Characterization of a Recombinant Pea 5-Aminolevulinic Acid Dehydratase and Comparative Inhibition Studies with the *Escherichia coli* Dehydratase[†]

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ABSTRACT: Pea 5-aminolevulinic acid dehydratase (ALAD) was purified 200-fold from a recombinant overproducing strain of *Escherichia coli*, yielding an octameric enzyme with a specific activity of 280 units mg^{-1}. Divalent metal ions were essential, Mg^{2+}, Mn^{2+}, and Co^{2+} ions all supporting activity, whereas Zn^{2+} ions could not. Equilibrium dialysis and atomic absorption studies revealed two Mg^{2+} ion binding sites per subunit. Pea ALAD bound the substrate 5-aminolevulinic acid covalently through a Schiff base at the P-site, electrospray mass spectrometry of the reduced enzyme−ALA Schiff base complex showing the presence of one P-site per subunit. The amino acid residue modified by ALA was identified by MALDI-MS and Edman sequencing as Lys-293, analogous to the active site Lys-247 of *E. coli* ALAD and Lys-252 of mammalian ALAD. Comparative studies of pea ALAD with *E. coli* ALAD using the inhibitors 3-acetyl-4-oxoheptane-1,7-dioic acid (AOHD) and succinylacetone (SA) indicated similar modes of inhibition, with the formation of a Schiff base complex between the inhibitors and the active site lysine. Studies with the ALA homolog, 4-amino-3-oxobutanoic acid (AOB), revealed that it is specific for the A-site of both the pea and *E. coli* ALADs. An interesting difference exists between the enzymes, however, pea ALAD being far more susceptible to inhibition with AOB than the *E. coli* enzyme. AOB bound 10 times better to the A-site of pea ALAD compared to the substrate, ALA. Despite the 2000 times lower K_i of AOB for pea ALAD, no abortive Schiff base intermediate, between enzyme-bound ALA at the P-site and AOB bound at the A-site, could be demonstrated.

5-Aminolevulinic acid dehydratase (ALAD;[1] porphobilinogen synthase; EC 4.2.1.24) catalyzes the condensation of two molecules of 5-aminolevulinic acid (ALA) to give one molecule of porphobilinogen (PBG) (see Jordan (1991) and Jaffe (1995) for reviews and Scheme 1 for structures). All dehydratases have been shown to require a divalent metal ion for activity. Mammalian and some bacterial enzymes require Zn^{2+} ions (Gibbs *et al*., 1983; Nandi *et al*., 1968). Dehydratases utilizing Zn^{2+} are found to be oxygen sensitive while those utilizing Mg^{2+} are oxygen insensitive (Boese *et al*., 1991). The molecular basis for metal ion selectivity and oxygen sensitivity has been proposed to reside in the nature of the metal ligands (Boese *et al*., 1991). Thus ALADs containing "soft" metal ligands, such as cysteine, prefer "soft" or "borderline" metal ions such as Zn^{2+} and are susceptible to oxidation by a process in which disulfide bonds form between the cysteine metal ligands. ALADs containing "hard" metal ligands, such as aspartate, prefer "hard" or "borderline" metal ions (e.g., Mg^{2+}) and are oxygen insensitive.

The two identical substrates bound by ALAD can be distinguished by the side chain they contribute to the final product porphobilinogen (PBG). The ALA molecule that contributes the propionate side chain binds at a site termed the P-site, while the ALA molecule that contributes the acetate side chain binds at the A-site as shown in Scheme 2a (Jordan & Seehra, 1980; Jordan & Gibbs, 1985). Unlike the bovine ALAD, where four A- and P-sites per octamer appear to exist (Cheh & Neilands, 1976; Jaffe & Hanes, 1986), *Escherichia coli* ALAD has been shown to possess eight A- and P-sites per octamer (Spencer & Jordan 1995), the P-site forming a Schiff base intermediate with a lysine residue (Gibbs & Jordan, 1986). The Schiff base lysine was identified as Lys-252 in human ALAD (Gibbs & Jordan, 1986), equivalent to Lys-247 in *E. coli* ALAD (Echelard *et al*., 1988). Although the initial steps in the reaction mechanism of ALAD involving ALA binding at the P-site have been characterized, the subsequent order of formation of the two bonds required to give the pyrrole ring are unknown.

Studies with a range of inhibitors of ALAD have been carried out (Lüönd *et al*., 1992; Ha *et al*., 1993) although these were not able to distinguish whether inhibitors were active specifically at the A- or P-site of the enzyme. More recent studies have delineated the individual properties of the A- and P-sites of *E. coli* ALAD and have determined the site of action of several inhibitors (Spencer & Jordan, 1995).

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[1] Abbreviations: ALA = 5-aminolevulinic acid; ALAD = 5-aminolevulinic acid dehydratase; AOB = 4-amino-3-oxobutanoic acid; AOHD = 3-acetyl-4-oxoheptane-1,7-dioic acid; EDTA = ethylenediaminetetraacetic acid; ESMS = electrospray mass spectrometry; FPLC = fast protein liquid chromatography; HPLC = high performance liquid chromatography; MALDI = matrix assisted laser desorption ionization; PBG = porphobilinogen; SA = succinylacetone;TFA = trifluoracetic acid.

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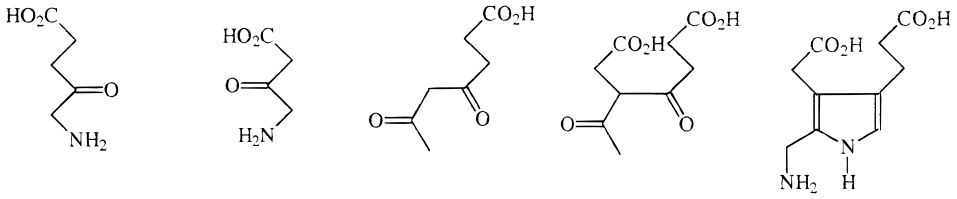
Scheme 1: Molecular Structures of ALAD Substrate, Product, and Inhibitors[a]



[a] From left to right: 5-aminolaevulinic acid (ALA); 4-amino-3-oxobutanoic acid (AOB); succinylacetone (SA); 5-acetyl-4-oxoheptane-1,7-dioic acid (AOHD); and porphobilinogen (PBG).

Characterization of the individual requirements for A-site and P-site binding (Spencer & Jordan, 1995) has provided information to assist in the design of new inhibitors of ALAD.

This paper concerns the purification and characterization of recombinant pea ALAD and for the first time provides an insight into the metal requirements of plant ALADs. The paper also describes studies on the mode of action of inhibitors differentially specific for the A- and P-sites of recombinant pea and *E. coli* ALADs.

EXPERIMENTAL PROCEDURES

Chemicals. Chemicals for organic synthesis were obtained from Aldrich or Fluka. 5-Aminolevulinic acid, 2-mercaptoethanol, V_8 protease, and Trizma base were purchased from Sigma Chemical Co. Ltd. Column chromatography supplies were obtained from Pharmacia Ltd. 5-Amino[5-^{14}C]levulinic acid was purchased from Amersham International, U.K. All other chemicals were purchased from BDH (now Merck).

