# Purification and characterization of 5-aminolaevulinic acid dehydratase from *Escherichia coli* and a study of the reactive thiols at the metal-binding domain

Paul SPENCER and Peter M. JORDAN*

School of Biological Sciences, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, U.K.

5-Aminolaevulinic acid dehydratase (ALAD) from a recombinant strain of *Escherichia coli* was purified to homogeneity. The enzyme is a homo-octamer of subunit $M_r$ 36554±17. Enzyme activity was dependent on the presence of $Zn^{2+}$ ions and an exogenous thiol. Two molar equivalents of $Zn^{2+}$ are bound/mol of subunit under reducing conditions. On exposure to the metal chelator EDTA, the two $Zn^{2+}$ ions are removed, giving an inactive metal-depleted apo-ALAD. On oxidation of holo-ALAD, two disulphide bonds are formed with the loss of 1 mol of $Zn^{2+}$/mol of subunit. The formation of the first disulphide led to the loss of catalytic activity. Replacement of the two bound $Zn^{2+}$ ions with $Co^{2+}$ resulted in the formation of a green protein with a spectrum indicative of the presence of charge-transfer bands from one or more cysteine–$Co^{2+}$ ligands. While $Mg^{2+}$ could not activate apo-ALAD alone, it was able to substitute for the second molar equivalent of bound $Zn^{2+}$, leading to a further 4-fold stimulation in activity. The four cysteine residues involved in the formation of the two disulphide bonds were identified by protein-chemistry studies and were all located in a region of the protein extending from amino acid residues 120–134. Protein sequence data obtained in the present study has permitted the resolution of several differences between the published gene-derived protein sequences for ALAD from *E. coli*.

## INTRODUCTION

5-Aminolaevulinic acid dehydratase (ALAD; E.C. 4.2.1.24) catalyses the synthesis of porphobilinogen (PBG) from two molecules of 5-aminolaevulinic acid (ALA) via a Schiff-base intermediate (Nandi and Shemin, 1968a; Jordan and Gibbs, 1985). The enzyme has been purified from bovine liver (Gibson et al., 1955), human erythrocytes (Anderson and Desnick, 1979; Gibbs et al., 1985b), members of the Athiorhodaceae [*Rhodopseudomonas capsulatus* (Nandi and Shemin, 1972) and *Rhodopseudomonas sphaeroides* (Nandi et al., 1968)] and spinach (*Spinacia oleracea*) (Liedgens et al., 1983).

All mammalian ALAD enzymes exist as homo-octamers (Bevan et al., 1980) and require $Zn^{2+}$ for activity (Gibbs et al., 1985a). One $Zn^{2+}$-binding site is present per subunit in both the bovine (Tsukamoto et al., 1979) and human (Gibbs, 1984) enzymes. The contradictory report by Tsukamoto et al. (1979) that bovine apo-ALAD retained 80% of the activity of the holoenzyme is possibly due to the presence of adventitious metal ions (1–2 $\mu$M) (Thiers, 1957) exceeding the concentration of apo-ALAD (0.5 $\mu$M) in the assay.

In contrast, the ALAD from spinach has been found to require $Mg^{2+}$ for activity. Unlike the eukaryotic enzymes, ALAD isolated from photosynthetic bacteria was not inhibited by the metal chelator EDTA, suggesting that zinc may not be involved in the function of this enzyme. However, in the absence of atomic-absorption data it is not possible to exclude the possibility of a tightly bound metal ion. The ALAD from *R. sphaeroides* requires $K^+$ for activity (Nandi and Shemin, 1968b), with evidence for the formation of more active oligomers in the presence of high concentrations of this ion.

The majority of ALAD enzymes isolated to date require a reductant such as 2-mercaptoethanol or dithiothreitol for the maintenance of activity, ALAD from spinach being an exception. Studies on the bovine and human enzymes have shown that oxidation results in inactivation and the formation of a disulphide bond which prevents metal binding (Tsukamoto, et al., 1979; Gibbs, 1984; Gibbs et al., 1985a). It has been reported that maximum activity is possible in the bovine enzyme when only one half of the metal-binding sites are occupied (Bevan et al., 1980; Jaffe et al., 1984). However, neither of these reports actually determined $Zn^{2+}$ bound to the enzyme by using atomic absorption. A linear correlation has been found between activity and metal bound for the human enzyme (Gibbs, 1984).

The availability of several nucleotide sequences specifying the ALAD enzymes from human (Wetmur et al., 1986), rat (Bishop et al., 1986) and *Escherichia coli* (Echelard et al., 1988; Li et al., 1989) has allowed the identification of a consensus zinc-binding domain (Berg, 1986), indicating a possible role for the oxidizable cysteine residues in this region for metal ligation. The spinach (Schaumburg et al., 1991) and pea (Boese et al., 1991) sequences have several cysteine residues replaced by aspartate residues, which may explain their requirement for $Mg^{2+}$ rather than $Zn^{2+}$ and their stability in the absence of an exogenous thiol.

The cloning and sequencing of the *hemB* gene specifying the ALAD from *E. coli* (Echelard et al., 1988; Li et al., 1988, 1989) and its overexpression from clones containing the gene has, for the first time, allowed a detailed study of the enzyme from a member of the Enterobacteriaceae. The present paper describes the isolation, metal requirement and status of the reactive cysteine residues with respect to $Zn^{2+}$ binding in the ALAD from *E. coli*.

## MATERIALS AND METHODS

### Chemicals

5-Aminolaevulinic acid (ALA), 2-mercaptoethanol, *Staphylo-*

Abbreviations used: ALAD, 5-aminolaevulinic acid dehydratase; PBG, porphobilinogen; ALA, 5-aminolaevulinic acid; DTNB, 5,5′-dithiobis-(2′-nitrobenzoic acid); TNB, thionitrobenzoate; CTNB, cyanothiolnitrobenzoate.

* To whom correspondence should be sent.

*coccus aureus* V8 protease and Trizma base were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Column-chromatography supplies were obtained from Pharmacia Ltd. Iodo[2-$^{14}$C]acetic acid was purchased from Amersham International. All other chemicals were purchased from BDH (now Merck), Poole, Dorset, U.K.

### Growth of cells

*E. coli* strain TB1 containing the plasmid pUC19 harbouring the *hemB* gene in a 2.85 kb *EcoR1–BamH* fragment as constructed by Li et al. (1988) was grown in 500 ml of Luria broth containing ampicillin (50 $\mu$g/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.

