for the reactions catalysed by the ALADs from pea, *E. coli* and yeast are shown in Figure 4, and values of the characterizing parameters (macroscopic pK_a values and pH-independent rate parameters, k_{cat}/K_m and k_{cat}) are compared in Table 3. The pH-dependence of k_{cat}/K_m provides pK_a values of the free reactant state, i.e. of the free enzyme molecule when the substrate is either non-ionizing or (perhaps less securely) as an ionizing group with a pK_a value far removed from those observed kinetically.

The results for the ALADs from both pea and *E. coli* can be fitted to classical bell-shaped curves. The two sets of free enzyme pK_a values are 8.4 and 7.5 for pK_1 and 9.5 and 8.9 for pK_2. For both of these enzymes, formation of the enzyme–substrate complexes results in a decrease in the value of pK_1 (by 0.5 for the pea enzyme and by 1.0 for the *E. coli* enzyme). The pK_a values of the free enzyme molecules, taken together with the results of the chemical modification studies described below, are consistent with the tentative assignment of pK_1 to a cysteinyl thiol group and of pK_2 to a lysine alkyl ammonium ion. In terms of these suggested assignments the formation of the enzyme–substrate complex would seem to stabilize the essential thiolate anion (with a decrease in pK_1) and stabilize the essential alkyl ammonium cation (with an increase in pK_2). These changes are consistent with the generation of a less hydrophobic environment and/or the provision of additional hydrogen-bonding capability consequent on formation of the complex. A pH/activity relationship of the *E. coli* enzyme together with two histidine mutant variants has recently been reported by Mitchell et al. [33], who concluded that pK_1 was not assignable to a histidine residue.

The pH-dependence characteristics of the yeast enzyme are noticeably different in that the fit of the results to simple bell-shaped curves, particularly for k_{cat} (Figure 4c) but also to a lesser extent for k_{cat}/K_m (Figure 4d), is not convincing. The additional pK_a suggested particularly by the results shown in Figure 4(c) might be associated with an essential histidine imidazolium ion, in agreement with the evidence from the chemical modification studies (see below).

If the three pK_a fits do describe the behaviour of the yeast enzyme, the apparent lack of change in pK_2 and pK_3 consequent on enzyme–substrate complex formation suggests that the change in environment deduced from the pea and *E. coli* enzymes might not occur in the yeast enzyme.

In view of the uncertainty in the analysis of Figure 4(d) and the probable presence of an additional pK_a in Figure 4(c), detailed interpretation of the pH-dependence characteristics of the yeast enzyme must await further investigation. In terms of specificity ratio the yeast enzyme is similar to the *E. coli* enzyme in being much larger than the pea enzyme.

The ionizing residues suggested by the pH-dependent kinetic study are residues that have been proposed from mechanistic studies with protein-modifying reagents. Thus roles for histidine, cysteine and lysine residues have been implied by previous work [34–37]. To confirm that these residues are important in the catalytic cycle of the three ALADs used in this study, the proteins were allowed to react with different concentrations of amino acid-modifying reagents as shown in Table 4. The results show clearly that DEP inhibits all three dehydratases, although the *E. coli* enzyme is more susceptible to modification. The effect of DEP was reversible if the modified protein was incubated with 800 mM hydroxylamine (results not shown).

All the dehydratases were susceptible to inactivation by the thiol-directed reagents IAM and NEM. As the role of cysteine groups in the spinach enzyme had been previously questioned [17], it was interesting to note that the pea enzyme was more susceptible to alkylation with IAM than was the yeast enzyme. The assignment of a pK_a to a cysteine residue in a plant dehydratase reaffirms the idea that such an active-site cysteine might be important in the catalytic mechanism as has been suggested for ALAD and other enzymes [34,38].

