Investigation of the nature of the two metal-binding sites in 5-aminolaevulinic acid dehydratase from *Escherichia coli*

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Two distinct metal-binding sites, termed α and β, have been characterized in 5-aminolaevulinic acid dehydratase from *Escherichia coli*. The α-site binds a Zn^{2+} ion that is essential for catalytic activity. This site can also utilize other metal ions able to function as a Lewis acid in the reaction mechanism, such as Mg^{2+} or Co^{2+}. The β-site is exclusively a transition-metal-ion-binding site thought to be involved in protein conformation, although a metal bound at this site only appears to be essential for activity if Mg^{2+} is to be bound at the α-site. The α- and β-sites may be distinguished from one another by their different abilities to bind divalent-metal ions at different pH values. The occupancy of the β-site with Zn^{2+} results in a decrease of protein fluorescence at pH 6. Occupancy of the α- and β-sites with Co^{2+} results in u.v.–visible spectral changes. Spectroscopic studies with Co^{2+} have tentatively identified three cysteine residues at the β-site and one at the α-site. Reaction with *N*-ethyl[^{14}C]maleimide preferentially labels cysteine-130 at the α-site when Co^{2+} occupies the β-site.

INTRODUCTION

5-Aminolaevulinic acid dehydratase [ALAD; also named porphobilinogen synthase (PBGS), EC 4.2.1.24] catalyses the synthesis of porphobilinogen (PBG) from two molecules of 5-aminolaevulinic acid (ALA), as shown in Scheme 1. The enzyme has been purified from many sources, including bovine liver (Gibson et al., 1955), human erythrocytes (Anderson and Desnick, 1979; Gibbs et al., 1985), bacteria, including *Rhodopseudomonas capuslatus* (Nandi and Shemin, 1972), *Rhodopseudomonas sphaeroides* (Nandi et al., 1968) and *Escherichia coli* (Spencer and Jordan, 1993; Mitchell and Jaffe, 1993) and spinach (*Spinacia oleracea*) (Liedgens et al., 1983).

Of the two molecules of 5-aminolaevulinic acid that make up porphobilinogen, the one that gives rise to the propionic acid side of the product binds first to the enzyme at the 'P' site (Jordan and Seehra, 1980) via a Schiff base with a highly conserved lysine residue (Jordan and Gibbs, 1985, 1986). All ALA dehydratases exhibit an absolute requirement for bivalent-metal ions for activity and are inhibited by the metal chelator EDTA. The ALAD from the photosynthetic bacterium *R. sphaeroides*, although not susceptible to immediate inhibition by EDTA (Nandi et al., 1968), does show Mg^{2+}-dependent activity following prolonged dialysis with EDTA (Shemin, 1972). It has been suggested that the metal ion may play a role in the binding of the second molecule of 5-aminolaevulinic acid at the 'A' site (Jordan, 1991).

The 5-aminolaevulinic acid dehydratases isolated appear to fall into two classes: those requiring Zn^{2+} [e.g. human (Gibbs et al., 1985); *Escherichia coli* (Spencer and Jordan, 1993)] and those requiring Mg^{2+} ions [e.g. spinach (Liedgens et al., 1983); pea (Boese et al., 1991)]. The structural basis for these differences appear to arise from the nature of the ligands in the proposed metal-binding domain (Boese et al., 1991; Jordan, 1991). Those ALADs requiring Zn^{2+} ions possess a region containing four cysteine residues in close proximity to one another that are implicated in metal binding. Oxidation of these cysteine residues to form disulphide bonds leads to reversible inactivation. In the case of *E. coli* ALAD, two such disulphide bonds are formed (Spencer and Jordan, 1993). The presence of these cysteine residues accounts for the requirement of an exogenous thiol to maintain activity of the Zn^{2+}-dependent dehydratases, although under anaerobic conditions the thiol is unnecessary. In the case of ALADs requiring Mg^{2+} ions, the cysteine residues implicated in metal binding are replaced by aspartic acid residues providing an explanation for the change in metal-ion requirement and for the stability of these dehydratases to oxidizing conditions (Boese et al., 1991; Jordan, 1991).

The stoichiometry of metal binding in bovine and human ALADs has been shown to be one per subunit (Tsukamoto et al., 1979; Gibbs, 1984). A linear correlation between metal content and activity was found for the human ALAD (Gibbs, 1984), whereas full activity was observed on addition of only half the stoichiometric amount of Zn^{2+} ions to bovine apo-ALAD (Bevan et al., 1980; Jaffe et al., 1984). In contrast, ALAD from *E. coli* has been shown to bind two Zn^{2+} ions per subunit (Spencer and Jordan, 1993; Mitchell and Jaffe, 1993), although the second molar equivalent of Zn^{2+} may be substituted for by Mg^{2+}, leading to greatly increased enzyme activity (Spencer and Jordan, 1993). Comparison of the *E. coli* protein sequence deduced from the *hemB* gene (Echelard et al., 1988; Li et al., 1989) with those deduced from the cDNAs from human ALAD (Wetmer et al., 1986) and plant ALAD (Boese et al., 1991) indicates substantial similarities. Notably, the sequence including for four cysteine residues of the proposed metal-binding domain are more closely related in the bacterial and mammalian enzymes. However, some residues in the proposed metal-binding region of the *E. coli* dehydratase are different from mammalian ALADs, but identical with conserved residues in plant ALADs. In particular, two alterations of a 'soft' (cysteine -SH) and a 'borderline' (histidine

Abbreviations used: ALAD, 5-aminolaevulinic acid dehydratase; PBGS, porphobilinogen synthase; PBG, porphobilinogen; ALA, 5-aminolaevulinic acid; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; NEM, *N*-ethylmaleimide; PTH, phenylthiohydantoin; ATZ, anilinothiazolinone; to specify metal binding, α- and β-sites are used in this paper rather than the A- and B-sites used elsewhere (Dent et al., 1990); this is to avoid possible confusion between the A and P substrate-binding sites.

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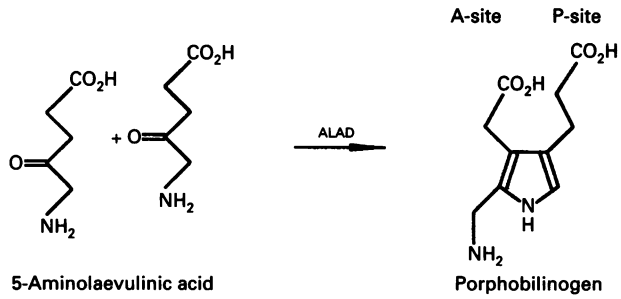


Scheme 1 The reaction catalysed by ALAD

Abbreviations: A, acetic acid side chain (-CH_2CO_2H); P, propionic acid side chain (-$CH_2CH_2CO_2H$).