### Purification of 5-aminolaevulinic acid dehydratase (ALAD) from *E. coli*

*E. coli* cell paste (4 g) was suspended in 20 ml of 50 mM potassium phosphate buffer, pH 6.0, containing 100 $\mu$M $ZnSO_4$ and 20 mM 2-mercaptoethanol and sonicated for a total of 4 min to disrupt the cells. Cell debris was removed by centrifugation at 1000 *g* for 20 min. The supernatant was treated with $(NH_4)_2SO_4$ to bring the concentration to 33% saturation, and the resulting precipitate was removed by centrifugation and discarded. Further $(NH_4)_2SO_4$ was added to give 40% saturation, and the precipitate, containing the enzyme, was collected by centrifugation. The enzyme pellet was resuspended in 3 ml of the above buffer and the solution was applied to a Sephacryl S-300 gel-filtration column. The column was developed in the same buffer, and fractions eluted with a specific activity greater than 30 were pooled and concentrated to 20 mg/ml (Table 1). The enzyme was judged to be pure by electrophoresis on PAGE (Laemmli and Favre, 1973).

### Assay of *E. coli* ALAD

ALAD was assayed at a minimum protein concentration of 10 $\mu$M subunit in 50 mM potassium phosphate buffer, pH 6.8, containing 50 $\mu$M $ZnSO_4$ and 10 mM 2-mercaptoethanol in a total volume of 500 $\mu$l. The reaction was initiated by the addition of ALA to give a final concentration of 5 mM. After incubation at 37 °C for 3 min, the reaction was terminated by the addition of an equal volume of 10% trichloroacetic acid containing 0.1 M $HgCl_2$ to precipitate protein and thiol. The solution was centrifuged, and an aliquot was removed and mixed with an equal volume of modified Ehrlich's reagent (Mauzerall and Granick, 1956). After 15 min the absorbance at 555 nm was determined ($\epsilon_{555}$ 60200 litres·mol$^{-1}$·cm$^{-1}$; Mauzerall and Granick, 1956). The absorbance at 280 nm of a 0.1% solution of ALAD from *E. coli* in 50 mM potassium phosphate, pH 6, was determined as 0.83. A unit of enzymic activity is 1 $\mu$mol PBG produced/h/mg of protein at 37 °C. The specific activity of the purified enzyme was found to be 33 $\mu$mol/h/mg at pH 6.8.

### $M_r$ determination

(a) Subunit $M_r$ determination

The purified enzyme was subjected to SDS/PAGE (Laemmli and Favre, 1973).

(b) Oligomeric $M_r$ determination

A Pharmacia f.p.l.c. G12 gel-filtration column was equilibrated in 200 mM potassium phosphate, pH 6.5, containing 7 mM 2-mercaptoethanol and 50 $\mu$M $Zn^{2+}$ at a flow rate of 0.2 ml/min. Standards of known $M_r$ (apoferritin, 443000; $\beta$-amylase, 200000; horse alcohol dehydrogenase, 150000; BSA, 66000) were applied (0.1 ml of a 5 mg/ml standard) and their elution detected at 280 nm. ALAD was chromatographed under identical conditions and its $M_r$ was compared with those of the standards (Laemmli and Favre, 1973).

### Isoelectric focusing

Isoelectric focusing of the purified ALAD was performed on LKB Ampholine PAGE plates (pH 3.5–9.5), using an LKB Multiphor apparatus. Protein samples (5 $\mu$g/5 $\mu$l) were applied on sample application papers (4 mm × 4 mm) to the gel (10 cm × 12 cm) 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 pI (range 4.2–9.3) and the pI of ALAD was then determined by comparison.

### Thiol-group determination

ALAD (50 $\mu$g) was denatured in 40 $\mu$l 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 ($\epsilon_{412}$ 14750 litre·mol$^{-1}$·cm$^{-1}$) was measured. The number of thiol groups was measured in all experiments by this method to confirm that the nature of the enzyme species under investigation was known.

### Modification of ALAD with iodoacetic acid

Enzyme (100 nmol) in 50 mM potassium phosphate buffer, pH 8, containing 6 M guanidinium chloride and 5 mM 2-mercaptoethanol, was treated with neutralized 15 mM iodoacetic acid. The reaction was allowed to proceed for 2 h at 20 °C, after which time the modified enzyme was dialysed and processed as described.

### Atomic-absorption studies

Samples for $Co^{2+}$, $Zn^{2+}$ and $Mg^{2+}$ determination 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 on to the linear-response region of the standard curve ($Zn^{2+}$ over the range 5–30 $\mu$M and $Co^{2+}$ and $Mg^{2+}$ over the range 5–40 $\mu$M).

## RESULTS AND DISCUSSIONS

### Purification of ALAD from *E. coli*

The amount of ALAD present in cell-free extracts from the recombinant strain of *E. coli* is approx. 10% of the total soluble protein, and therefore the purification of the enzyme was greatly facilitated compared with the wild-type strain in which the enzyme is barely detectable, even after a 20 min assay. Consequently the enzyme was isolated by a single 33–40% satd. $(NH_4)_2SO_4$ fractionation with gel filtration as the only additional

**Table 1 Purification of ALAD from recombinant *E. coli***

ALAD was purified from an *E. coli* cell paste as described in the Materials and methods section.

| Sample | Volume (ml) | Total activity (units) | Total protein (mg) | Specific activity (units · $mg^{-1}$) |
|---|---|---|---|---|
| Initial extract | 18 | 4932 | 880 | 5.6 |
| $(NH_4)_2SO_4$ precipitation | | | | |
| 33% satn. pellet | 3 | 192 | 213 | 0.9 |
| 40% satn. pellet | 2 | 780* | 115 | 6.8 |
| 40% satn. supernatant | 16 | 1650 | 660 | 2.5 |
| Gel filtration | 30 | 2640 | 80 | 33.0 |

* Activity was always found to be lower than expected after $(NH_4)_2SO_4$ precipitation, but could be fully regained after gel filtration or dialysis against 50 mM potassium phosphate buffer, pH 6, containing 20 mM $\beta$-mercaptoethanol and 100 $\mu$M $Zn^{2+}$. Overall yield of the enzyme was found to be 54%. The specific activity is increased by the inclusion of 5 mM $Mg^{2+}$.

step necessary to yield an enzyme in excess of 95% purity. Homogeneous enzyme was obtained by anion-exchange chromatography using DEAE-Sephacryl or f.p.l.c. using a MonoQ 5HR column. The enzyme was applied to the column in 50 mM potassium phosphate buffer, pH 7.0, containing 100 $\mu$M $ZnSO_4$ and 20 mM 2-mercaptoethanol and eluted with a gradient of KCl in the same buffer. The purified enzyme was stored, after filter sterilization, at 4 °C in 50 mM potassium phosphate buffer, pH 6, containing 100 $\mu$M $ZnSO_4$ and 20 mM 2-mercaptoethanol. The purification of the enzyme is summarized in Table 1.