Lastly, all three enzymes were inhibited by PLP. This reagent is known to react with lysine residues and PLP is a known inhibitor of the active-site lysine of ALAD. Because the active site lysine residue is conserved in the primary structure of all ALADs it is likely that the pK_a of 9.5–10 can be attributed to the

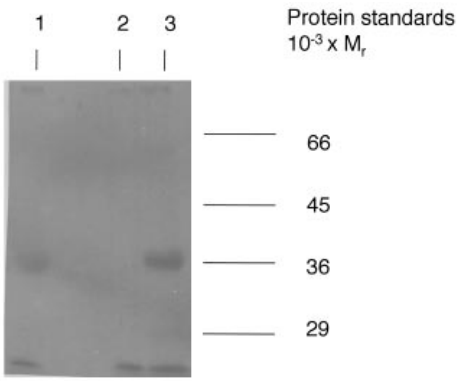


Figure 5 Evidence for a Schiff base by reduction of the yeast ALA/ALAD complex with NaB^3H_4

The figure shows an autoradiogram of an SDS-polyacrylamide gel containing 10 μg of an *E. coli* ALAD/ALA mix that had been reduced with NaB^3H_4 (lane 1), 10 μg of yeast ALAD that had been reduced with NaB^3H_4 (lane 2) or 10 μg of yeast ALAD/ALA mix that had been reduced with NaB^3H_4 (lane 3). Only enzyme in the presence of ALA incorporated radioactivity (lanes 1 and 3).

deprotonation of the lysine group. Evidence that the yeast enzyme mechanism proceeds via Schiff-base formation with ALA was obtained from an autoradiogram of the enzyme–substrate complex that had been reduced with NaB^3H_4 (Figure 5).

Identification of inhibitors

Systematic studies with inhibitors have been performed on ALADs isolated from *R. sphaeroides* [39] and bovine liver [40], and studies on lone inhibitors [36,41,42] have also been reported with ALADs from these and other sources. However, there are differences between these studies because investigators have used different experimental approaches to characterize the inhibitory mechanism, and no comparison of the effects of inhibitors on ALADs from different sources has previously been made. In this study we looked at the effect of a range of inhibitors that had previously been identified by others, as well as a number of compounds that were identified from a screening of potential inhibitors, as outlined in the Materials and methods section.

The list of inhibitors used in this study is shown in Table 5. Laevulinic acid had been previously shown to act as a competitive inhibitor of the ALADs from, among others, *R. sphaeroides* (K_i 1 mM) [36,39] and bovine liver (K_i 2 mM) [40]. When assayed with the three enzymes (from yeast, pea and *E. coli*), laevulinic acid acted as a competitive inhibitor with a similar K_i value for each enzyme. This result indicated that the presence of a terminal amino group on the substrate molecule is not essential for binding to the enzyme. Studies were also conducted with 3,5-dibromolaevulinic acid: similar results were found to those obtained with laevulinic acid, demonstrating competitive inhibition of the enzymes with no apparent alkylation because inhibition was fully reversible. The effect of haloketone analogues has been investigated previously [43–45]. Such derivatives were found to act as active-site alkylating agents and studies by Jaffe [45] showed that 5-chlorolaevulinic acid had a differential effect on binding bovine and *E. coli* ALADs, presumably reflecting differences in the arrangement of cysteine residues at their active sites.

Succinylacetone (4,6-dioxoheptanoic acid) also acts as a competitive inhibitor but displays an enormous differential inhibitory activity. The inhibitor is much more potent against the pea enzyme with a K_i of 0.3 nM, which is three orders of magnitude smaller than the K_i observed with the *E. coli* ALAD. The differential inhibitory effect might reflect the binding of the inhibitor to Mg^{2+} but inhibition is not the result of Mg^{2+} chelation alone because an increase in the concentration of Mg^{2+} in the buffer from 1 mM to 20 mM did not significantly affect the amount of inhibition obtained. In addition, no change was seen between the inhibition of the Zn^{2+}/Zn^{2+} and Zn^{2+}/Mg^{2+} species of the yeast and *E. coli* enzymes. Furthermore dioxovaleric acid, which is also a potential Mg^{2+} chelator, is not as potent an inhibitor as succinylacetone nor does it show the same preference for the pea enzyme. Inhibition by succinylacetone is time-dependent and the inhibitor was able to form a covalent bond with the enzyme that was reducible with sodium borohydride (results not shown). Succinylacetone was originally identified as an inhibitor of haem biosynthesis in malignant murine erythroleukaemia cells [42] but it has since been suggested that succinylacetone pyrrole, formed by reaction of succinylacetone with ALA, rather than succinylacetone, is the active compound [46]. To investigate this, the pyrrole analogue was synthesized and its effect on the three ALADs tested. The pyrrole showed no inhibitory action, suggesting that succinylacetone is the inhibitory molecule.