Synthesis of Inhibitors. *Benzyl 4-Bromo-3-oxobutanoate (Benzyl 4-Bromoacetoacetate)*. Bromine (5.2 mL, 0.1 mol) in $CHCl_3$ (10 mL) was added to benzyl acetoacetate (19.22g, 0.1 mol) in $CHCl_3$ (100 mL) at 0 °C, and the mixture was stirred at room temperature overnight. A stream of air was then passed through the solution for 1 h to remove excess bromine gas. The solution was dried over anhydrous Na_2SO_4 and filtered, and the solvent was removed to give a dark oil (26.11 g). The more volatile material in the mixture was distilled off at 45−80 °C, 0.6 mmHg, leaving benzyl 4-bromo-3-oxobutanoate (11.60 g, 42.8%). ^{1}H-NMR ($CDCl_3$): δ 7.36 (5H, s), 5.18 (2H, s), 4.00 (2H,, s), 3.75 (2H, s).

Benzyl 4-Azido-3-oxobutanoate (Benzyl 4-Azidoacetoacetate). Benzyl 4-bromo-3-oxobutanoate (11.60 g, 4.28×10^{-2} mol) was dissolved in acetone (10 mL) and mixed with NaN_3 (5.04 g, 7.75×10^{-2} mol) in water (30 mL). The mixture was stirred vigorously at room temperature for 6 h, and the lower organic layer was then separated and dried over anhydrous Na_2SO_4. After filtration and evaporation of the solvent, a dark red oil was obtained that was purified by chromatography through a silica gel column using $CHCl_3$ as the eluting solvent. An orange oil (4.60 g, 46.1%) was obtained ($R_f = 0.24$ in $CHCl_3$). ^{1}H-NMR ($CDCl_3$): δ 7.38 (5H, s), 5.19 (2H, s), 4.08 (2H, s), 3.55 (2H, s). IR (LF): 3062, 3032, 2958, 2108 (strong), 1731, 1000 cm^{-1}.

4-Amino-3-oxobutanoic Acid Hydrochloride (AOB). Benzyl 4-azido-3-oxobutanoate (0.52 g, 2.24×10^{-3} mol) was dissolved in a mixture of 0.5 M HCl (7 mL) and 1,4-dioxane (3 mL) and hydrogenated in the presence of 10% Pd-C (0.05g) at 1 atm pressure and room temperature for 24 h. After filtration, the solution was diluted with water (10 mL) and washed with ether (3 × 20 mL). The aqeuous layer was then freeze-dried, yielding light brown sticky crystals (0.24 g, 90.7%). ^{1}H-NMR (DMSO-d_6): δ 8.35 (−COO*H*, s, broad), 7.35 (−N$^+$*H*$_3$, t), 4.03 (−NC*H*$_2$CO−, broad, q), 3.65 (−COC*H*$_2$COOH, s). ^{13}C-NMR (DMSO-d_6): 197.78 (−*C*O−), 166.02 (−*C*OOH), 47.23 (−N*C*H$_2$CO−), 46.69 (−COCH$_2$COOH). IR (LF): 3440, 3160−2940, 1722 (broad), 1612, 1169 cm^{-1}. FAB (MS): M$^+$ (118, 100), (74, 35.9), (58, 11.4).

Ethyl 4,6-Dioxoheptanoate. Mg turnings (39 g, 1.6 mol) were added, carefully, in portions into dry MeOH (200 mL) and CCl_4 (1 mL) in a 2 L round-bottomed flask. The mixture became hot and H_2 gas was evolved so that cooling in a water bath was required. Additional MeOH (500 mL) was added slowly, and the reaction was allowed to continue until all the Mg had reacted. The solution was then refluxed for 10 min. After cooling, *tert*-butyl acetoacetate (250 g, 1.58 mol) was added with stirring over 10 min, yielding a precipitate. The suspension was refluxed for 1 h, after which the solid Mg complex (368.6 g) was collected in two large Buchner funnels, washed with MeOH, and dried in high vacuum for *ca*. 8 h.

Ethyl succinyl chloride (300 g, 1.82 mol) was added over 20 min to a stirred suspension of the Mg complex (368.6 g) in dry ether (600 mL). The thick suspension was refluxed for 30 min, cooled in ice, and acidified with 2 M H_2SO_4. The organic layer was separated, and the aqeuous layer was extracted with ether (3 × 300 mL). After the combined ethereal solutions had been washed with water (2 × 200 mL), dried with anhydrous Na_2SO_4, and evaporated, an orange oil was obtained (437.27 g). Toluenesulfonic acid (1.5 g) was added to the oil, and the whole mixture was heated, over *ca*. 1 h, to 180 °C. After cooling the resulting oil, ether (1 L) was added and extraction was carried out quickly four times with solutions of ice-cold 2 M NaOH (350, 200, 100, and 80 mL, respectively). Each extract was poured directly into ice-cold 1.8 M H_2SO_4 (500 mL), and the aqeuous solutions were extracted with CH_2Cl_2 (3 × 300 mL). The combined organic layers were washed with satd $NaHCO_3$ and dried over anhydrous Na_2SO_4. After filtration and evaporation of the solvent, a dark orange oil was obtained (168.8 g). Ethyl 4,6-dioxoheptanoate (138 g, 47%) was obtained as a colourless oil, by distillation over a boiling range of 80−100 °C, 0.03 mmHg. From ^{1}H-NMR, it was revealed that a mixture of *keto*−*enol* tautomers is present in a ratio of *ca*. 1:3. ^{1}H-NMR ($CDCl_3$): *keto* tautomer, δ 4.13 (2H, q), 3.60 (2H, s), 2.80 (2H, t), 2.60 (2H, t), 2.25 (3H, s), 1.25 (3H, t); *enol* tautomer, δ 15.2 (1H, s, broad), 5.52 (1H, s), 4.15 (2H, q), 2.60 (4H, m), 2.04 (3H, s); 1.25 (3H, t). IR (LF): 3628, 3442, 2980, 2929, 1722, 1625 (strong), 1186, 1022 cm^{-1}.

Diethyl 3-Acetyl-4-oxoheptanedioate. A mixture of ethyl 4,6-dioxoheptanoate (138 g, 0.74 mol), ethyl chloroacetate (75.8 g, 0.62 mol), K_2CO_3 (85.5 g, 0.62 mol) and KI (30.8 g, 0.19 mol) was refluxed in dry acetone (500 mL) for 4 h. The cooled suspension was filtered in a Buchner funnel, the solids collected were dissolved in a minimum amount of water, and this solution was extracted with ether (3 × 150 mL). The filtrate was evaporated, and the residual oil was dissolved in the combined ether extracts. This combined ethereal solution was washed with water (200 mL) and brine (2 × 200 mL) and dried over anhydrous Na_2SO_4. After filtration and evaporation of the solvent, an orange oil (160 g) was obtained. Diethyl 3-acetyl-4-oxoheptanedioate (84.3 g, 42%) was distilled as a pale yellow oil over a boiling range of 134−140 °C, *ca*. 0.02 mmHg. The main product is the *keto* tautomer. ^{1}H-NMR ($CDCl_3$): δ 4.2 (1H, t), 4.12 (4H, q), 2.90 (2H, d), 2.85 (2H, t), 2.60 (2H, t), 2.28 (3H, s). IR (LF): 2980, 2936, 1722, 1625 (weak), 1201, 1030 cm^{-1}.