N) to 'hard' ligands (aspartate -COOH) are apparent (Boese et al., 1991), which may provide the binding site for the Mg^{2+} ion (Glusker, 1991).

Investigation of the possible metal ligands in the bovine ALAD by e.x.a.f.s. suggests the presence of two distinct metal-ion-binding sites, one of which is cysteine-rich (Dent et al., 1990). It has been shown that Co^{2+} ions can substitute for Zn^{2+} in *E. coli* ALAD, resulting in a characteristic charge-transfer spectrum indicative of cysteine co-ordination (Spencer and Jordan, 1993). The present paper investigates the nature and function of the two metal-binding sites, termed α- and β-, in *E. coli* ALAD, using the techniques of protein fluorescence, chemical modification and visible spectroscopy using Co^{2+} ion as a probe for the metal-binding sites.

MATERIALS AND METHODS

Chemicals

5-Aminolaevulinic acid, 2-mercaptoethanol, *Staphylococcus aureus* V8 proteinase and Trizma base were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Column chromatography supplies were obtained from Pharmacia Ltd. *N*-Ethyl[2,3-$^{14}C_2$]maleimide and iodo[2-^{14}C]acetic acid were purchased from Amersham International. All other chemicals were purchased from BDH, Poole, Dorset, U.K.

Isolation of *E. coli* ALAD and enzymic assay

E. coli ALAD was purified and assayed according to the method of Spencer and Jordan (1993). A recombinant strain of *E. coli* harbouring the *hemB* gene was constructed and generously provided by Dr. C. Roessner (Chemistry Department, Texas A&M University, College Station, TX, U.S.A.). The specific activity of purified *E. coli* ALAD was 33 units·mg^{-1} at pH 6.8.

Thiol-group determination

ALAD (50 μg; 1.4 nmol) was denatured in 40 μl of 50 mM potassium phosphate buffer, pH 8, containing 4 M guanidinium chloride and 5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was then added. The solution was diluted in the same buffer and the absorbance at 412 nm (ϵ_{412} = 14750 $M^{-1}\cdot cm^{-1}$) was determined. The number of thiol groups was measured in all experiments by this method to confirm that the thiol status of the enzyme species under investigation was known.

Metal substitution

Apo-ALAD was generated from the holoenzyme by overnight incubation at 4 °C with 10 mM EDTA in 50 mM potassium phosphate buffer, pH 6, containing 20 mM β-mercaptoethanol. Subsequent gel filtration of apo-ALAD in potassium phosphate buffer, pH 6 or 8, under nitrogen removed EDTA and β-mercaptoethanol. Different metal-ion solutions (under nitrogen) were then added to apo-ALAD at various molar excesses (see the Figures). The resulting enzyme species were then rapidly gel-filtered using a Sephadex G-25 (medium) column (10 cm × 1 cm) in the above buffer, under nitrogen, to remove excess unbound metal ion before metal-ion analysis. Addition of Co^{2+} ions to solutions containing β-mercaptoethanol resulted in the formation of a brown precipitate. Therefore the addition of Co^{2+} ions to apo-ALAD necessitated the use of an anaerobic environment (N_2 gas) to protect the sensitive ALAD thiol groups from aerial oxidation and to prevent the formation of Co^{2+}–mercaptoethanol complexes.

Atomic-absorption studies

Samples for metal-ion determination (Co^{2+} and Zn^{2+}) were injected into an atomic-absorption spectrometer (Instrumentation Laboratory 157) and the metal-ion concentrations were determined against the appropriate standard curves. Samples for analysis were adjusted by dilution to bring the ion concentration into the linear response region of the standard curve (Zn^{2+} in the 5–30 μM range and Co^{2+} in the 5–40 μM range).

Fluorimetry

Apo-ALAD (8–50 μM, 0.5–2 mg·ml^{-1}) was titrated with Zn^{2+} ions at 15 °C with excitation at 280 nm (slit width 10 nm) and emission at 330 nm (slit width 10 nm) in a Hitachi F-2000 fluorimeter.

Determination of 5-thio-2-nitrobenzoic acid (TNB) content and spectral properties of DTNB-modified Co^{2+}-ALAD

The amount of TNB bound to DTNB-modified Co^{2+}-ALAD (see the Results and discussion section) was determined by reduction using β-mercaptoethanol under denaturing conditions (4 M guanidinium chloride in 100 mM potassium phosphate buffer, pH 8). The increase in absorbance at 412 nm resulting from the release of bound TNB from the ALAD was 65% of that initially observed on reaction of DTNB with ALAD, indicating that some disulphide bond formation may have occurred (maximum 0.17 mol equiv.). Knowing the amount of TNB bound to the DTNB-modified ALAD allowed the absorbance of the bound TNB to be corrected for. The absorbance correction was achieved by subtraction of the absorbance of TNB (determined as one half that of DTNB) from that of the DTNB-modified CO^{2+}-ALAD.

N-Ethyl[2,3-$^{14}C_2$]maleimide ([^{14}C]NEM) modification of Co^{2+}-ALAD

A sample (3.8 ml) of Co^{2+}-ALAD (see the Results and discussion section) containing 3.4 mg of protein/ml (equivalent to 0.36 μmol) was allowed to react with 0.8 mol-equiv. of [^{14}C]NEM (sp. radioactivity 6 μCi·$\mu$$mol^{-1}$; 10560 d.p.m.·$nmol^{-1}$ in 50 mM potassium phosphate, pH 6, under nitrogen. After 1 h, the sample was gel-filtered to remove excess NEM, giving 0.22 μmol of protein with 1.6 × 10^6 d.p.m. of incorporated label (0.7 mol of NEM per subunit).

Iodoacetic acid modification of ALAD

The sample of ALAD, previously modified with NEM as described above, was then denatured in 200 mM potassium phosphate, pH 8, containing 10 mM β-mercaptoethanol for 30 min, and the remaining cysteine residues were modified by the addition of 20 mM iodoacetic acid for 1 h. The sample was then dialysed against 2 x 4 litres of 2 mM potassium phosphate buffer, pH 8, and freeze-dried.

V8 proteinase digestion of ALAD

The radiolabelled freeze-dried sample of ALAD was redissolved in 50 mM potassium phosphate buffer, pH 8, containing 1 mM EDTA, and was digested with V8 proteinase (1 %, w/w) for 16 h at 37 °C. After digestion, 89 % of the ^{14}C radioactivity remained in the soluble fraction (1×10^6 d.p.m.).

Purification of peptides by h.p.l.c.

The soluble peptides (in 100 μl aliquots containing 20 nmol of label) from V8 protease digestion were applied to a C_{18} reverse-phase h.p.l.c. column using a linear gradient of 0.5 % trifluoroacetic acid in water/0.1 % trifluoroacetic acid in acetonitrile.