A common epitope shared between these inhibitors and the substrate (ALA) is the succinyl moiety and, with this in mind, succinic acid was tested against the enzymes, but minimal inhibition was found: the K_i for all three enzymes was in excess of 20 mM. In addition, a difluoro derivative of succinic acid was also tested that showed competitive inhibition of the enzymes, with a small preference for the pea enzyme, but the affinity of the inhibitor for the enzyme was not very high.

The effect of protoporphyrin IX on the activity of the enzymes was investigated because this late-porphyrin-pathway intermediate has been reported to act as a pathway regulator in previous studies [47]. When incubated with the ALADs, protoporphyrin was indeed found to act as a competitive inhibitor of the enzymes, with K_i values for the *E. coli* and yeast enzymes of 0.25 and 2.50 mM respectively, but its K_i for the pea enzyme, 0.012 mM, was significantly less. This would suggest that tetrapyrrole biosynthesis, and particularly the biosynthesis of chlorophyll in the pea plant, may be controlled partly by feedback inhibition by protoporphyrin IX.

By selecting from the databases a range of compounds that fitted some of the substrate characteristics it was possible to perform a rapid screening to identify new inhibitors. In this way 4-keto-5-aminohexanoic acid was identified as an inhibitor. This compound displays uncompetitive inhibitory kinetics yet covalently modifies the protein. In fact, close scrutiny of the literature reveals that 4-keto-5-aminohexanoic acid was identified as a competitive inhibitor of the *Propionibacterium shermanii* enzyme, with a K_i of 12.1 μM [48]. This inhibitor seems to behave differently with the *P. shermanii* enzyme. The monomethyl ester of succinic acid was also found to inactivate the ALADs in an uncompetitive way. These are the first reports of uncompetitive inhibitors for ALADs.

The screening identified a number of other inhibitors, including a variety of dicarboxylic acids, that acted as competitive inhibitors. It was observed that as the chain length of the molecule was increased to ten carbons from the original five carbons, the kinetics changed from competitive to non-competitive inhibition. The long-chain inhibitors probably act in a non-specific way because they are able to bind to hydrophobic parts of the enzymes in a surfactant-like manner. The competitive dicarboxylic acid inhibitors have broadly similar inhibitory properties. It could be that the dicarboxylic acids are able to

Table 5 List of inhibitors tested with the three ALADs

A number of compounds that were found to inhibit the enzymes are shown. The K_i for each inhibitor is given together with the type of inhibition that was observed kinetically. Covalent binding of inhibitors to enzymes was investigated by dilution experiments and the presence of an imine linkage was investigated by reduction with $NaBH_4$.

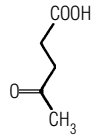
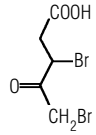
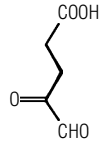
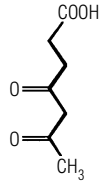
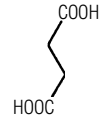
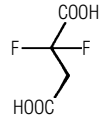
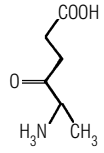
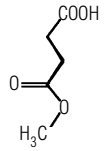
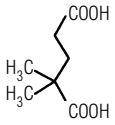
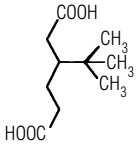
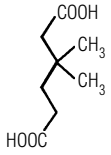
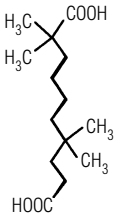
Inhibitor	Structure	Pea K_i (mM)	Yeast K_i (mM)	*E. coli* K_i (mM)	Kinetics	Nature of bond	Evidence for imine
Laevulinic acid		1.0	2.0	2.7	competitive	covalent	yes
DL-3,5-Dibromo-laevulinic acid		1.0	1.2	2.0	competitive	covalent	yes
Dioxovaleric acid		1.0	not determined	8.0	not determined	not determined	not determined
Succinyl acetone (4,6-dioxohept-anoic acid)		0.3×10^{-3}	0.78	0.80	competitive	covalent	yes
Succinic acid		> 20	> 20	> 20	not applicable	not applicable	not applicable
2,2-Difluoro-succinic acid		7.0	7.5	7.5	competitive	non-covalent	not applicable
Proto-porphyrin IX		0.0125	0.25	2.50	competitive	non-covalent	no
4-Keto-5-amino-hexanoic acid (L-isomer)		0.8	0.2	0.2	un-competitive	covalent	yes
Succinic acid monomethyl ester		1.5	3.0	3.0	un-competitive	not determined	not determined