### $K_{cat.}$, $K_m$ and pH optimum of ALAD from *E. coli*

The pH optimum of *E. coli* ALAD, determined in either 50 mM potassium phosphate buffer or 50 mM Tris/HCl buffer with 5 mM ALA, is at pH 8.5. In phosphate buffer the specific activity of ALAD increased from 8 units/mg at pH 6 to 80 units/mg at pH 9, corresponding to a change in $K_{cat.}$ from 0.25 to 2.5 $s^{-1}$ respectively with an apparent $pK_a$ of 7.3. The $K_m$ varied little over this pH range, being 800 $\mu$M at both pH 6 and 8.5 and 1 mM at pH 6.8. This increased activity at higher pH is similar to that reported for other ALADs isolated from bacterial sources (Nandi et al., 1968).

### Determination of pI, subunit and oligomeric $M_r$ and N-terminal sequence

On performing isoelectric focusing on purified ALAD, as described in the Materials and methods section, a single protein band was observed corresponding to a pI of 5.0. The subunit $M_r$ was $36000 \pm 3000$ by SDS/PAGE in agreement with an electro-spray m.s.-determination of $36554 \pm 17$. The subunit $M_r$ predicted by the gene sequence of Echelard et al. (1988) is 11 residues shorter at the N-terminal compared with that of the sequence given by Li et al. (1989), giving predicted $M_r$ values of 35600 and 36700 respectively. To determine which of the two suggested N-terminal sequences was correct, Edman degradation was carried out with the purified enzyme. The sequence obtained was

$NH_2$-T-(D)-L-I-Q-

in agreement with the translation start proposed by Echelard et al. (1988). The N-terminal methionine thus appears to have been removed during post-translational processing as noted with many other proteins. As a result of these findings, the residue numbering of *E. coli* ALAD proposed by Echelard et al. (1988) should be adopted, and this has been used throughout the present paper.

The oligomeric $M_r$ was determined by comparing the elution volume of native ALAD with that of known $M_r$ standards, using a Pharmacia f.p.l.c. gel-filtration column. The $M_r$ was determined to be $270000 \pm 20000$, which, with a subunit $M_r$ of 36000, would indicate an octameric species similar to that found for ALADS from human (Anderson and Desnick, 1979; Gibbs et al. 1985b) and bovine (Bevan et al. 1980) sources.

### Determination of the number of thiol groups per subunit in *E. coli* ALAD

Titration of reduced ALAD with DTNB under denaturing conditions, as described in the Materials and methods section, led to the release of $6.0 \pm 0.3$ mol. equiv. of thionitrobenzoate (TNB) indicating a probable total of six free cysteine residues in the protein. On blocking the six cysteines with unlabelled iodoacetic acid, reducing the modified protein with 10 mM 2-mercaptoethanol and treating with 20 mM iodo[2-$^{14}$C]acetic acid, only 0.2 mol. equiv. of $^{14}$C radioactivity could be incorporated, indicating the absence of any disulphide bonds in the native protein.

Addition of 4 mM DTNB to native holoenzyme, or apoenzyme ($Zn^{2+}$ removed with EDTA), resulted in the release of 4.4 mol. equiv. of TNB, indicating that only four of the six cysteine residues are accessible for modification (Figure 1a). The release of TNB on addition of DTNB was found to proceed faster with apo-ALAD than with the holoenzyme, as found with the human enzyme (Gibbs et al. 1985b). The disulphide bonds thus formed were shown to be intrasubunit, since non reductive SDS/PAGE showed no evidence of dimers.

The number of cysteine residues determined above agrees with the *E. coli* gene-derived protein sequence of Li et al. (1989), but not with that of Echelard et al. (1988), which predicts a total of nine cysteine residues. The three 'extra' cysteine residues fall in the region of residues 18–42, which is at variance with the sequence (29–53) of Li et al. (1989) and other dehydratases. The sequence adopted will thus be that of Li et al. (1989) minus eleven, to allow for the N-terminus being 11 residues shorter, as predicted by Echelard et al. (1988).

### Time course of metal loss and correlation of enzyme activity with metal content

Excess unbound $Zn^{2+}$ was removed from holo-ALAD by Sephadex G-50 gel filtration in 50 mM potassium phosphate buffer, pH 6, and the $Zn^{2+}$ bound was determined by atomic absorption as described in the Materials and methods section. The holo-ALAD contained $2 \pm 0.3$ mol of $Zn^{2+}$/mol of subunit, but no $Mg^{2+}$.

Addition of 10 mM EDTA to holo-ALAD resulted in the immediate loss of all activity and, following gel filtration in 50 mM potassium phosphate buffer, pH 6, containing 20 mM 2-mercaptoethanol and 1 mM EDTA, only 0.63 mol of $Zn^{2+}$/mol of subunit remained bound. This remaining $Zn^{2+}$ was lost over a much longer time period (Figure 1b), so that, after 21 h, the enzyme contained only 0.06 mol of $Zn^{2+}$/subunit.