RESULTS AND DISCUSSION

Effect of pH on the correlation of Zn^{2+} content with ALAD activity

It has been shown previously that metal-depleted *E. coli* ALAD (apo-ALAD) is inactive and that activity can be restored on the addition of Zn^{2+} ions to the reduced apoenzyme (Spencer and Jordan, 1993). To obtain preliminary information about the nature of interaction of Zn^{2+} with ALAD, a study of the restoration of activity on metal-ion binding at different pH values was conducted. Figure 1 shows the effect of pH on the relationship between enzymic activity and Zn^{2+} content of ALAD from *E. coli*. Re-addition of Zn^{2+} ions in 50 mM potassium phosphate buffer, pH 6, containing 10 mM β-mercaptoethanol, was found to restore full activity only after the binding of two Zn^{2+} ions per subunit. In contrast, at pH 8, the binding of only one mol of Zn^{2+}/mol of ALAD subunit was sufficient to restore near maximum (81 %) specific activity. A possible explanation for the same Zn^{2+} stoichiometry restoring different activities is that there are two distinct Zn^{2+}-binding sites present, only one of which, when filled, restored catalytic activity (termed the 'α-site'). This α-site may be occupied preferentially by Zn^{2+} at higher pH values, while the other, 'non-catalytic' site (termed the β-site), is preferentially filled at the lower pH. The change in the relationship between Zn^{2+} content and ALAD activity at pH 6 compared with pH 8 may therefore indicate a change in the relative affinities of the two metal-ion-binding sites, possibly resulting from the protonation of histidine residues, which can often act as Zn^{2+} ligands (Christianson, 1991). Addition of half-stoichiometric amounts of Zn^{2+} to bovine ALAD at pH 7 has been reported to restore maximum specific activity (Bevan et al., 1980; Jaffe et al., 1984), whereas a linear correlation between metal content and activity was reported for human ALAD at pH 7 (Gibbs, 1984).

Protein fluorescence decrease on Zn^{2+} binding to apo-ALAD at pH 6

Addition of Zn^{2+} ions to apo-ALAD in 50 mM potassium phosphate buffer, pH 6, containing 10 mM β-mercaptoethanol, at 15 °C, resulted in a decrease in protein fluorescence monitored at 330 nm after excitation at 280 nm. Klotz analysis (Stinson and Holbrook, 1973) indicated the presence of one binding site per subunit with a K_d of 6 μM (± 2 μM) (Figure 2). To investigate the relationship between Zn^{2+} binding and the increase in enzymic activity, a correlation between the fluorescence signal change

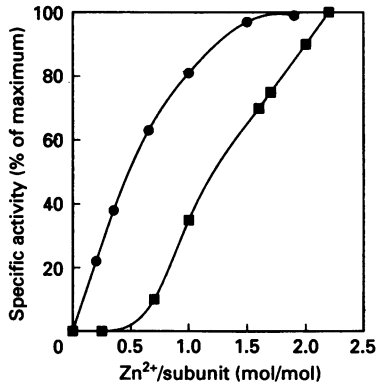


Figure 1 Correlation of *E. coli* ALAD activity with Zn^{2+} content at pH 6 and pH 8

E. coli apo-ALAD (150 μM) was titrated with Zn^{2+} ions (0–750 μM) in either 50 mM potassium phosphate buffer, pH 8 (●), or pH 6 (■), containing 10 mM β-mercaptoethanol. Samples were removed and gel-filtered in 50 mM potassium phosphate buffer, pH 8 or pH 6, containing 10 mM β-mercaptoethanol. Enzyme activity was then determined by assay and Zn^{2+} content by atomic absorption.

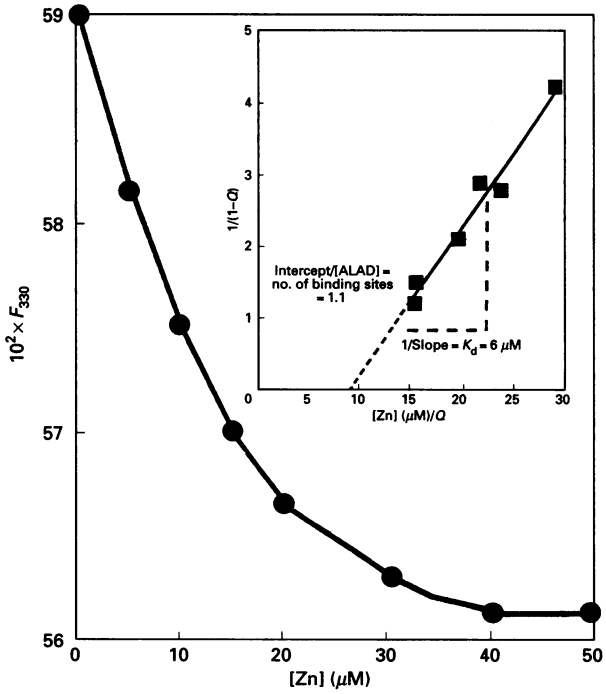


Figure 2 Reduction of protein fluorescence of apo-ALAD on titration with Zn^{2+} at pH 6

E. coli apo-ALAD (8 μM) was titrated with Zn^{2+} ions in 50 mM potassium phosphate buffer, pH 6, containing 10 mM β-mercaptoethanol at 15 °C and protein fluorescence was monitored (excitation at 280 nm; emission at 330 nm). The inset shows the Klotz analysis where Q = fractional saturation indicating one Zn^{2+} binding site with a K_d = 6 μM.

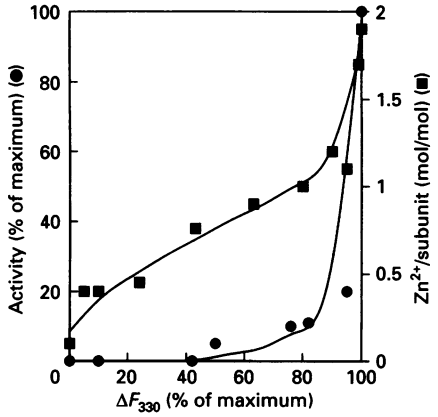


Figure 3 Correlation of fluorescence change at 330 nm with enzyme activity and Zn^{2+} content of ALAD

E. coli apo-ALAD (50 μM) was titrated with Zn^{2+} ions in 50 mM potassium phosphate buffer, pH 6, containing 10 mM β-mercaptoethanol at 15 °C, and protein fluorescence was monitored (excitation at 280 nm; emission at 330 nm). Samples were taken at various F_{330} values and gel-filtered to remove unbound Zn^{2+}; their enzyme activity (●) and metal content (■) were then determined.