Table 5. (*cont.*)

Inhibitor	Structure	Pea K_i (mM)	Yeast K_i (mM)	*E. coli* K_i (mM)	Kinetics	Nature of bond	Evidence for imine
2,2-Dimethyl-glutaric acid		3.0	4.0	6.0	competitive	not determined	not determined
DL-3-*tert*-Butyladipic acid		1.0	2.0	2.0	competitive	non-covalent	not applicable
3,3-Dimethyl-adipic acid		1.0	1.5	2.5	competitive	non-covalent	not applicable
2,2,7,7-Di-methyl sebacic acid		1.5	0.5	1.5	non competitive	non-covalent	not applicable

cross-link the carboxylic acid-binding groups associated with the A and P substrate-binding sites. A complete understanding of the inhibitory action of these compounds will be obtained only when a three-dimensional model for the enzyme becomes available.

Crystallization

With the vapour-diffusion technique it was possible to reproducibly grow large single crystals of the yeast ALAD. The crystals were tetragonal bipyrimidal in shape with dimensions of approx. 0.5 mm × 0.5 mm × 0.3 mm (Figure 6). The crystallization opens the possibility of determining the three-dimensional structure of the protein by X-ray diffraction. As the yeast ALAD primary structure is similar to the human sequence (56 % identity), such a three-dimensional structure might also allow the modelling of the human enzyme and give an insight into the molecular basis of Doss porphyria [49,50] as well as the dual functionality of ALAD as the CF-2 component of the proteasome–inhibitor complex [51].

Conclusions

The yeast ALAD has been overexpressed and purified and found to be a Zn^{2+}-dependent, Mg^{2+}-binding enzyme that is similar in behaviour to, but not identical with, the ALAD from *E. coli*. Comparative studies with the ALADs from three different sources have given an insight into some of the features required for molecular recognition, demonstrating that there are real differences both between and within the different classes of ALADs. These differences are sufficient to enable the selective inhibition of the enzymes. It will not be possible to rationalize all of the inhibition results collected until the three-dimensional structure of the yeast enzyme has been solved and substantial progress has been made towards this goal with the reported crystallization of the yeast enzyme.

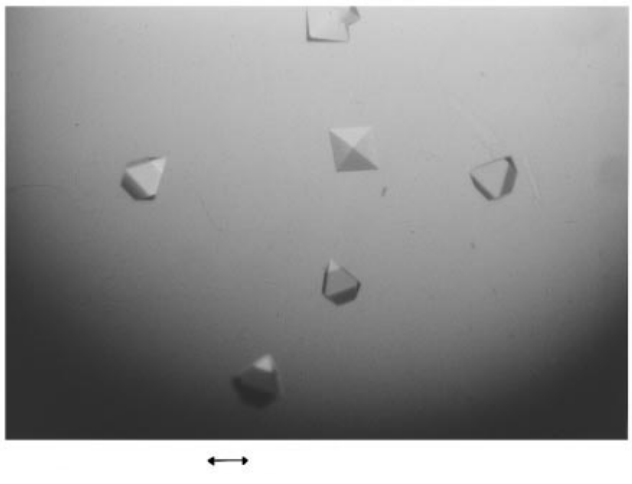


Figure 6 Crystals of purified recombinant yeast ALAD

The crystals were grown by the vapour-diffusion method. The crystals have dimensions of 0.5 mm × 0.5 mm × 0.3 mm. Scale bar, 0.5 mm.

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