3-Acetyl-4-oxoheptanedioic Acid (AOHD). Diethyl 3-acetyl-4-oxoheptanedioate (0.97 g, 3.57×10^{-3} mol) was mixed in 5 M HCl (3 mL) and stirred at 50 °C for 18 h. After the evaporation of the HCl and rinsing the residue with ether (3 × 20 mL), a yellow oil was obtained that yielded white crystals at room temperature over a period of 24 h. The crystals were washed with $CHCl_3$ and dried (0.5 g, 64.9%), mp = 110−115 °C. ^{1}H-NMR revealed that the compound is mainly present as a *keto* tautomer. ^{1}H-NMR (DMSO-d_6): δ 12.2 (2H, broad, s), 4.25 (1H, t), 2.80 (2H, t), 2.70 (2H, d), 2.45 (2H, t), 2.20 (3H, s). ^{13}C-NMR (DMSO-d_6): 204.83, 203.08, 173.73, 172.71, 61.76, 37.42, 32.39, 29.94, 27.89. FAB (MS): $(M + 1)^+$ (217, 30.2), (157, 13.7), (115, 54.8).

Expression, Purification, and Assay of ALAD from E. coli. *E. coli* ALAD was expressed in strain CR261 generated by transforming TB1 with pCAR261 harboring the *hemB* gene (Roessner *et al*., 1995). The strain was constructed by Dr. C. Roessner (Texas A&M University) and was a generous gift. *E. coli* ALAD was purified and assayed as described in Spencer and Jordan (1993).

Expression of Pea ALAD. Pea ALAD was expressed in an ALAD deficient *E. coli hemB*$^-$ mutant, Y10-90, transformed with pKK223-3 (Pharmacia) containing the cDNA encoding pea ALAD (Boese *et al*., 1991). The pea ALAD overexpression strain was grown in 500 mL batches of Luria broth containing ampicillin (100 μg/mL) for 24 h. The cells were harvested by centrifugation at 1000*g* for 15 min and stored as a cell paste at −20 °C until required.

Assay of Pea 5-Aminolevulinic Acid Dehydratase. In contrast to the Zn^{2+} dependent *E. coli* ALAD, the pea enzyme requires Mg^{2+} for activity. Pea ALAD was therefore assayed in 50 mM Tris-HCl buffer, pH 8.5, containing 10 mM $MgSO_4$ in a total volume of 250 μL at a minimum protein subunit concentration of 10 μM. The reaction was initiated by the addition of ALA to give a final concentration of 5 mM. After incubation at 37 °C for 2 min, the reaction was terminated by the addition of an equal volume of 10% trichloroacetic acid containing 0.1 M $HgCl_2$. The solution was centrifuged, and an aliquot of the supernatant was removed and mixed with an equal volume of modified Ehrlich's reagent (Mauzerall & Granick, 1956). After 15 min, the absorbance at 555 nm was determined (ϵ_{555} = 60 220 M L^{-1}). The absorbance at 280 nm of a 0.1% solution of pea ALAD in 50 mM potassium phosphate, pH 6, was determined as 1.5. One unit of enzymatic activity = 1 μmol of PBG produced/(h·mg of protein) at 37 °C. The specific activity of purified pea ALAD was found to be 280 units mg^{-1} at pH 8.5.

Purification of Pea 5-Aminolevulinic Acid Dehydratase. *E. coli* cell paste (4 g) was suspended in 20 mL of 50 mM potassium phosphate buffer, pH 6.0, containing 10 mM $MgSO_4$ and was sonicated for 4 min at 0 °C to disrupt the cells. Cell debris was removed by centrifugation at 1000*g* for 20 min. Ammonium sulfate was then added to give 30% saturation, and the resulting precipitate was removed by centrifugation and discarded. Further ammonium sulfate was then added to give 50% saturation, and the precipitate, containing the enzyme, was collected by centrifugation. The pellet was resuspended in 3 mL of 50 mM Tris-HCl buffer, pH 8.4, containing 10 mM $MgSO_4$, and the solution was applied to a Sephacryl S-200 gel filtration column (100 cm × 2.5 cm). The column was developed in the same buffer at 1 mL/min and fractions eluting with a specific activity greater than 30 were pooled and applied to a DEAE Sephacel ion-exchange column (30 cm × 2.5 cm). ALAD was eluted with a 0−1 M gradient of KCl in 50 mM Tris-HCl buffer, pH 8.2, containing 10 mM $MgSO_4$. ALAD eluted at 200 mM KCl, and fractions of specific activity greater than 70 were pooled and concentrated to 10 mg/mL using an Amicon cell fitted with a PM20 membrane. The concentrated protein solution was then reapplied to a Sephacryl S-200 column as described above. Fractions eluting with a specific activity of greater than 260 units mg^{-1} were pooled and judged to be greater than 90% pure by SDS−PAGE (Laemmli & Favre, 1973). The maximum specific activity for the homogeneous enzyme was 280 units mg^{-1}. A solution 1 mg/mL of the pure protein gave an absorbance of 1.5 at 280 nm. Protein concentration was also determined by reaction with DTNB (as detailed below).

Sulfhydryl Group Determination. The total number of free thiol groups present in pea ALAD were determined by prior denaturation of the enzyme (50 μg) in 40 μL of 50 mM potassium phosphate buffer, pH 8, containing 4 M guanidine hydrochloride. A solution of 5 mM 5,5′-dithiobis-(2′-nitrobenzoic acid) (DTNB) was then added, and after dilution in the same buffer, the absorbance was determined at 412 nm (ϵ_{412} = 14 750 M L^{-1}). Protein concentration could then be determined based on four SH groups per subunit detected in the gene-derived amino acid sequence (Boese *et al*., 1991). This was consistent with the M_r determined by ESMS (see below).

M_r Determination. (a) Subunit M_r Determination. The purified enzyme was subjected to polyacrylamide gel electrophoresis in the presence of SDS (Laemmli & Favre, 1973).

(b) Oligomeric M_r Determination. A Pharmacia FPLC Superose G12 HR10/30 gel filtration column was equilibrated in 50 mM Tris-HCl buffer, pH 8.5, containing 300 mM KCl and 10 mM $MgSO_4$ at a flow rate of 0.4 mL/min. Standards of known M_r (apoferritin, 443 000; β-amylase, 200 000; horse alcohol dehydrogenase, 150 000; bovine serum albumin, 66 000) were applied to the column (0.1 mL of 5 mg/mL standard), and their elution volumes were detected at 280 nm. Pea ALAD was chromatographed under identical conditions, and the M_r was compared to those of the

standards by plotting elution volume against log M_r (data not shown).

Isoelectrofocusing. Isoelectrofocusing of the purified pea ALAD was performed on LKB Ampholine polyacrylamide gel plates (pH 3.5−9.5) using an LKB Multiphor apparatus. Protein samples (5 μg in 5 μL of 20 mM Tris-HCl, pH 7) were applied to the gel (10 cm × 12 cm) on sample application papers (4 mm × 4 mm) and electrofocused for 2 h at 15 W (limited at 1500 V and 25 mA). The protein bands were detected by staining the gel with Coomassie brilliant blue. A standard curve was constructed from proteins of known p*I* (range 4.2−9.3), and the p*I* of pea ALAD was then determined by comparison.

Generation of Metal-Depleted Pea ALAD (ApoALAD). Pea ALAD (3 mg/mL) was dialyzed against 40 mM Tris-HCl buffer, pH 8.5, containing 50 mM Na_2SO_4 and 5 mM EDTA for 4 h at 4 °C. Assay of pea apoALAD in the absence of $MgSO_4$ gave less than 0.5% of the activity exhibited in the presence of 10 mM $MgSO_4$. All experiments on pea apo-ALAD were carried out using 40 mM Tris-HCl buffer, pH 8.5, containing 50 mM Na_2SO_4, after removal of excess EDTA by gel filtration or dialysis using the same buffer.