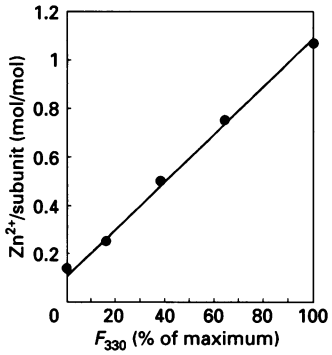


Figure 4 Correlation of fluorescence at 330 nm with Zn^{2+} content of ALAD in the presence of excess Mg^{2+} at pH 6

E. coli apo-ALAD (50 μM) was titrated with Zn^{2+} ions in 50 mM potassium phosphate buffer, pH 6, containing 10 mM β-mercaptoethanol at 15 °C in the presence of 300 μM Mg^{2+}. Protein fluorescence was monitored (excitation at 280 nm; emission at 330 nm). Samples were taken at various F_{330} values and gel-filtered to remove unbound metal ions; their Zn^{2+} contents were then determined.

against activity and Zn^{2+} content was established (Figure 3). The binding of 1 equiv. of Zn^{2+} was found to restore only 25% of the activity, yet gave nearly the maximum reduction (94%) of the fluorescence signal at 330 nm. This suggested that Zn^{2+} ion binding to a metal-binding site that does not restore activity (termed the β-site) can be monitored conveniently by using protein fluorescence.

The exact cause of the protein fluorescence change has not been established, although similar results were obtained on excitation at 295 nm, which indicates the probable involvement of a tryptophan residue. This indicates that the decrease in protein fluorescence is arising from a conformational change rather than from a direct interaction with tyrosine, a potential metal ligand. The binding of Mg^{2+} to ALAD has been shown to be dependent on the prior binding of Zn^{2+} (Spencer and Jordan, 1993). Therefore the binding of Mg^{2+} to ALAD may be reliant on this conformational change (promoted by Zn^{2+} binding initially to the β-site) and may thus account for the inability of apo-ALAD to bind Mg^{2+} alone.

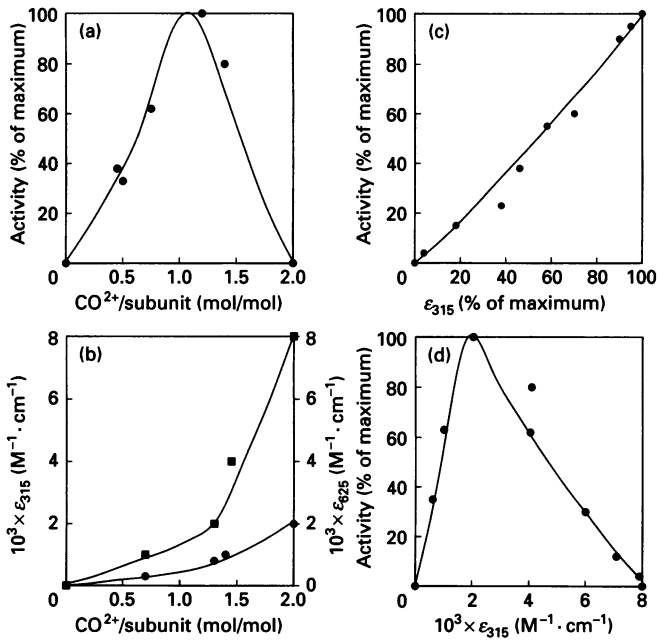


Figure 5 Interaction of Co^{2+} with *E. coli* ALAD

(**a**) Correlation of activity against Co^{2+} content for ALAD. *E. coli* apo-ALAD (150 μM) was titrated with Co^{2+} ions (0–500 μM) in 50 mM potassium phosphate buffer, pH 8. Samples were removed for gel filtration in 50 mM potassium phosphate buffer, pH 8, under nitrogen. Enzyme activity was then determined by assay and Co^{2+} content by atomic absorption. (**b**) Correlation of ϵ_{315} and ϵ_{625} with Co^{2+} content of ALAD. *E. coli* apo-ALAD (150 μM) was titrated with Co^{2+} ions (0–500 μM) in 50 mM potassium phosphate buffer, pH 8. The absorption coefficients per mol of ALAD are shown at 315 nm (■) and 625 nm (●). Samples were removed for gel filtration in 50 mM potassium phosphate buffer, pH 8, under nitrogen. The Co^{2+} content was then determined by atomic absorption. (**c**) Correlation of activity with ϵ_{315} for Co^{2+}-ALAD at pH 6. *E. coli* apo-ALAD (160 μM) was titrated with Co^{2+} ions (0–1 mM) in 50 mM potassium phosphate buffer, pH 6. The percentage of the maximum absorption coefficient per mol of ALAD is shown at 315 nm (100% = 1500 $M^{-1} \cdot cm^{-1}$). Samples were removed for determination of enzymic activity. (**d**) Correlation of activity with ϵ_{315} for Co^{2+}-ALAD at pH 8. *E. coli* apo-ALAD (150 μM) was titrated with Co^{2+} ions (0–500 μM) in 50 mM potassium phosphate buffer, pH 8, under nitrogen. The absorption coefficient per mol of ALAD at 315 nm is shown. Samples were removed for determination of enzymic activity.

Repeating the Zn^{2+} titration of apo-ALAD (50 μM) in the presence of excess Mg^{2+} (300 μM), conditions that allow the immediate filling of the α-site with Mg^{2+} after Zn^{2+} has bound at the β-site, did not alter significantly the magnitude of the fluorescence decrease at 330 nm. A linear correlation of fluorescence decrease against the binding of 1 mol-equiv. of Zn^{2+} was found, indicating that fluorescence decrease resulted solely from β-site occupancy (Figure 4).

Correlation of Co^{2+} content and absorbance with ALAD activity at pH 8

The addition of Co^{2+} ions to apo-ALAD was undertaken under anaerobic conditions (see the Materials and methods section). *E. coli* ALAD has been shown previously to be inactive with two Co^{2+} ions bound and exhibits a charge-transfer absorption band characteristic of cysteine residues in the Co^{2+} ion ligation sphere (Spencer and Jordan, 1993). Further studies revealed that the full spectral characteristic of the two Co^{2+} species developed over a

Table 1 Properties of various metallo-species of *E. coli* ALAD

The activity of $0_{\alpha}Zn_{\beta}$-ALAD and $Zn_{\alpha}0_{\beta}$-ALAD species generated at pH 6 and pH 8 respectively was not determined (nd) outside this pH, as ion mobility to the other metal-ion-binding site may have occurred. The asterisk (*) indicates that some activity was detected, due to very low occupancy of the α-site; see Figure 3. Although the occupancy of the α- and β-sites is referred to as either '0' or '1' because of difficulty in loading the two sites precisely, there is likely to be some degree of variation from these 'ideal' values.