Holo-ALAD could be regenerated from apo-ALAD by the

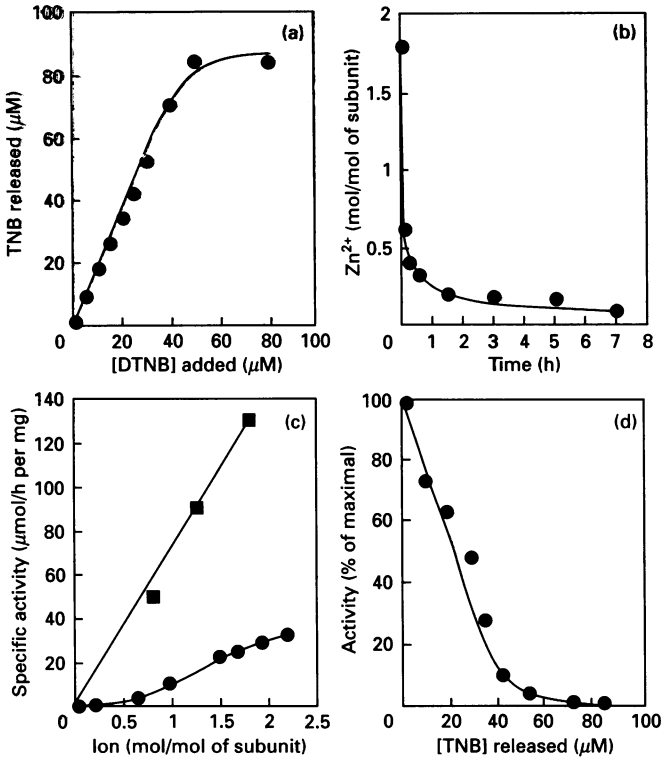


**Figure 1 Status of *E. coli* ALAD with respect to $Zn^{2+}$ content, enzyme activity and thiol groups**

(**a**) Correlation of TNB released with DTNB added to ALAD. Apo-ALAD (23.5 $\mu$M) was titrated with DTNB, and the amount of TNB (mol) liberated was determined by spectroscopy ($\epsilon_{412}$ 14750 litre·mol$^{-1}$·cm$^{-1}$). (**b**) Time course of $Zn^{2+}$ loss from holo-ALAD in the presence of EDTA. EDTA (as shown) was added to holo-ALAD (333 $\mu$M) in potassium phosphate buffer, pH 6, containing 50 $\mu$M $Zn^{2+}$ and 20 mM $\beta$-mercaptoethanol. Aliquots (100 $\mu$l) were then removed and gel-filtered in the above buffer containing 1 mM EDTA to remove excess $Zn^{2+}$. The $Zn^{2+}$ content was then analysed by atomic absorption as described in the Materials and methods section. (**c**) Correlation of ALAD activity with $Zn^{2+}$ and $Mg^{2+}$ content. Various molar excesses (up to 5-fold) of $Zn^{2+}$ were added to apo-ALAD (180 $\mu$M) in 50 mM potassium phosphate buffer, pH 6, containing 20 mM 2-mercaptoethanol and incubated for 15 min at 15 °C. Samples were then gel-filtered in the above buffer without $Zn^{2+}$, and the molar $Zn^{2+}$ content and enzymatic activity were determined (●) as described in the Materials and methods section. Alternatively, various molar amounts of $Zn^{2+}$ (up to 1 mol equiv.) were added to apo-ALAD (180 $\mu$M) in 50 mM potassium phosphate buffer, pH 8, containing 20 mM 2-mercaptoethanol and $Mg^{2+}$ (6 mol equiv.). After 1 h at 15 °C, samples were gel-filtered in the above buffer, but without either $Zn^{2+}$ or $Mg^{2+}$. The molar $Zn^{2+}$ and $Mg^{2+}$ contents were determined to be equimolar. The sum of ion content is shown against enzymic activity (■), determined as described in the Materials and methods section, but in the presence of 50 $\mu$M $Mg^{2+}$ alone. (**d**) Correlation of TNB release with activity of ALAD. Apo-ALAD (23.5 $\mu$M) was titrated with DTNB, and the amount of TNB (mol) liberated was determined by spectroscopy ($\epsilon_{412}$ 14750 litre·mol$^{-1}$·cm$^{-1}$). Aliquots (50 $\mu$l) were re-activated by the addition of 1 mM $Zn^{2+}$ for 15 min and then assayed.

addition of various molar excesses of $Zn^{2+}$ (0.2–5; 35–900 $\mu$M) to apo-ALAD under reducing conditions (20 mM 2-mercaptoethanol) in 50 mM potassium phosphate buffer pH 6. Metal content and activity was determined after gel filtration in the above buffer. The original specific activity of 33 units/mg was recovered on the binding of 2 $Zn^{2+}$ ions/subunit (Figure 1c). However, restoration of activity was not completely linear with respect to metal content (Figure 1c), possibly indicating a difference in the function of the two metal-binding sites. A further indication of a difference in the nature of the two binding sites was the ability of $Mg^{2+}$ to substitute for the second mol. equiv. of $Zn^{2+}$ bound, giving a 4-fold increase in specific activity over the 2 $Zn^{2+}$ species (Figure 1c). However, $Mg^{2+}$ alone could not restore activity to apo-ALAD or bind to the protein.

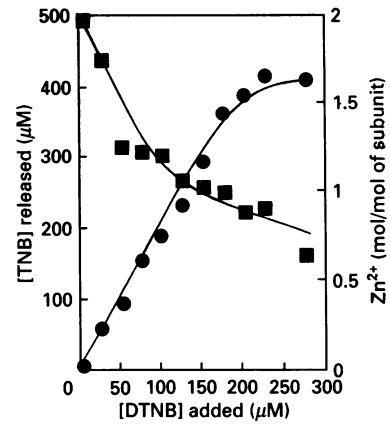


**Figure 2 Correlations of the oxidation state of cysteine residues with $Zn^{2+}$ content**

Holo-ALAD (100 $\mu$M) was titrated with DTNB in 50 mM potassium phosphate buffer, pH 7.8. Disulphide-bond formation was monitored by TNB release (●). Samples (0.4 ml) were taken at various states of oxidation and gel-filtered. The $Zn^{2+}$ content was then determined by atomic absorption (■).

## Correlation of enzyme activity and metal content with free cysteine content

Titration of apo-ALAD with 2 mol. equiv. of DTNB resulted in the release of 4 mol. equiv. of TNB, indicating the formation of two disulphide bonds (Figure 1a). When DTNB-titrated enzyme was passed through a gel-filtration column to remove excess TNB and exposed to excess 2-mercaptoethanol (20 mM), less than 0.1 mol of TNB/mol of subunit was released, confirming the formation of the two disulphide bonds with DTNB. Correlation of TNB release with the ability to form the holo-ALAD from the apo-ALAD with $Zn^{2+}$ in the absence of 2-mercaptoethanol showed that virtually all ability to form the active holoenzyme is lost after the release of 2 mol. equiv. of TNB. This indicates that the formation of only one disulphide bond per subunit prevents re-activation with $Zn^{2+}$ (Figure 1d).

Titration of holo-ALAD with DTNB also revealed the formation of disulphide bonds with, again, 90% of the activity lost on the formation of one disulphide bond per subunit. Determination of zinc content indicated that on formation of the first disulphide, 0.9 mol of $Zn^{2+}$/mol of subunit was lost, whereas after the formation of the second disulphide bond, 0.8 mol of $Zn^{2+}$/mol of subunit still remained bound to the enzyme (Figure 2). This difference in DTNB-sensitivity of the two metal-binding sites may indicate a possible difference in the ligands at each metal site.

## Incorporation of $Co^{2+}$

When $Co^{2+}$ ions are ligated to cysteine residues they are known to exhibit distinct absorption spectra (Garbett et al. 1972). To investigate the possibility that cysteine acted as a metal-ion ligand in ALAD, $Co^{2+}$ (1 mM) was added to apo-ALAD (100 $\mu$M) in 50 mM potassium phosphate, pH 7.8, in the absence of 2-mercaptoethanol, but under anaerobic conditions. The resulting protein was green in colour and exhibited two major peaks on difference spectroscopy against $Zn^{2+}$-holo-ALAD (Figure 3). The absorbance maxima ($\lambda_{max.}$ = 315 nm and 625 nm) and absorption coefficients ($\epsilon$ 8000 and 1900 litre·mol$^{-1}$·cm$^{-1}$ respectively) observed in the $Co^{2+}$-ALAD are suggestive of a

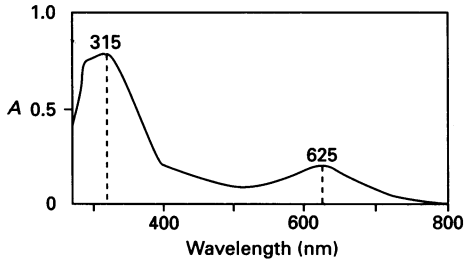


**Figure 3 Difference spectrum of $Co^{2+}$-substituted ALAD**

Difference scan of $Co^{2+}$-ALAD (100 $\mu$M) against $Zn^{2+}$-ALAD (100 $\mu$M) in 50 mM potassium phosphate, 7.8, showing the absorbance peak at 625 nm ($\epsilon$ 1900 litre·mol$^{-1}$·cm$^{-1}$) and 315 nm ($\epsilon$ 8000 litre·mol$^{-1}$·cm$^{-1}$).