Equilibrium Dialysis Studies. Samples of pea apoALAD (0.5 mL of 50−100 μM) were dialyzed twice against 1 L of 40 mM Tris-HCl buffer, pH 8.5, containing 50 mM Na_2SO_4 and various concentrations of $CoCl_2$ (20−150 μM) for 4 h at 4 °C. Metal concentrations, both inside and outside the dialysis bag, were then determined as described below. Similar experiments were undertaken by the addition of various concentrations of $CoCl_2$ (100−700 μM) to apo-ALAD (0.3 mL; 20−35 μM) in the above buffer. After 30 min at 4 °C, the samples were then centrifuged in a Centricon P-10 microconcentration ultrafiltration system (Amicon) for 15 min, resulting in the filtration of 100−150 μL of buffer. Metal content of the filtrate and the concentrate were then determined as described below.

Ultrafiltration Experiments. Samples of pea apoALAD (200−400 μL; 0.4−0.6 mg/mL; 10−15 μM), to which various concentrations of metal ions had been added, were placed in a Centricon P-10 microconcentration tube and centrifuged for 1−2 h at 1000*g* at 4 °C to give a 4−6-fold concentration of sample. Samples of the concentrate and filtrate were then analyzed for metal content by atomic absorption (see below). Protein concentrations were determined by the method of Bradford (1976).

Atomic Absorption Studies. Samples for Mg^{2+}, Mn^{2+}, and Co^{2+} ion determinations were injected into an atomic absorption spectrometer (Instrumentation Laboratory 157), and the metal concentration was determined by comparison with a standard curve constructed from known metal ion concentrations. Samples for analysis were adjusted by dilution to bring them into the linear response region (5−40 μM) of the standard curve.

Formation of ALAD-ALA P-Site Adducts by Reduction of ALAD with $NaBH_4$ in the Presence of ALA. Pea ALAD (0.3 mL; 0.8 mg/mL) in 300 mM Tris-HCl buffer, pH 8, containing 10 mM $MgSO_4$ was mixed with various concentrations of [^{14}C]ALA (5 μM−10 mM; specific activity 1−10 × 10^{11} dpm mol^{-1}) followed immediately by reduction with 0.5 mg of solid $NaBH_4$. The resulting labeled samples were then dialyzed against 2 × 2 L of 50 mM Tris-HCl buffer, pH 7, containing 50 mM Na_2SO_4 and 10 mM $MgSO_4$. Samples were analyzed for protein content, enzyme activity, and [^{14}C]ALA incorporation. Reduced *E. coli* ALAD-ALA adducts were prepared as described in Spencer and Jordan (1995). For comparative experiments with the inhibitor AOB (4 mM), the active site peptide was labeled with [^{14}C]ALA (2 mM; specific activity 2 × 10^{12} dpm) with and without AOB.

Proteolytic Degradation of Modified ALAD and Purification of Peptides by HPLC. Samples of ALAD, modified by reduction with $NaBH_4$ in the presence of 5-[^{14}C]ALA (see above), were treated with trypsin (1% w/w ratio), and the resulting peptides were purified by HPLC using a Waters C_{18} Microbondapak column (30 cm × 4mm) developed using a linear gradient from 0.5% TFA in H_2O to 0.1% of TFA in acetonitrile at a flow rate of 1 mL/min (Spencer & Jordan, 1994).

Alternatively, ALAD samples labeled with [^{14}C]ALA were digested with V_8 protease, and the labeled peptides were purified by reverse-phase HPLC as described above. The reduced pea ALAD-[^{14}C]ALA adduct yielded two peaks containing 35% of the radiolabel in the first peak and 65% in the second whereas enzyme, reduced in the presence of ALA and AOB, had 70% of the radiolabel in the first peak and 30% in the second. Further HPLC purification of the labeled peptides was carried out, and they were then analyzed by MALDI-MS and Edman degradation.

A-Site Binding Determination by Rate-of-Dialysis Measurements. ALA binding at the A-site of the reduced ALAD-ALA P-site adduct (unlabeled) was followed by the displacement of [^{14}C]ALA from the A-site by unlabeled ALA (Spencer & Jordan, 1995). Similarly, inhibitor binding at the A-site was also monitored by the displacement of [^{14}C]-ALA from the A-site of this adduct in the presence of the inhibitor.

N-Terminal Sequencing of Native Pea ALAD and Peptides by Edman Degradation. Recombinant pea ALAD (1400 pmol) in 5 mM Tris-HCl buffer, pH 8.0, was applied to polybrene filter discs and sequenced using an Applied Biosystems 477A sequencer linked to a 120A analyzer for the detection of the phenylthiohydantoin (PTH) derivatives. Peptides generated from proteolytic digests (1000 pM) were isolated by HPLC, as described above, and sequenced similarly.

Electrospray Mass Spectrometry. Electrospray mass spectrometry data were collected using a Quattro II triple-quadrapole (VG Biotech, Altringham, Cheshire, U.K.). The analyses were carried out using 10 μL of ALAD (10 pmol/μL) in acetonitrile−water (1:1) containing 1% formic acid. Peptides (5 pmol/μL) generated from ALAD and its adducts, after purification by HPLC (as described above), were prepared in the same solvent. Samples were injected into the electrospray source *via* a Rheodyne injection valve. The source voltage was 40 V, the source temperature was 80 °C, and the injector flow rate was 10 μL min^{-1}. Typically, 40 scans were used to accumulate data. Sample solutions were prepared immediately prior to analysis unless otherwise stated. Samples were scanned over the mass range 700−1400 Da. The instrument was calibrated using horse heart myoglobin (M_r 16 951.5) at a concentration of 10 pmol μL^{-1}.

Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS). Because some samples did not give acceptable spectra using ESMS and to obtain confirmatory

Table 1: Purification of Recombinant Pea ALAD

step	vol (mL)	units mL^{-1}	mg mL^{-1}	total mg	total units	sp act.	% yield
initial extract	100	20	50	5000	2000	0.4	100
30−50% satd. $(NH_4)_2SO_4$ pellet	4	415	250	1000	1660	1.7	83
S-200 gel filtration	31	43	1.1	34	1333	39	67
DEAE Sephacel	25	35	0.33	8.2	872	106	44
S-200 gel filtration	8	87.5	0.34	2.7	700	260[a]	35

[a] Fractions with specific activity greater than 260 were pooled. The highest activity obtained was 280 units mg^{-1}.

data, MALDI-MS was also utilized for analysis. Peptides generated from ALAD and its adducts (1 μL;10 mg/mL), after purification by HPLC, were diluted 3−5 times with an equal volume of 30% acetonitrile containing 0.1% TFA saturated with the matrix 3,5-dimethoxy-4-hydroxycinnamic acid (sinipinic acid). An aliquot (4 μL) was then dried onto the MALDI-MS plate, and laser desorption mass spectrometry was carried out at a potential of 30 000 V. Protein samples were treated similarly. The system was calibrated using the singly and doubly charged peaks of bovine insulin B-chain. M_r determinations of adduct species were compared to unlabeled species with the same matrix ion peak intensities (10 000−15 000). The peak centroid was used to determine the M_r of all peaks, and an average of at least six laser desorption determinations were taken in each case.