Metal bound		Specific activity		Absorption coefficient (ϵ)		Available cysteine residues
α-	β-	pH 6.8	pH 8	315 nm	625 nm	
0	Zn	< 7*	nd	–	–	4
Zn	Zn	33	80	–	–	4
Mg	Zn	130	120	–	–	4
Co	Zn	110	100	1600	200	4
0	Co	Inactive		6400	1600	1
Zn	Co	Inactive		6400	1600	1
Co	Co	Inactive		8000	2000	1
Co	0	125	100	1600	200	4
Zn	0	nd	66	–	–	4

period of time after exposure of apo-ALAD to 1 mM Co^{2+} ions. During this time the catalytic activity, although decreasing, was still detectable. A more detailed investigation correlating Co^{2+} content with enzymic activity (Figure 5a) indicated that maximum specific activity (100 units·mg^{-1}), similar to that supported by Zn^{2+} (80 units·mg^{-1} at pH 8; Table 1) could be restored to apo-ALAD by the binding of 1.2 mol of Co^{2+}/mol of subunit, predominantly at the α-site (Figure 5a). The subsequent binding of a second mol of Co^{2+}/mol of subunit, presumably to the β-site, resulted in the complete loss of ALAD activity (Figure 5a). Determination of the absorbance spectrum of ALAD containing between 0 and 2 mol of Co^{2+}/mol of subunit indicated that cysteine ligands may be associated with both the α- and the β-sites (Figure 5b). The magnitude of the absorption coefficients at 315 nm and 625 nm, with 1 mol-equiv. of Co^{2+} bound, increased markedly from 1500 and 200 $M^{-1}\cdot cm^{-1}$ respectively to 8000 and 2000 $M^{-1}\cdot cm^{-1}$ respectively on the binding of the second mol-equiv. of Co^{2+} to the β-site (Figure 5b). The disproportionate increase in the magnitude of the absorption coefficients at 315 and 625 $M^{-1}\cdot cm^{-1}$ indicates that several cysteine ligands are involved in the binding of the second mol-equiv. of Co^{2+} to ALAD at the β-site. Substitution of Co^{2+} into proteins of known cysteine content indicates an absorption coefficient at 310–350 nm of between 1500 and 2000 per cysteine ligand (Garbett et al., 1972; Maret et al., 1979). Applying this value, the absorption coefficient of 1600 observed for Co^{2+} binding to the α-site of *E. coli* ALAD would suggest the involvement of a single cysteine residue. The further increase in the absorbance at 315 nm on the binding of the second mol of Co^{2+} at the β-site suggests the involvement of three or four additional cysteine ligands. These results are comparable with the number of cysteine residues contributing to the catalytic and structural sites (one and four respectively) in bovine ALAD as determined by e.x.a.f.s. studies (Dent et al., 1990).

The interaction between *E. coli* ALAD and Co^{2+} ions has been shown in the present study to be sensitive to the presence of exogenous thiols and the pH of the buffer. Whether, or to what extent, enzyme activity is exhibited after the addition of Co^{2+} ions to *E. coli* apo-ALAD is dependent on how much Co^{2+} can bind under a given set of conditions. The variation in activity found may help to explain the conflicting reports of the level of activation on bovine ALAD by Co^{2+} ions (Bevan et al., 1980; Cheh and Neilands, 1976). The inability to regenerate active bovine Co^{2+}-ALAD (Jaffe et al., 1984) may also be attributable to the addition of Co^{2+} ions in the presence of β-mercaptoethanol, since addition of Co^{2+} ions to β-mercaptoethanol alone results in the formation of a brown colloidal precipitate. Table 1 summarizes some of the properties of the metallo-species of ALAD studied here.

Effect of pH on the correlation between the absorbance spectrum of Co^{2+}-ALAD and enzymic activity

Initial binding of Co^{2+} to apo-ALAD was predominantly at the α-site, both at pH 6 and pH 8, although a much lower affinity for Co^{2+} at pH 6 was apparent from the requirement of exogenous Co^{2+} to maintain activity. The increase in activity observed at both pH 6 and 8 on addition of Co^{2+} ions to the apo-ALAD correlated with an increase in absorbance at 315 and 625 nm resulting in absorption coefficients of about 1500 $M^{-1}\cdot cm^{-1}$ and 300 $M^{-1}\cdot cm^{-1}$ respectively (Figures 5c and 5d). The subsequent decline of ALAD activity at pH 8 on the further addition of Co^{2+} ions correlated with the further increase in the magnitude of the absorption coefficients at 315 nm and 625 nm (Figure 5c) resulting from the binding of the second mol-equiv. of Co^{2+} to the β-site (as discussed above). Lowering the pH of ALAD with 2 mol-equiv. of Co^{2+} bound (from pH 8 to 6), in the presence of 500 μM exogenous Co^{2+}, did not result in any significant change in the absorption coefficient (the ϵ_{315} decreased by less than 500 $M^{-1}\cdot cm^{-1}$). The virtual pH-independence of the absorption coefficients at 315 and 625 nm indicates that the lower absorbance at 315 and 625 nm of Co^{2+}-ALAD at pH 6 is due to the inability to bind Co^{2+} at the second, cysteine-rich, β-site, at least in the presence of a 7-fold molar excess (1 mM) of Co^{2+}.

The ability of Co^{2+} to bind to the β-site at pH 6 and its slow kinetics of binding at pH 8 permitted the determination of the K_d for Co^{2+} at the α-site with little interference from metal binding at the β-site. The extent of Co^{2+} bound to the α-site could be determined by assessing the percentage maximum change in absorbance with maximum occupancy at the α-site resulting in an ϵ_{315} value of 1600 $M^{-1}\cdot cm^{-1}$. The K_d for Co^{2+} for the α-site fell from 260 μM at pH 6 to around 2 μM at pH 8 (Figure 6). This marked pH-dependence on K_d of the α-site is similar to that noted for the affinity of Zn^{2+} in glycerol dehydrogenase (Spencer et al., 1989), where two histidine residues have been implicated in Zn^{2+} binding (Spencer et al., 1991). The findings are also consistent with the e.x.a.f.s. study of bovine ALAD, which indicated that two histidine ligands may be present at the

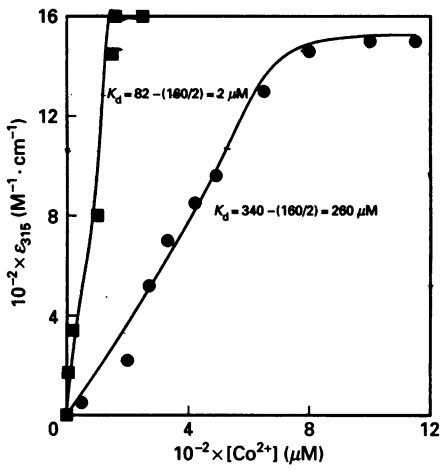


Figure 6 Titration of apo-ALAD with Co^{2+} at pH 8 and pH 6

E. coli apo-ALAD (160 μM) was titrated with Co^{2+} ions (0–1 mM) in 50 mM potassium phosphate buffer pH 6 (●) and pH 8 (■), and the absorption coefficient per mol of ALAD at 315 nm was determined. Only absorbance readings correlating with an increase (indicating Co^{2+} binding to the α-site) are shown for pH 8.

catalytic metal-binding site, termed the A-site by Dent et al. (1990) (see the Abbreviations footnote). Since *E. coli* ALAD can exhibit activity with Zn^{2+} or Co^{2+} bound only at the α-site, the occupancy of the β-site may not be mechanistically important.