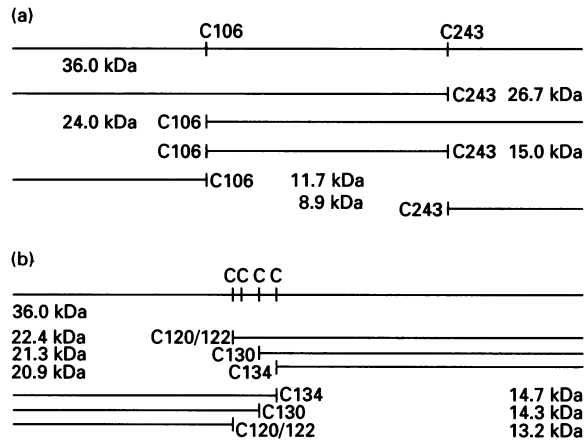


**Figure 4 Peptides produced on CTNB cleavage of ALAD from *E. coli***

(a) Pattern of bands produced by CTNB cleavage at available cysteines in ALAD after oxidation of cysteines able to form disulphide bonds. (b) Pattern of bands produced on cleavage by CTNB at available cysteines following reduction of ALAD containing one equivalent of disulphide bond per subunit. Cysteines not involved in disulphide bonds were blocked by iodoacetic acid prior to reduction. The ALAD is represented by the top horizontal line.

charge-transfer-band cysteine -$S^-$–$Co^{2+}$ in a tetrahedral environment (Garbett et al., 1972). The spectrum is similar to those obtained with $Co^{2+}$-substituted alcohol dehydrogenase ($\lambda_{max.}$ = 390, $\epsilon$ 3000; $\lambda_{max.}$ = 670, $\epsilon$ 1000), as described by Garbett et al. (1972) and Maret et al. (1979). The $Co^{2+}$-substituted alcohol dehydrogenase is known to utilize cysteine residues as ligands for the bound $Co^{2+}$ ions.

Subsequent gel filtration of $Co^{2+}$-substituted ALAD in 50 mM potassium phosphate buffer, pH 7.8, to remove excess $Co^{2+}$ did not alter the observed spectrum, and atomic-absorption studies revealed 2.1 mol of $Co^{2+}$ bound/mol of subunit and a maximum of 0.17 mol of $Zn^{2+}$/mol of subunit. All cysteine residues remained in the reduced form in the $Co^{2+}$-substituted ALAD. The resulting $Co^{2+}$-substituted ALAD did not exhibit enzyme activity, in contrast with the findings of Cheh and Neilands (1976), but in agreement with Jaffe et al. (1984), with bovine ALAD, although in both these cases actual $Co^{2+}$ bound to the enzyme was not determined.

## Cleavage of *E. coli* 5-ALAD at cysteine residues by treatment with cyanothiolnitrobenzoate (CTNB)

To determine the position of the cysteine residues involved in the formation of the S–S bonds, apo-ALAD (2 mg/ml) containing two disulphide bonds was exposed to 5 mM CTNB in 50 mM potassium phosphate buffer, pH 8, containing 6 M guanidinium chloride HCl and 1 mM EDTA for 2 h (Jacobson et al., 1973). Following dialysis against water the sample was denatured in SDS denaturing buffer with, or without, 2-mercaptoethanol.

No apparent difference in the peptide cleavage patterns was obtained on SDS/PAGE for the apo-ALAD with two disulphide bonds, with or without 2-mercaptoethanol in the denaturing buffer (results not shown), indicating that neither disulphide bond is spanning across a free cysteine residue. Comparison of the $M_r$ of peptides produced with those predicted by the gene sequence of Li et al. (1989), allowing for the 'extra' 11 residues at the N-terminus (Fig. 4a), establishes that C106 (i.e. Cys-106) and C243 are not involved in disulphide-bond formation.

A second sample of ALAD containing one disulphide bond (prepared by DTNB treatment) was allowed to react with 20 mM iodoacetic acid under denaturing conditions to modify the four remaining free cysteine residues. After dialysis, the cysteine residues in the disulphide bond were liberated by reduction with 10 mM 2-mercaptoethanol for 30 min under denaturing conditions. Cleavage at these cysteine residues was then achieved by the addition of 15 mM CTNB for 2 h. After dialysis against water the sample was denatured in SDS containing 2-mercaptoethanol for SDS/PAGE.

If the 'first' disulphide bond formed is only between C120 and C122, this would appear effectively as one cleavage site (SDS/PAGE being unable to resolve a single amino acid residue), giving two visible bands on SDS/PAGE. If a unique disulphide is formed by any of the remaining combinations, then the two partially cleaved sites would result in the presence of four bands on SDS/PAGE. If the process was completely random, then cleavage could occur at three sites (C130, C134 and C120+122), giving six visible bands (Figure 4b).

From the cleavage pattern of ALAD containing one disulphide bond, six bands were observed (results not shown) of $M_r$ consistent with those predicted for a random reactivity (Figure 4b). This indicates that the formation of the first disulphide bond is not uniquely between any two of the four possible cysteine residues.

## Labelling studies

To confirm further which cysteine residues were participating in the formation of the disulphides, two labelling experiments were performed with 2-iodo[$^{14}$C]acetic acid. In the first experiment, (a), the cysteine residues remaining after the formation of two disulphide bonds per subunit were labelled, while in the other experiment, (b), the cysteine residues involved in the 'first' disulphide were labelled.

### (a) Labelling of the free cysteines in ALAD containing two disulphides

Apo-ALAD (1.9 mg/ml; 200 nmol), with two disulphide bonds, was treated with 3 mM 2-iodo[$^{14}$C]acetic acid (sp. radioactivity 8.4 Ci/mol) in 50 mM potassium phosphate buffer, pH 8, containing 6 M guanidinium chloride and 1 mM EDTA to modify the two remaining free cysteine residues. A total of 300 nmol of $^{14}$C label was found to have been incorporated out of a maximum of 400 nmol, giving a labelling efficiency of 75%. After dialysis against potassium phosphate buffer (3 × 2 litres) the two disulphide bonds were reduced with 5 mM 2-mercaptoethanol for 30 min and the resulting free cysteine residues were modified with non-radioactive iodoacetic acid (10 mM) as described above.