RESULTS AND DISCUSSION

Purification of Pea ALAD. Pea ALAD was purified as described in the Experimental Procedures section. The stages of purification are shown in Table 1. The maximum specific activity of the homogeneous enzyme, as judged by SDS−PAGE, was 280 units mg^{-1}. Fractions of specific activity greater than 260 were pooled from the final stage and used for experimentation.

N-Terminal Sequence of Pea ALAD. The subunit M_r predicted from the pea gene sequence (Boese *et al*., 1991) is 43 793; however, the *hemB* gene in the overexpressed clone used in this study had been modified at the N-terminus by the removal of 34 amino acids and the alteration of the 35th residue, serine, to the initating methionine. The N-terminal sequence of the purified enzyme was confirmed by Edman degregation. The first seventeen amino acids were found to be MDSDSEAAVVAGKVPER in agreement with the gene-derived protein sequence (Boese *et al*., 1991), with the exception of residue five where a serine residue, not the expected phenylalanine residue, was found. The alteration of the 5th amino acid residue could theoretically be accounted for by a single DNA base change from TTC to TCC. The predicted M_r of the purified recombinant pea ALAD in this study was therefore expected to be 39 900. Also detected was an additional minor amino acid sequence beginning SDSEAAV, representing an N-terminus starting at position 3 of the expected recombinant ALAD. This minor sequence, varying from <10−30% of the total, has probably arisen by partial N-terminal posttranslational modification, resulting in the loss of methionine and aspartate from positions 1 and 2. All mass spectrometric measurements were conducted using samples where this minor sequence was <10%.

Determination of pI and Subunit and Oligomeric M_r of Pea ALAD. Isoelectric focusing of purified pea ALAD (described in the methods) gave a single major band corresponding to a p*I* of 5.2. The subunit M_r determined by SDS−PAGE was 43 000 (±4300) and was in approximate agreement with the predicted M_r of 39 900. The oligomeric M_r was determined as 309 000 (±20 000) by gel filtration (see methods), indicating an octameric species, as found for most mammalian and bacterial dehydratases, but in contrast to ALAD from spinach that is reported to be hexameric (Liedgens *et al*., 1983). Both the apo-ALAD and the Mg^{2+} species of pea ALAD gave octameric species on gel filtration, indicating that the metal ion is not essential for quaternary structure.

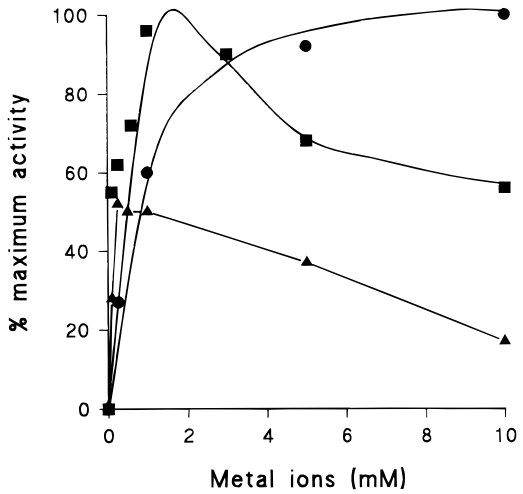


FIGURE 1: Stimulation of pea ALAD by metal ions. Various concentrations of Mg^{2+} (●), Mn^{2+} (■), and Co^{2+} (▲) ions were added to pea apo-ALAD in 100 mM Tris-HCl buffer, pH 8.5. The enzyme was then assayed by the addition of 5 mM ALA. Activity restored was determined as a % maximum of Mg^{2+}-stimulated activity.

Metal Requirement for Activity of Pea ALAD. The activity of purified pea ALAD was dependent on the presence of exogenous Mg^{2+} ions. Atomic absorption spectroscopy showed that the enzyme contained no bound Zn^{2+} ions, unlike dehydratases from mammalian and many bacterial sources. Addition of 25 mM EDTA to pea holo-ALAD resulted in the immediate loss of all activity, and following gel filtration in 50 mM Tris-HCl buffer, pH 8.5, no bound Mg^{2+} could be detected by atomic absorption spectroscopy. Readdition of Mg^{2+} to the above apoenzyme resulted in the complete restoration of the original specific activity (280 units mg^{-1}) with a K_d of 800 μM (Figure 1). However, in 100 mM potassium phosphate buffer, pH 8.5, the K_d for Mg^{2+} ion was 2 mM. Further studies indicated that phosphate buffer was acting as a competitive inhibitor against Mg^{2+} ion binding with a K_i of 100 mM at 1 mM Mg^{2+}, possibly reflecting the ability of phosphate ions to form complexes with metal ions. Often this phenomenon is not significant as the affinity of a metalloenzyme for its metal ion is usually several orders of magnitude greater than the affinity of the phosphate ion for metal ions. However, in the case of pea ALAD, with its relatively low affinity for Mg^{2+}, the competing affinity of the metal ion for the phosphate ion appears to be significant.

Pea apo-ALAD activity could also be restored by the addition of Mn^{2+} or Co^{2+} ions with K_d values of 100 μM (Figure 1), somewhat lower than that for Mg^{2+} ions. While Mn^{2+} restored enzyme activity to levels observed with Mg^{2+}, Co^{2+} only restored activity to 50% of this value (Figure 1).

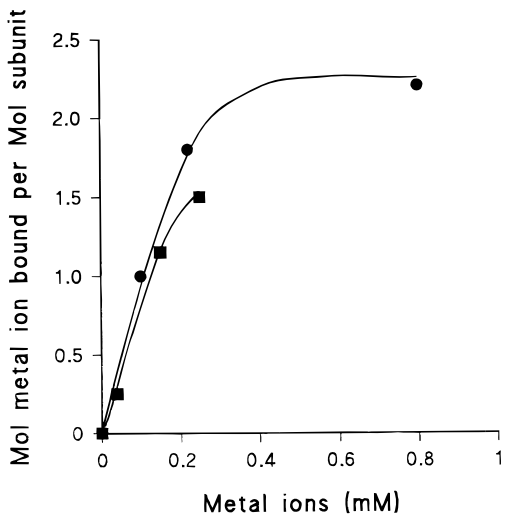


FIGURE 2: Correlation of metal ion content against metal ion concentration for pea ALAD. Various concentrations of Mn^{2+} (●) were added for ultrafiltration experiments (see methods) and Co^{2+}-(■) for equilibrium dialysis experiments (see methods)). Free metal ion concentration was subtracted from the metal ion concentration present with pea ALAD to give bound metal ion concentration expressed as mol of metal ion/mol of subunit. Metal ion concentrations were determined by atomic absorption (see methods).

Concentrations of Co^{2+} and Mn^{2+} ions higher that 1 mM were found to be inhibitory with K_i values of 6 mM and 10 mM, respectively.