Unfortunately, the much lower affinity of the α-site for Co^{2+} at pH 6 prevented isolation (by subsequent gel filtration in phosphate buffer at pH 6) of ALAD with Co^{2+} bound only at the α-site. Gel filtration of ALAD, with two Co^{2+} ions bound at pH 8, termed '$Co_\alpha Co_\beta$-ALAD', in phosphate buffer at pH 6, resulted in the loss of 0.8 mol-equiv. of Co^{2+} and a decrease in the absorption coefficients at 315 and 625 nm of 1100 $M^{-1} \cdot cm^{-1}$ and 200 $M^{-1} \cdot cm^{-1}$ respectively (1400 $M^{-1} \cdot cm^{-1}$ and 250 $M^{-1} \cdot cm^{-1}$/mol of Co^{2+} ion), indicating that Co^{2+} had been lost preferentially from the α-site. The resulting $0_\alpha Co_\beta$-ALAD (0_α = no metal bound at the α-site) was still inactive (Table 1). Although Co^{2+} did not bind to the β-site at pH 6, once bound to the β-site at pH 8, subsequent lowering of the pH to 6 did not result in the loss of Co^{2+} (as determined above by the characteristic spectrum and inactivity of this Co^{2+} species), presumably as a result of kinetic factors.

The ability of Zn^{2+}, Co^{2+} and Mg^{2+} to support enzyme activity by binding to the α-site suggests strongly that this site is directly involved in the catalytic mechanism (Scheme 2). Since all three metal ions are well known to participate as Lewis acids at the active sites of a range of metalloenzymes (Glusker, 1991), it is proposed that the role of the metal ion in ALADs is to polarize the carbonyl group of the 5-aminolaevulinic acid molecule at the 'A' site, facilitating both the aldol condensation and the Schiff-base formation (Scheme 2).

Mixed Zn/Co metallo-species of ALAD

Zn^{2+} was found to be able to compete with Co^{2+} for both the α- and β-metal-ion-binding sites. The two mixed metallo-ALAD species of Zn^{2+} and Co^{2+} were constructed with Zn^{2+} at the α-site and Co^{2+} at the β-site and vice versa. ALAD with Zn^{2+} at the α-site and Co^{2+} at the β-site (termed '$Zn_\alpha Co_\beta$-ALAD') was constructed by exposure of apo-ALAD (400 μM) to 1 mM Co^{2+} in 50 mM potassium phosphate, pH 8 under nitrogen, giving $Co_\alpha Co_\beta$-ALAD. Gel filtration of this species in 50 mM potassium phosphate, pH 6, under nitrogen, resulted in the loss of Co^{2+} from the catalytic α-site (as discussed above). Subsequent addition of excess Zn^{2+} (200 μM), followed by gel filtration at pH 6, under nitrogen, yielded a species containing 1.0 mol (±0.1) of Co^{2+} and 1 mol (±0.1) of Zn^{2+} per mol of subunit and having an absorption coefficient of 6400 $M^{-1} \cdot cm^{-1}$ at 315 nm. This $Zn_\alpha Co_\beta$-ALAD species was devoid of enzymic activity.

The other mixed metallospecies, containing Co^{2+} at the α-site and Zn^{2+} at the β-site (termed '$Co_\alpha Zn_\beta$-ALAD'), was constructed by exposure of apo-ALAD (400 μM) to 1 mM Co^{2+} in 50 mM potassium phosphate, pH 8, under nitrogen, but, before the second equivalent of Co^{2+} was allowed to bind (due to slower kinetics of binding as discussed above), 1 mol-equiv. of Zn^{2+} ions (400 μM) was added and the sample gel was gel-filtered in phosphate buffer, pH 8, under nitrogen. Analysis of the resulting species revealed an absorption coefficient, at 315 nm, of 1600 $M^{-1} \cdot cm^{-1}$ and a metal content of 1 mol (±0.1) of Co^{2+} and 1 mol (±0.1) of Zn^{2+} per mol of subunit. This species was found to exhibit a specific activity similar to that of the $Co_\alpha 0_\beta$-ALAD species (Table 1).