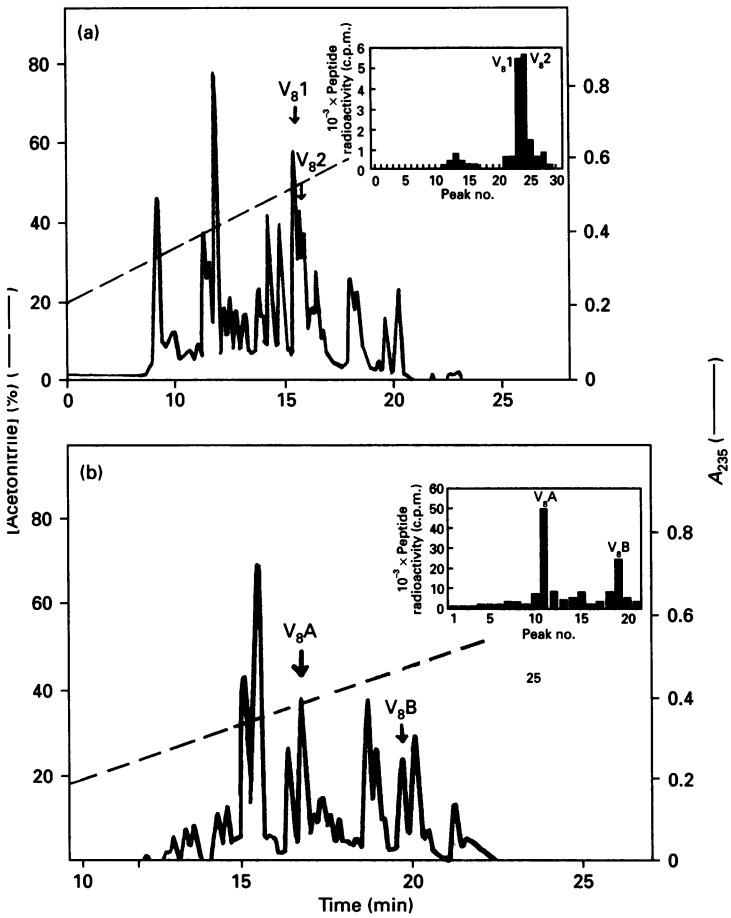


**Figure 5 H.p.l.c. separation of $^{14}$C-labelled peptides derived from *S. aureus* $V_8$ protease digestion of modified ALAD**

The Figure shows initial separations, on a $C_{18}$ reverse-phase column, of peptides from *S. aureus*-V8-protease digestions. The gradients are shown by a broken line. The insets show the radioactivity recovered in each peak. (**a**) Separation of $^{14}$C-labelled cysteine peptides not involved in disulphide formation. The peptides were generated, after modification with iodo[$^{14}$C]acetic acid of the cysteine residues available after the formation of the two disulphide bonds, as described in the Results section. (**b**) Separation of $^{14}$C-labelled peptides containing cysteines involved in the formation of the first disulphide. The peptides were generated, after modification with iodo[$^{14}$C]acetic acid of the cysteine residues available after the formation of the first disulphide bond, as described in the Results section.

The sample was then dialysed against 50 mM potassium phosphate buffer, pH 8, containing 1 mM EDTA and digested with *S. aureus* V8 proteinase (100:1, w/w) for 16 h at 37 °C. All the $^{14}$C label (180 nmol; $3.3 \times 10^6$ d.p.m.) was in the soluble fraction after digestion.

The resulting peptides were purified by h.p.l.c. on a reverse-phase $C_{18}$ column using a gradient from 0.5% trifluoroacetic acid in water to 70% of 0.1% trifluoroacetic acid in acetonitrile (Figure 4a). Two peptides, $V_8$1 and $V_8$2, containing about a third of the total radioactivity each, were then further purified by a subsequent pass down the reverse-phase column using a shallow gradient (1%/min) that was isocratic in the region of elution. In both cases at least 90% of the radioactivity was associated with a single peptide peak. Recovery of peptides at each stage is shown in Table 2. Both peptides were then subjected to amino acid analysis, electro-spray m.s. spectrometry and Edman protein sequence determination.

(b) Labelling of cysteines involved in the initially formed disulphide

A further labelling experiment was undertaken with ALAD containing 0.88 disulphide bonds per subunit (formed by DTNB treatment and followed by gel filtration under nitrogen to remove excess TNB). Iodoacetic acid treatment was carried out as above, except that the initial modification was carried out with non-radioactive iodoacetic acid in order to block the four free cysteine residues. The sample was then dialysed, freeze-dried and reduced with 10 mM 2-mercaptoethanol to liberate the free cysteine residues that were then labelled by reaction with 20 mM 2-iodo[$^{14}$C]acetic acid (sp. radioactivity 2.5 Ci/mol). A total of 585 nmol of $^{14}$C label was incorporated out of a maximum expected of 850 nmol, giving a labelling efficiency of 69%. After digestion with *S. aureus* V8 proteinase, as above, 93% (541 nmol) of label was present in solution.

The resulting peptides were purified by h.p.l.c. on a reverse-phase $C_{18}$ column using a gradient from 0.5% trifluoroacetic acid in water to 70% of 0.1% trifluoroacetic acid in acetonitrile (Figure 5b). Two labelled peptides, $V_8$A and $V_8$B, containing 50% and 25% of the total radioactivity respectively, were purified further by an additional pass down the reverse-phase column as before. In both cases at least 90% of the radioactivity was associated with a single peak. Recovery of label at each stage is shown in Table 2. Both labelled peptides were then subjected to amino acid analysis, electro-spray m.s. and Edman protein sequence determination.

## Peptide analysis

Purified peptides were subjected to Edman sequence determination and residues were identified as the phenylthiohydantoin derivatives; however, very little $^{14}$C radioactivity ($\approx$ 0.3%) could be detected after this derivatization, as previously noted with

**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 h.p.l.c. reverse-phase column are shown.

| | First passage | | | Second passage | | | |
|---|---|---|---|---|---|---|---|
| Peptide | Radioactivity (% of total) | Label (nmol) | Yield (%) | Radioactivity (% of total) | Label (nmol) | Yield (%) | Final yield (%) |
| $V_8$1 | 33 | 64 | 36 | 90 | 52 | 81 | 29 |
| $V_8$2 | 35 | 49 | 27 | 95 | 27 | 55 | 15 |
| $V_8$A | 50 | 146 | 27 | 90 | 98 | 67 | 18 |
| $V_8$B | 25 | 33 | 10 | 80 | 19 | 57 | 4 |

**Table 3 Peptide sequencing of cysteine-containing peptides from *E. coli* ALAD by Edman degradation as phenylthiohydantoin (PTH) and anilinothiazolinone (ATZ) derivatives**

Asterisks indicate residues not identified. The radioactivity recovered at each turn were determined by a second sequencing experiment where the amino acid released was derivatized to the anilinothiazolinone. Radioactivity recovered at each turn is expressed as a % of the total counts recovered. For comparison, the relevant sequences derived from the gene sequence given by Li et al. (1989) and Echelard et al. (1988) are shown with the assigned residue number of the cysteine(s) in the primary sequences.