Determination of the Number of Metal Ion Binding Sites of Pea ALAD. As a consequence of the low affinity of pea ALAD for its activating metal ions, gel filtration to determine the stoichiometry of metal ion binding could not be performed. The use of equilibrium dialysis using Mg^{2+} was also made technically difficult by the high K_d, requiring prohibitively large amounts of protein to perform these experiments reliably. However, the lower K_d of pea ALAD for Co^{2+} and Mn^{2+} did permit equilibrium dialysis and ultrafiltration experiments to be performed with the small amounts of protein available (as described in the methods). The maximum number of Co^{2+} and Mn^{2+} ions bound was determined to be approximately 2 mol/mol of subunit, both sites having similar affinities with K_d values of 100 μM ± 10% (Figure 2). Maximum enzyme activity was restored on the binding of 2 mol equiv of Mn^{2+}. The enzyme appeared to be activated by the binding of only 1 mol equiv of Co^{2+}, the second mol equiv of Co^{2+} resulting in no further increase in activity. Although these findings should be looked upon with some caution, because the small amount of enzyme made detailed studies difficult, they may nevertheless be compared to those for the ALAD from *E. coli* where a maximum binding of 2 mol equiv of Zn^{2+} was determined (Spencer & Jordan, 1994). Interestingly, activity of the *E. coli* ALAD was also supported by the binding of one Co^{2+} ion.

pH Dependence of ALA Binding at the A- and P-Sites of Pea ALAD. The K_m of ALA for pea ALAD varied little from pH 7.5 to pH 9 (800 μM−1 mM), and inactivation of pea ALAD by reductive incorporation of ALA with $NaBH_4$ at various pHs indicated that the K_d for ALA binding to the P-site was 25 μM over the pH range 7.5−9. By comparison, the K_d for Schiff base formation at the P-site is well below the value for the K_m, implying that the affinity of ALA binding to the A-site of the pea ALAD is limiting the K_m over this pH range, as found for *E. coli* $Mg_\alpha Zn_\beta$ (Spencer & Jordan, 1995).

Correlation of ALA Incorporation, on Reduction with $NaBH_4$ at the P-Site, Against Activity for Pea ALAD. Reduction of the Schiff base formed by [^{14}C]ALA binding at the P-site (Scheme 2a) of pea ALAD (as described in the methods) inactivated pea ALAD. Inactivation correlated linearly with [^{14}C]ALA incorporated, virtually all the activity being lost on incorporation of 1 (±0.1) mol of 5-aminolevulinic acid per mol of subunit. Therefore, as with human and *E. coli* ALADs (Gibbs & Jordan 1986; Spencer & Jordan, 1994), pea ALAD does not exhibit half site reactivity as reported for bovine ALAD (Cheh & Neilands, 1976; Jaffe & Hanes, 1986).

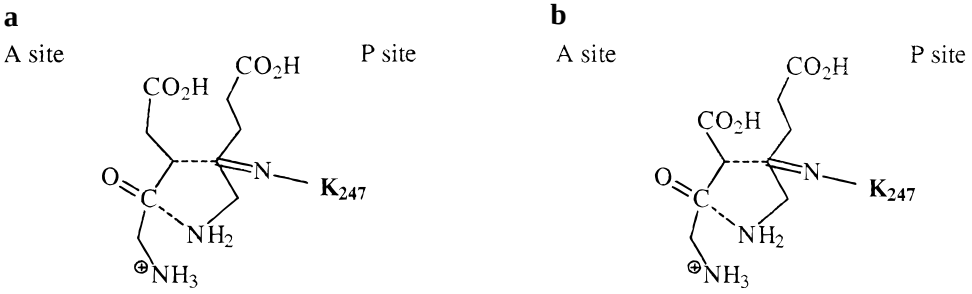
Determination of the M_r of pea ALAD and its reduced ALA adduct by electrospray mass spectrometry gave species of M_r 39 900 (±5) and 40 014 (±4), respectively, showing, as with *E. coli* ALAD, the binding of 1 mol of ALA/mol of ALAD.

Determination of the Mode of Inhibition of E. coli ALAD by 4-Amino-3-oxobutanoic acid (AOB). AOB, a lower homolog of ALA (structure in Scheme 1), was found to be a competitive inhibitor of *E. coli* ALAD with a K_i of 20 mM at 75 μM ALA (Figure 3). Reduction of the ALAD with $NaBH_4$ in the presence of 30 mM AOB resulted in only a 10% loss of activity, compared to a 95% loss when the enzyme was reduced in the presence of 7 mM ALA, suggesting that the inhibitor did not act by binding to the P-site. The possible competition by AOB with ALA for the P-site was investigated by determining the extent of Schiff base formation between ALAD and 75 μM [^{14}C]ALA (specific activity 1.06×10^{14} dpm mol^{-1}) by trapping with $NaBH_4$ in the presence and absence of 30 mM AOB. The incorporation of [^{14}C]ALA into the *E. coli* enzyme, as the reduced Schiff base, was essentially unaffected by AOB, increasing marginally from 0.05 mol of ALA/mol of subunit in the absence of AOB to 0.07 mol of ALA/mol of subunit in the presence of 30 mM AOB. This confirmed that AOB was not preventing ALA from binding to the P-site of *E. coli* ALAD, suggesting that inhibition by AOB was mediated primarily through binding to the A-site (see Scheme 2b).

Addition of AOB to *E. coli* ALAD, that had been previously reductively labeled with ALA at the P-site, resulted in the displacement of [^{14}C]ALA from the A-site as determined by rate-of-dialysis experiments giving a K_i of 13 mM for the A-site (Figure 3). The K_i of 13 mM for the AOB at the A-site of the reduced ALA-ALAD adduct was similar to that determined kinetically, at the same concentration of ALA, indicating that the inhibitory effect of AOB on *E. coli* ALAD is mediated almost exclusively through binding at the A-site. The ratio [ALA]/K_i of 5×10^{-3} determined at several concentrations of ALA (75−500 μM) indicated that the affinity of the natural substrate, ALA, for the A-site is approximately 200 times greater than that of AOB.

Determination of the Mode of Inhibition of Pea ALAD by 4-Amino-3-oxo-butanoic acid (AOB). Pea ALAD was also inhibited by AOB, although at much lower concentrations of inhibitor than those observed for *E. coli* ALAD, the K_i being 10 μM when assayed with 100 μM ALA. On incubation of pea ALAD with 10 mM AOB, followed by reduction with $NaBH_4$, less than 10% of the enzyme activity was lost, suggesting that, like *E. coli* ALAD, AOB inhibition

Scheme 2: Proposed Binding of ALA and AOB to the Catalytic Site of ALAD[a]



[a] (a) Proposed binding of 2 molecules of ALA to the A-site and P-sites of ALAD. The ALA molecule bound at the P-site interacts through a Schiff base with Lys-247 (*E. coli*) or Lys-293 (pea). The ALA bound at the P-site may be reduced by reaction with $NaBH_4$. (b) Proposed binding of AOB to the A-site of ALAD in the presence of ALA at the P-site.

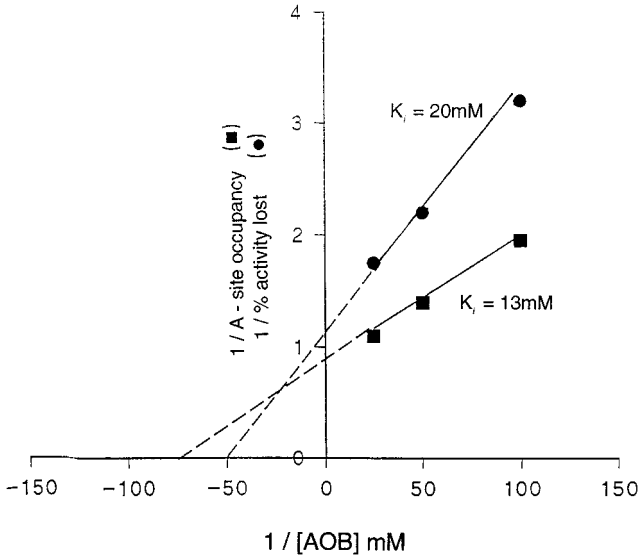


FIGURE 3: Determination of the K_i for AOB inhibition of *E. coli* $Mg_\alpha Zn_\beta$ALAD by kinetic and "A-site" binding assay. The K_i for AOB inhibition of *E. coli* $Mg_\alpha Zn_\beta$ALAD determined by steady state kinetics (●) in 75 μM ALA is compared to the K_i determined for AOB binding for the A-site (■) of ALAD by rate-of-dialysis experiments (see methods) in the presence of 75 μM ALA.

of the pea enzyme was due primarily to interaction at the A-site with 10 times the affinity of ALA (see Scheme 2b). In comparison to *E. coli* ALAD, AOB was 2000 times more effective against pea ALAD.