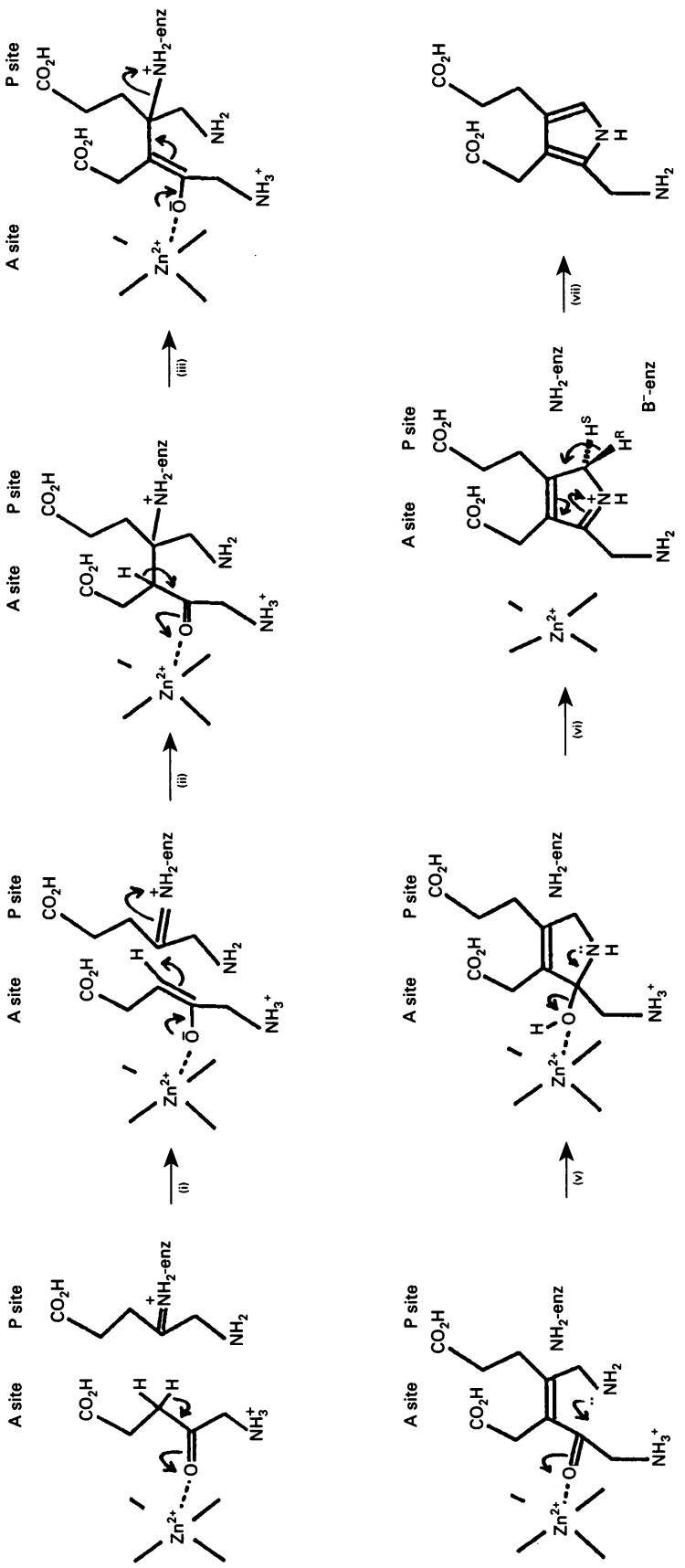
Effect of DTNB on the Co^{2+} content and spectrum of $Co_\alpha Co_\beta$-ALAD

Addition of DTNB (4-fold molar excess; 600 μM) to $Co_\alpha Co_\beta$-ALAD in 50 mM potassium phosphate buffer, pH 8, resulted in the rapid liberation of 0.8–1.0 mol of TNB/mol of subunit. This result is in sharp contrast with the reaction of $Zn_\alpha Zn_\beta$-ALAD with DTNB, when 4 mol of TNB/mol of subunit are released on oxidation (Spencer and Jordan, 1993). Determination of Co^{2+} bound to ALAD (after gel filtration at pH 8 to remove excess Co^{2+}) indicated that of the 2 mol-equiv. of Co^{2+} initially bound to ALAD, 1 mol-equiv. was lost as a result of DTNB modification. Analysis of the absorbance spectrum of DTNB-modified ALAD showed retention of the two absorbance peaks at 315 and 625 nm, although the magnitude of the absorption coefficient at 315 nm was increased, while that at 625 nm had decreased by 200 $M^{-1} \cdot cm^{-1}$. The increase in the absorption coefficient at 315 nm was a result of the covalent modification of ALAD by TNB. The absorbance increase resulting from TNB modification was determined and the absorption coefficient corrected accordingly (see the Materials and methods section). The adjusted absorption coefficient indicated that the absorbance at 315 nm had fallen by 2300 $M^{-1} \cdot cm^{-1}$/mol of cysteine modified, suggesting that the $0_\alpha Co_\beta$-ALAD species had been formed. The availability of essentially one cysteine for modification in $Co_\alpha Co_\beta$-ALAD, resulting in the loss of one mol of Co^{2+}/mol of subunit, is consistent with the α metal-ion-binding site contributing a cysteine residue as one of the Co^{2+} ligands.

Modification of the reactive cysteine of $Co_\alpha Co_\beta$-ALAD by NEM

A sample of $Co_\alpha Co_\beta$-ALAD was gel-filtered at pH 6 to remove the metal from the α-site to give $0_\alpha Co_\beta$-ALAD. On reacting a sample of this species with DTNB, 1 mol of TNB/mol of subunit was released. This reaction with DTNB could be abolished by prior exposure to 1 mol.-equiv. of NEM under nitrogen. Subsequent removal of any unchanged NEM by gel filtration revealed only 0.2 mol of free cysteine available after NEM treatment.

To identify the modified cysteine residue a further sample of $0_\alpha Co_\beta$-ALAD was labelled with [^{14}C]NEM as described in the Materials and method section. The remaining cysteine residues were then modified with iodoacetic acid, and the carboxymethylated protein was digested with V8 proteinase (see the Materials and methods section). The soluble peptides (accounting



Scheme 2 Proposed role of a metal ion as a Lewis acid in the mechanism of action of ALAD

The molecule of 5-aminolaevulinic acid initially bound to the enzyme at the P site does so through a Schiff base (Jordan and Gibbs, 1986). This is lysine-247 in the *E. coli* enzyme. The second molecule of 5-aminolaevulinic acid is bound at the A site. Reactions (i)–(iv) involve the aldol condensation, and reactions (v) and (vi) involve the formation of a Schiff base between the two substrate molecules. Though less favourable for stereoelectronic reasons, the Schiff-base formation could precede the aldol condensation (Jordan and Seehra, 1980). Reaction (vii) involves the stereospecific loss of H^R (Jordan, 1991).

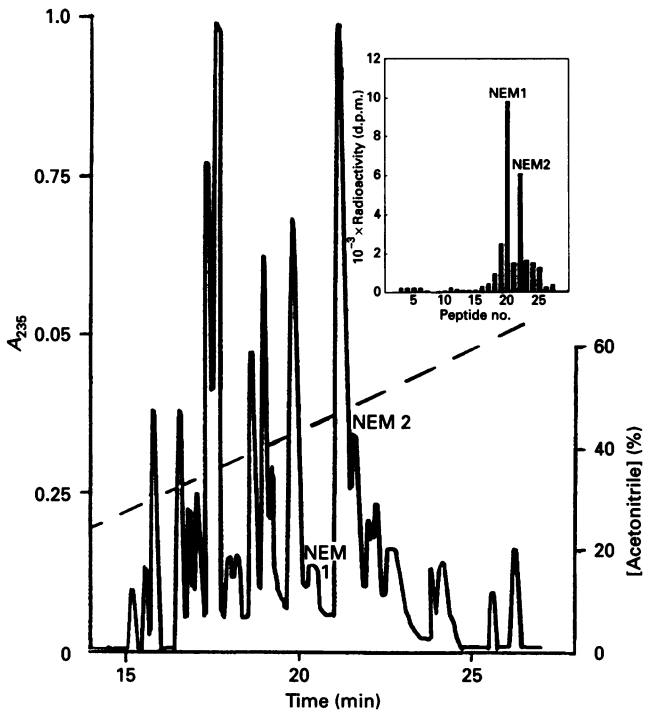


Figure 7 Separation of [^{14}C]NEM-labelled peptides containing the available cysteine in $0_\alpha Co_\beta$-ALAD

Initial separation, on a reverse-phase C_{18} column, of peptides from a V8-proteinase digestion after incorporation of [^{14}C]NEM label into the available cysteine of $0_\alpha Co_\beta$-ALAD. The acetonitrile gradient is shown by the broken line. The insert shows the radioactivity (d.p.m.) recovered in each peak.

for 89% of the label incorporated) were then subjected to h.p.l.c. (see the Materials and methods section). Two labelled peptide peaks, termed NEM1 and NEM2, were detected, containing 40 and 25% of the applied radioactivity respectively (Figure 7). These peptides were subsequently purified further, by a second passage down the C_{18} column, each giving a single labelled peptide containing over 80% of the recovered radioactivity. Yields of NEM1 and NEM2 during purification are shown in Table 2.