(a) Peptide $V_81$

Samples of this peptide (103 pmol of residues 1–8) and 350 pmol of residues 8–17 were sequenced as phenylthiohydantoin derivatives. A total of 46600 d.p.m. were loaded, of which 32% were recovered in the anilinothiazolinone derivatives. Another peptide derived from ALAD which co-purified with the above peptide was also present giving the sequence AMPGVMRIPI. Further purification of $V_81$ by an additional h.p.l.c. reverse-phase step confirmed that counts were associated with $V_81$ alone and that the first and fourth residues were alanine.

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sequence (PTH)... | A | Q | G | A | D | C243 | L | M | V | K | P | A | G | A | Y | L | D |
| Amount (pmol) (PTH)... | 45 | 16 | 27 | 19 | 14 | – | 10 | 13 | 60 | 41 | 71 | 52 | 44 | 56 | 46 | 31 | 5 |
| Radioactivity (%) (ATZ)... | – | – | – | – | – | 70 | 16 | – | – | – | 16 | – | – | – | – | – | – |
| Gene (Li)... | A | Q | G | P | D | C254 | L | M | V | K | P | A | G | A | Y | L | N |
| Gene (Echelard)... | A | Q | G | A | D | C243 | L | M | V | K | P | A | G | A | Y | L | D |

(b) Peptide $V_82$

A 300 pmol portion of this peptide was sequenced as phenylthiohydantoin derivatives. Only a single sequence was present. A total of 48000 d.p.m. were loaded, of which 28% were recovered in the anilinothiazolinone derivatives.

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sequence (PTH)... | D | G | L | V | A | R | M | S | R | I | C106 | K | * | * | V | P | E |
| Amount (pmol) (PTH)... | 150 | 214 | 241 | 178 | 190 | 89 | 170 | 68 | 130 | 126 | – | 40 | – | – | 50 | 20 | 9 |
| Radioactivity (%) (ATZ)... | – | 4 | – | – | – | 5 | – | – | 4 | 5 | 74 | 9 | – | – | – | – | – |
| Gene (Li)... | D | G | L | V | A | R | M | S | P | I | C117 | K | Q | T | V | P | E |
| Gene (Echelard)... | D | G | L | V | A | R | M | S | R | I | C106 | K | Q | T | V | P | E |

(c) Peptide $V_8A$

A 495 pmol portion of this peptide was sequenced as phenylthiohydantoin derivatives. Only a single sequence was present. A total of 10000 d.p.m. were loaded, of which 37% were recovered in the anilinothiazolinone derivatives.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sequence (PTH)... | Y | T | S | H | G | H | C130 | G | V | L | C134 | E |
| Amount (pmol) (PTH)... | 240 | 132 | 116 | 58 | 185 | 63 | – | 141 | 135 | 145 | – | 24 |
| Radioactivity (%) (ATZ)... | – | – | – | – | – | – | 51 | 14 | – | – | 27 | 8 |
| Gene (Li)... | Y | T | S | H | G | H | C141 | G | V | L | C145 | E |
| Gene (Echelard)... | Y | T | S | H | G | H | C130 | G | V | L | C134 | E |

(d) Peptide $V_8B$

A 495 pmol portion of this peptide was sequenced as phenylthiohydantoin derivatives. Only a single sequence was present. A total of 1300 d.p.m. were loaded, of which 46% were recovered in the anilinothiazolinone derivatives.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sequence (PTH)... | M | I | V | M | S | D | T | C120 | F | C122 | E |
| Amount (pmol) (PTH)... | 69 | 84 | 80 | 79 | 23 | 32 | 30 | – | 38 | – | 6 |
| Radioactivity (%) (ATZ)... | – | – | – | – | – | – | – | 33 | 17 | 24 | 25 |
| Gene (Li)... | M | I | V | M | S | D | T | C131 | F | C133 | E |
| Gene (Echelard)... | M | I | V | M | S | D | T | C120 | F | C122 | E |

tritiated carboxymethylcysteine (Spencer et al., 1991). A second sequence determination was therefore performed in which the amino acids were converted into their anilinothiazolinone derivatives for radioactivity counting, giving a 30–50% recovery of $^{14}C$ (40–60% allowing for the repetitive yield). The results of the peptide sequencing are given in Table 3. With the exception of three positions, the protein sequences determined for the peptides agree with both of the published gene-derived protein sequences for *E. coli* ALAD. The residues 104, 241 and 254 were found to be arginine, alanine and aspartate respectively, in accord with the sequence given by Echelard et al. (1988), but at variance with that given by Li et al. (1989), who found proline, proline and asparagine at these positions.

Peptide analysis by electro-spray m.s., using a VG BioQ mass spectrometer, gave masses in agreement with the protein sequences determined by Edman degradation, $V_81$ giving a mass of $1782 \pm 3$ (theoretical 1781), $V_82$ a mass of $1961 \pm 3$ (theoretical 1962), $V_8A$ giving a mass $1423 \pm 2$ (theoretical 1426) and $V_8B$ giving a mass $1396 \pm 3$ (theoretical 1399).

Determination of the amount of peptide present using the reagent trinitrobenzoylsulphate (Fields, 1972) allowed the specific activity of the isolated peptide to be calculated. From Edman-degradation studies the distribution of label within peptides $V_8A$ and $V_8B$ between the two cysteine residues was about equal, while the specific radioactivity of $V_8A$ was 2.5 times that of $V_8B$, indicating a possible preference towards cysteine residues 130 and 134 being involved in the first disulphide. However, the determined specific radioactivity of $V_82$ was 1.8 times that of $V_81$, where an equal distribution would be expected. A comparison of these two results indicates that any preference of the residues involved in the 'first' disulphide is not significant.

The two cysteine residues labelled by iodo[$^{14}C$]acetic acid (in peptides $V_81$ and $V_82$), after the formation of the two disulphide bonds, are those at positions 106 and 243 in the primary sequence, indicating that these residues do not participate in disulphide-bond formation. The sequences of these peptides determined by Edman degradation agreed well with the electro-spray m.s. determinations described above if A is the fourth and D is the last amino acid of $V_81$, and A is the ninth amino acid of $V_82$ (see Table 3). Amino acid analysis of $V_82$ revealed a T and an extra G, which are likely to account for the two unidentified residues. Electro-spray m.s. analysis indicated that the extra E was derived from a Q on acid hydrolysis and this would correlate with the predicted sequence of Q–T as predicted by both the gene sequences.

The labelling of all the cysteine residues in peptides $V_8A$ and $V_8B$ with $^{14}C$ after the formation of one equivalent of disulphide per subunit again indicates that the 'first' disulphide is not formed from a unique pair of cysteine residues. This was further confirmed by determination of the specific radioactivity of the

labelled peptides, which was not significantly different from a random distribution of label.