No enzyme-bound intermediates *en route* to porphobilinogen have hitherto been detected with any ALAD, using the natural substrate, ALA. The specific A-site inhibition exhibited by AOB with pea ALAD therefore raised the possibility that an intermediate analog formed between ALA (at the P-site) and AOB (at the A-site) could be trapped by treatment of the pea ALAD with $NaBH_4$ (Scheme 2b). Table 2 shows when pea ALAD was reduced with $NaBH_4$ in the presence of [^{14}C]ALA (2 mM) and AOB (4 mM), only the ALAD-ALA adduct was detected by ESMS (M_r = 40 014 ± 5). Proteolytic digestion of the modified, labeled pea enzyme with V_8 protease, followed by MALDI-MS of the purified peptide, gave a M_r = 1435, indicative of a K^+ adduct of the predicted active site peptide ILLVKPAGAYLD (M_r = 1393) with one ALA bound through a reduced Shiff base to the active site lysine. The identity of the peptide was confirmed by N-terminal sequencing.

Determination of the Mode of Inhibition of ALAD by Succinylacetone (SA). Preincubation of *E. coli* ALAD with 4 mM SA (structure shown in Scheme 1) led to the loss of all enzyme activity. The ALAD-SA adduct was not disrupted by gel filtration, indicating that a stable complex had formed. However, this adduct was not stable to the concentration of acid required for ESMS, suggesting the presence of an acid-sensitive covalent link. Subsequent reduction of the ALAD-SA adduct by $NaBH_4$ allowed the identification, by ESMS, of a species with M_r 35 641 (±5), indicating that the linkage was likely to be *via* a reduced Schiff base (Table 2). The M_r of the reduced ALAD-SA adduct was consistent with the incorporation of 1 mol equiv of SA per subunit.

Preincubation of pea ALAD with 4 mM SA also led to the complete loss of all activity, again, with the formation of a tight complex between the ALAD and SA. As with

Table 2: ESMS and MALDI-MS Determinations of *E. coli* and Pea ALAD and Their Inhibitor Adducts

species	M_r by MALDI-MS	M_r by ESMS	predicted
	E. coli ALAD		
ALAD	35 500 (±11)	35 500 (±4)[b]	35 494
ALAD + AOB + $NaBH_4$	35 509 (±14)	nd	35 494 or 35 597
ALAD + ALA + $NaBH_4$	35 646 (±27)	35 614 (±8)[b]	35 611 (35 494 + 117)
ALAD + ALA + AOB + BH_4	nd	nd	
ALAD + SA	nd	35 500 (±6)	
ALAD + SA + $NaBH_4$	35 672 (±86)	35 641 (±5)	35,643[c] (35,494 + 143)
ALAD + AOHD	35 500 (±11)	35 500 (±7)	
ALAD + AOHD + BH_4	35 653 (±29)	35 700 (±8)	35 695[c] (35 494 + 201)
		35 600 (±6)	
	Pea ALAD		
ALAD	39 900 (±30)	39 900 (±5)	39 900
ALAD + AOB + $NaBH_4$	39 905 (±25)	nd	39 900 or 40 003
ALAD + ALA + $NaBH_4$	40 011 (±19)	40 014 (±4)	40 014 (39 900 + 117)
ALAD + ALA + AOB + BH_4	40 011 (±19)	40 014 (±5)	40 014
ALAD + SA + $NaBH_4$	40 029 (±25)	40 066 (±8)	40 043[c] (39 900 + 143)
ALAD + AOHD	39 900 (±30)	nd	
ALAD + AOHD + BH_4	40 010 (±25)	40 079 (±8)	40 101[c] (39 900 + 201)

[a] All enzyme samples were prepared as described in the methods. Standard deviations are shown in parentheses. [b] Predicted M_rs have been determined previously by ESMS (Spencer & Jordan, 1995). [c] Predicted M_r represents incorporation of simple multiples of the M_r of the inhibitor used allowing for the loss of 16 on the formation of a Schiff base, where appropriate (SA 158; AOHD 216: AOB 100).

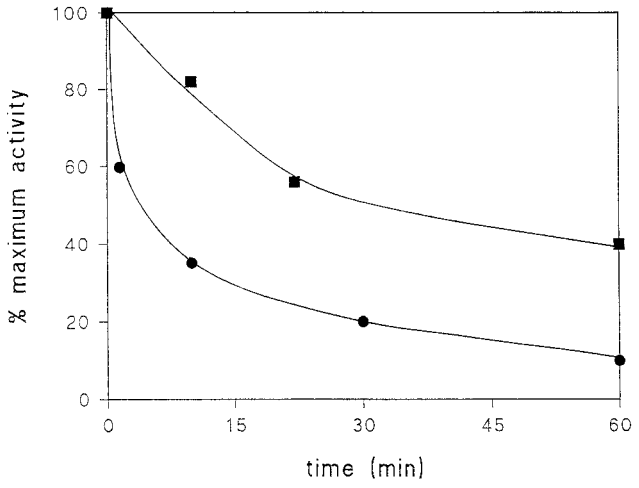


FIGURE 4: Time-dependent inhibition of ALAD by AOHD. *E. coli* ALAD in 100 mM potassium phosphate buffer, pH 7.8, containing 20 μM Zn^{2+} and 15 mM β-mercaptoethanol treated with 16 mM AOHD (■) and pea ALAD in 100 mM Tris-HCl buffer, pH 7.8, containing 10 mM Mg^{2+} treated with 4 mM AOHD (●).

the *E. coli* ALAD-SA complex, reduction with $NaBH_4$ was required to observe the adduct species of M_r 40 066 (±8), using ESMS, indicating modification by 1 mol SA per mol of pea ALAD subunit.

Determination of the Mode of Inhibition of ALAD by 3-Acetyl-4-oxoheptane-1,7-dioic Acid (AOHD). 3-Acetyl-4-oxoheptane-1,7-dioic acid (AOHD) (structure shown in Scheme 1) acted as a competitive inhibitor of *E. coli* ALAD; however, curvilinear kinetic traces of AOHD inhibition prevented the determination of a meaningful K_i. At low concentrations of AOHD (10 μM), increasing concentrations of ALA could overcome the inhibition. On further investigation, a time-dependent inactivation of *E. coli* ALAD by 16 mM AOHD was observed (Figure 4). The activity of the AOHD-inactivated *E. coli* ALAD was not restored by gel filtration at pH 7, indicating a tight complex had formed. The presence of ALA during incubation with AOHD was found to protect ALAD partially from inactivation as judged by 50% maximum activity remaining after subsequent gel filtration, compared with 12% activity remaining after incubation with AOHD alone.