Peptide analysis and identification of the reactive cysteine residue of $Co_\alpha Co_\beta$-ALAD

Purified peptides were subjected to Edman sequence analysis, and amino acid residues were identified as the phenylthiohydantoin (PTH) derivatives. A second sequencing experiment was performed in which the amino acids were derivatized to their anilinothiazolinone (ATZ) derivatives and then collected for radioactivity counting, giving a 30–50% recovery of label (40–60% allowing for the repetitive yield). The results of the peptide sequencing are given in Table 3. The sequence of both peptides NEM1 and NEM2 (YTSHGHCGVLCE) agreed with the published gene-derived sequences for *E. coli* ALAD (Echelard et al., 1988; Li et al., 1989) (Table 3). Background sequences were obtained from NEM1 (RTELIGAYQVSG) and NEM2 (DGLVARMSPICKQ) that agreed with the known sequence, and although the contaminating peptide in NEM2 contained a cysteine residue, this was not labelled. The radioactivity obtained in NEM1 was equally distributed between cysteine (C)-130 and C-134 (Table 3). NEM1 comprised 40% of the total radioactive label initially incorporated, therefore each cysteine labelled represents 20% of the initial label incorporated. The label in NEM2 was found only at C-130, therefore, as NEM2 comprised 25% of the initial radioactivity incorporated, this site represents 25% of the initial label incorporated. Combining the total radioactive label at each site indicates that most (70%) had been incorporated at C-130, with a smaller amount (30%) at C-134. Therefore it would appear that the reactive cysteine residue, C-130, may be acting as an α-site Co^{2+} ligand. It should, however, be stressed that the presence of a single cysteine residue in the enzymically inactive $0_\alpha Co_\beta$-ALAD species does not guarantee that it is acting as an α-site ligand in the catalytically active $Co_\alpha 0_\beta$-ALAD species. Furthermore, in the $Mg_\alpha Zn_\beta$-ALAD species, the interaction of Mg^{2+}, a 'hard' metal ion, with a 'soft' cysteine ligand would not be ideal.

Chemical modification studies on bovine ALAD using 5-chlorolaevulinic acid, a substrate analogue, have identified possible ligands to the catalytic metal. Modification of a cysteine residue on incorporation of 0.5 mol of 5-chlorolaevulinic acid/mol of subunit resulted in the inactivation of bovine ALAD (Seehra and Jordan, 1981). Further work by Jaffe et al. (1992) identified this cysteine residue in the primary sequence of bovine ALAD and also identified a modified histidine residue. However, in the absence of any reported stoichiometry data for 5-chlorolaevulinic acid incorporation, it is unclear whether both these residues were equally labelled or if one or other was the major modification site. The residues in *E. coli* ALAD analogous to those modified in bovine ALAD by 5-chlorolaevulinic acid are Ser-229 (in place of the cysteine) and Gln-270 [in place of the histidine; numbering as in Spencer and Jordan (1993)]. Ser-229 is replaced by Thr in plant ALADs (utilizing Mg^{2+}) and Cys in mammalian ALAD (utilizing Zn^{2+}), indicating a possible role for this residue in metal binding and selectivity. As *E. coli* ALAD can utilize Zn^{2+} or Mg^{2+} (as well as Co^{2+}) at the α-site, alteration of Cys-229, a 'soft' ligand, to Ser, a 'hard' ligand, may assist in allowing the additional acceptance of Mg^{2+} at the α-site. Such a ligand substitution has been suggested for the Cys replacement

Table 2 Recovery of ^{14}C-labelled peptides from h.p.l.c.

The amount (nmol) and percentage recovery of labelled peptides after each passage down the C_{18} reverse-phase h.p.l.c. column is shown.

	First passage			Second passage			
Fraction	Total d.p.m. (%)	Label (nmol)	Yield (%)	Total d.p.m. (%)	Label (nmol)	Yield (%)	Final yield (%)
NEM1	40	12	13	85	4	33	4
NEM2	25	11	12	80	3	27	3

Table 3 Peptide sequencing of [^{14}C]NEM-labelled peptides from *E. coli* ALAD by Edman degregation as PTH and ATZ derivatives

(a) Peptide NEM1

Samples of this peptide (200 pmol) were sequenced as PTH derivatives. A total of 10000 d.p.m. were loaded, of which 40% was recovered in the ATZ derivatives.

Sequence (PTH)...	Y	T	S	H	G	H	C-130	G	V	L	C-134	E
Amount (pmol) (PTH)...	92	–	33	–	40	27	–	30	16	22	–	4
Radioactivity (%) (ATZ)...	–	–	–	–	–	–	27	13	4	–	30	19

(b) Peptide NEM2

Samples of this peptide (100 pmol) were sequenced as PTH derivatives. A total of 2700 d.p.m. were loaded, of which 25% was recovered in the ATZ derivatives.

Sequence (PTH)...	Y	T	S	H	G	H	C-130	G	V	L	C-134	E
Amount (pmol) (PTH)...	48	45	23	52	28	32	–	14	5	6	–	–
Radioactivity (%) (ATZ)...	–	–	–	–	–	8	60	16	8	–	–	–

by 'hard' aspartyl ligands at the proposed metal-binding domain in plant ALAD (Jordan, 1991; Boese et al., 1991). Whether plant ALADs also have both structural and catalytic binding sites remains to be established.

The Science and Engineering Research Council and Agriculture and Food Research Council are gratefully acknowledged for financial support to M.R.I. and P.M.B. respectively. Thanks are due to Dr. M. G. Gore and Mr. L. Hunt at the Protein Sequencing Unit, University of Southampton, for carrying out the Edman sequencing and radioactive analysis of ATZ derivatives. We are grateful to Dr. C. Roessner (Texas A&M University) for providing a recombinant strain of *E. coli* containing the *hemB* gene.

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Received 22 October 1993/29 December 1993; accepted 10 January 1994

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