## DISCUSSION

Cheh and Neilands (1976) attempted to classify ALADs, on the basis of their bivalent-transition-metal requirements for activity, into prokaryotic (no transition metal required) and eukaryotic (bivalent transition metal required, largely $Zn^{2+}$). As shown in the present work, the ALAD from *E. coli* does require a bivalent transition metal for activity and so argues against a classification on this basis. With increasing structural knowledge becoming available and with the characterization of ALADs from several additional sources, it appears that a different classification of ALAD into two alternative groups may be more instructive, one group requiring $Zn^{2+}$ and the other $Mg^{2+}$ for activity. However, other properties such as whether the pH optimum is 'high' (pH 8.5) or 'low' (pH 6.8) do not appear to be dependent on which of these metals are used. For instance, both human and *E. coli* ALAD require $Zn^{2+}$ for activity, yet their pH optima are 6.8 (Gibbs et al., 1985a,b) and 8.5 (the present study) respectively.

The structural basis for the difference in metal ion requirement may be determined by the nature of possible metal ligands in the putative zinc binding region of *Escherichia coli* ALAD (Jordan, 1991). In *Escherichia coli* ALAD, where $Zn^{2+}$ alone or $Zn^{2+}$ and $Mg^{2+}$ can support activity, there are four cysteines present in this region whereas in ALAD from plants, that require only $Mg^{2+}$, these residues are replaced by aspartic acid residues (Schaumburg et al., 1991; Boese et al., 1991). Similarly, the presence of an added thiol such as 2-mercaptoethanol for maintaining activity of ALAD also appears to be related to the presence of cysteine residues in this region of the protein. Thus the ALAD from *Escherichia coli*, along with other ALADs containing cysteine in this region, requires the presence of free thiols for maintaining activity whereas the $Mg^{2+}$-dependent ALADs from spinach (Liedgens et al., 1983) and *Arabidopsis* (Jones and Jordan, unpublished) do not.

Several types of divalent transition metals are reported to support the catalytic activity of $Zn^{2+}$ containing ALADs (Cheh and Neilands, 1976) although the metal and the amounts bound to the enzyme were not determined. *E. coli* ALAD with 2 $Co^{2+}$ ions bound per subunit was found to be inactive. To date little is known about the role, if any, of metal ions in ALAD in relation to catalysis.

The generally accepted stoichiometry of metal ion binding to ALAD isolated from bovine liver (Tsukamoto et al., 1979) and human erythrocytes (Gibbs, 1984) is one per subunit. However, the nature of this binding site may not be unique since EXAFS data from experiments with the bovine enzyme indicate the presence of two types of metal binding site, one of which is cysteine rich (Dent et al., 1990). The finding that ALAD from *Escherichia coli* can bind 2 moles of $Zn^{2+}$ per subunit at sites, each of which have differing DTNB sensitivity, may reflect the proposed differences between the metal ligands. This difference is further reinforced by the finding that $Mg^{2+}$ can substitute for one of the $Zn^{2+}$ ions. The presence of two types of metal binding site may indicate more than one role for the metal ions, possibly both catalytic and structural. This possible difference in metal ion function may underlie the reports of full activity exhibited by the bovine enzyme with only half of its potential metal content. The differences observed for correlation of metal content with activity for ALAD from different species may then be a reflection of the relative affinities of the proposed two types of metal binding sites. The ability of $Co^{2+}$ to substitute into these sites and

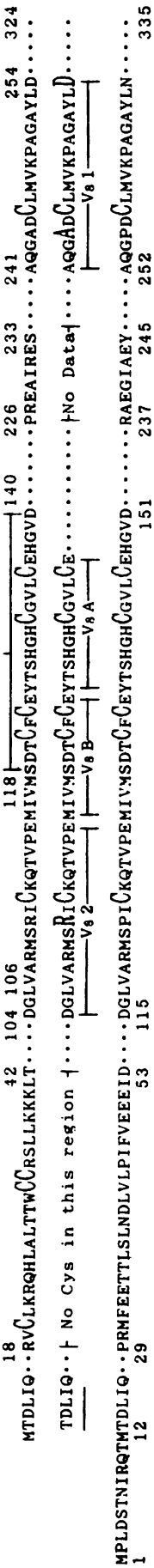


**Figure 6 Comparison of protein sequences of ALAD from *E. coli*, determined by Edman degradation, with those derived from the two published gene sequences**

The middle protein sequence shows data obtained in this study from the characterization of the four peptides containing cysteine residues by Edman degradation. Differences observed with the gene-derived protein sequences are highlighted together with all cysteine residues. The gene-derived protein sequences of Echelard et al. (1988) (upper) and Li et al. (1989) (lower) are shown with their respective original residue numbering.

the resulting spectrum, consistent with the metal ion interacting with one or more cysteine ligands, may be of help in elucidating the nature and function of the different metal binding sites in ALAD from *Escherichia coli*.

A possible location for the $Co^{2+}$ binding sites in the protein accounting for the cysteine charge transfer bands may be the region extending from 118–140 in the primary sequence which contains 4 cysteines, among other possible metal ligating residues. The cysteines in this region have been shown, by this work, to be capable of forming disulphides with each other. The labelling experiments indicated that disulphide formation was not ordered, i.e. one specific disulphide does not form before the other. The apparent randomness of disulphide bond formation may thus indicate that the four reactive cysteines are all in close proximity to one another in the 3-dimensional structure and can yield the six possible combinations of disulphide bonds. However, the data do not discount the possibility that two specific pairs of disulphide bonds are forming simultaneously. Disulphide bond formation is known to occur in proteins containing zinc finger motifs, although attempts at labelling the cysteines involved has been unsuccessful (Henderson et al., 1981).

The protein sequence information gained from this study has allowed the resolution of 4 of the 6 discrepancies between the two published gene sequences such that the N-terminus and residues found at positions 104, 241 and 254 are in agreement with the gene sequence of Echelard et al. (1988) as shown in Figure 6. The remaining two differences are regions of no sequence identity between residues 18–42 and 226–233. Whilst protein sequence data is not available for these regions, it is known that the region 18–42 cannot contain three cysteine residues, as predicted by Echelard et al. (1988), as these were not detected by radiolabelling of cysteine residues and subsequent peptide isolation. Therefore the sequence given by Li et al. (1989) should be adopted in this region [corresponding to residues 29–53 (Li et al.'s numbering)]. No additional information is available to resolve the difference in gene sequences in the remaining region, 226–233 (Echelard et al.'s numbering). The clarification of these remaining discrepancies is obviously important to allow interpretation of the crystal structure from X-ray-scattering data when they become available and for any meaningful site-directed mutagenic studies. Whether some of these discrepancies are the result of the presence of microheterogeneity or misinterpretation of gene sequence data is as yet unclear.

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