As with SA modification, reduction of this tight complex with $NaBH_4$ was required to observe the adduct species by ESMS, indicative of an acid-sensitive Shiff base linkage. ESMS revealed two major species of M_r 35 700 (±8) and 35 600 (±6), the former being due to the ALAD-AOHD Schiff base adduct giving the predicted M_r of 35 700, since the M_r of AOHD = 216. The latter species represents a bound species of M_r = 100 that may be formed from enzymic cleavage of the AOHD-Schiff base. The modification site of AOHD in the *E. coli* ALAD was identified as Lys-247 by MALDI-MS analysis of peptides generated after trypsin digestion of the reductively modified ALAD-AOHD complex. Two active site peptides with increased M_rs of 100 and 144 were observed.

Preincubation of pea ALAD with 4 mM AOHD also gave a time-dependent inhibition (Figure 4), resulting in a rapid loss of activity. As with *E. coli* ALAD, a tight Schiff base complex was formed with AOHD which required reduction by $NaBH_4$ before the adduct of M_r 40 079 (±8) could be detected by ESMS.

DISCUSSION

The requirement of Mg^{2+} ions for activity, the value of K_m for ALA, and the high pH optimum for pea ALAD are similar to the well characterized plant ALAD isolated from spinach (Liedgens *et al*., 1983). However, the oligomeric structures appear to differ, the pea ALAD being an octamer while the spinach enzyme is reported to be a hexamer (Liedgens *et al.*, 1983). As with *E. coli* ALAD, two metal binding sites per subunit of pea ALAD were found, although their much lower affinities prevented a more detailed characterization of the two individual metal binding sites, as have been achieved with *E. coli* and bovine ALAD (Spencer & Jordan, 1994; Dent *et al*., 1990). The preference for Mg^{2+} ions by plant ALADs is thought to arise from the presence of aspartic acid in place of cysteine residues as metal ligands (Boese *et al*., 1991). Why ALADs from plant sources utilize aspartyl ligands in place of cysteine is unclear, although aspartyl residues would be stable to the presence of high concentrations of O_2 produced during photosynthesis. Such a proposal is not, however, supported by the report that cysteine metal ligands exist in the ALAD from the photosynthetic Cyanobacteria species (Jones *et al*., 1994). An alternative explanation is that Mg^{2+} ions may act to control the activity of the dehydratase, especially as the K_m for Mg^{2+} is so high (1 mM). Indeed the concentration of Mg^{2+} within the chloroplast has been shown to rise from 1 mM in the dark to 10 mM in the light (Portis & Heldt, 1976) although exactly how much free Mg^{2+} these levels correspond to is uncertain.

Comparison of the K_m (800 μM) and K_d values for the P-site (25 μM) of pea ALAD indicated that the affinity of ALA for the A-site had the major influence on the K_m. ALA binding at the A-site appeared to be some 10 times weaker (800 μM) for pea ALAD ($2Mg^{2+}$) than for *E. coli* ALAD $Mg_\alpha Zn_\beta$ (60 μM) and $Zn_\alpha Zn_\beta$ALAD (45 μM) (Spencer & Jordan, 1994). The affinity of ALA binding for the P-site of pea ALAD (25 μM) is of the same order as that of *E. coli* $Mg_\alpha Zn_\beta$ALAD (70 μM); (Spencer & Jordan, 1994) but lower than for the $Zn_\alpha Zn_\beta$ALAD (600 μM). The tighter binding of ALA at the A-site of *E. coli* ALAD implies that the A-site is "better" designed for ALA and may explain why the inhibitor AOB was 2000 times worse at competing with ALA for the A-site of *E. coli* ALAD when compared to the pea ALAD. Despite the greater affinity of AOB for the A-site of pea ALAD, no formation of any abortive Shiff base intermediate between P-site ALA and A-site bound AOB was detected.

The inhibitor SA was able to form a stable adduct with both pea and *E. coli* ALADs although reduction by $NaBH_4$ was required to observe the adduct species under the acidic conditions required for ESMS. Both pea and *E. coli* ALAD exhibited a time-dependent inactivation with AOHD, giving rise to a stable inactive complex; however, this could only be demonstrated by MALDI-MS after prior reduction with $NaBH_4$, implying that inhibition by AOHD was being mediated *via* a Schiff base. The competition between SA, AOHD, and ALA for enzyme modification was attributable to reaction with the same residue, the active site Lys-247 in *E. coli* ALAD and Lys-293 in the pea enzyme.

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REFERENCES

Boese, Q. F., Spano, A. J., Li, J., & Timko, M. P. (1991) *J. Biol. Chem. 266*, 17060−17066.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Cheh, A. M., & Neilands, J. B. (1976) *Struct. Bond. 29*, 123−169.
Dent, A. J., Beyersmann, D., Block, C., & Hasnain, S. S. (1990) *Biochemistry 26*, 7822−7828.
Echelard, Y., Dymetryszyn, J., Drolet, M., & Sasarman, A. (1988) *Mol, Gen. Genet. 214*, 503−508.
Gibbs, P. N. B., & Jordan, P. M. (1986) *Biochem. J. 236*, 447−451.
Gibbs, P. N. B., Gore, M. G., & Jordan, P. M. (1985) *Biochem. J. 225*, 573−580.
Ha, H.-J., Park, J. W., Oh, S.-J., Lee, J.-C., & Song, C. (1993) *Bull. Korean Chem. Soc. 14*, 652−654.
Jaffe, E. K. (1995) *J. Bioenerg. Biomembr. 27*, 169−179.
Jaffe, E. K., & Hanes, D. (1986) *J. Biol. Chem. 261*, 9348−9353.
Jones, M. E., Jenkins, J. M., Smith, A. G., & Howe, C. J. (1994) *Plant. Mol. Biol. 24*, 435−448.
Jordan, P. M. (1991) *New Comprehen. Biochem. 19*, 1−66.
Jordan, P. M., & Seehra, J. S. (1980) *FEBS Lett. 114*, 283−286.
Jordan, P. M., & Gibbs, P. N. B. (1985) *Biochem. J. 227*, 1015−1020.
Laemmli, U. K., & Favre, M. (1973) *J. Mol. Biol. 80*, 573−599.
Liedgens, W., Lutz, C., & Schneider, H. A. W. (1983) *Eur. J. Biochem. 135*, 75−79.
Lüöng, R. M., Walker, J., & Neier, R. W. (1992) *J. Org. Chem. 57*, 5005−5013.
Mauzerall, D., & Granick, S. (1956) *J. Biol. Chem. 216*, 435−446.
Nandi, D. L., Baker-Cohen, K. F., & Shemin, D. (1968) *J. Biol. Chem. 243*, 1224−1230.
Portis, A. R., & Heldt, H. W. (1976) *Biochim. Biophys. Acta 449*, 434−446.
Roessner, C. A., Spencer, J. B., Ozaki, S., Min, C., Atshaves, B. P., Nayar, P., & Anousis, N. (1995) *Protein Expression Purif. 6*, 155−163.
Spencer, P., & Jordan, P. M. (1993) *Biochem. J. 290*, 279−287.
Spencer, P., & Jordan, P. M. (1994) *Biochem. J. 300*, 373−381.
Spencer, P., & Jordan, P. M. (1995) *Biochem. J. 305*, 151−158.

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