Extended-X-ray-absorption-fine-structure investigations of zinc in 5-aminolaevulinate dehydratase

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The zinc co-ordination in 5-aminolaevulinate dehydratase (5-aminolaevulinate hydro-lyase, EC 4.2.1.24) was investigated by recording and interpreting the extended X-ray-absorption fine structure (e.x.a.f.s.) associated with the zinc *K*-edge. The enzyme has a molecular mass of 280 000 Da and consists of eight subunits of 35 000 Da each; the samples studied contained approx. 1 g-atom of zinc/mol of subunit. Four forms of the enzyme were investigated and details of the zinc environment were elucidated, as follows. (a) In the native enzyme, zinc is considered to be co-ordinated to three sulphur atoms at 0.228(2) nm [2.28(2) Å] and a lower-Z atom at 0.192(5) nm [1.92(5) Å] (if nitrogen) or 0.189(5) nm [1.89(5) Å] (if oxygen). (b) Reaction of the enzyme with the inhibitor 2-bromo-3-(imidazol-5-yl)propionic acid produced significant changes in the e.x.a.f.s., the nature of which are consistent with co-ordination by about three sulphur atoms at 0.222(2) nm [2.22(2) Å], a nitrogen atom at 0.193(5) nm [1.93(5) Å] and a nitrogen atom from the inhibitor at 0.214(5) nm [2.14(5) Å]. (c) Inactivation of the enzyme by air-oxidation of essential thiol groups and binding of the substrate produce slight changes in the e.x.a.f.s. consistent with slight re-arrangement of ligands with additional lighter ligands (nitrogen or oxygen). These results, when combined with previous findings, are taken to indicate that zinc has a structural rather than a direct catalytic role in 5-aminolaevulinate dehydratase.

5-Aminolaevulinate dehydratase (ALAD, 5-aminolaevulinate hydro-lyase, EC 4.2.1.24) catalyses the synthesis of the pyrrole porphobilinogen from 2 molecules of 5-aminolaevulinic acid. The enzyme from bovine liver has a molecular mass of 280 000 Da and consists of eight subunits of 35 000 Da each (Shemin, 1976). Lysine, histidine and cysteine have been identified as amino acid constituents of the enzyme protein involved in the active site (Shemin, 1972; Chaudhry *et al.*, 1976; Tsukamoto *et al.*, 1979). Two cysteine residues essential for the enzyme activity are rapidly oxidized by air, and the maintenance of the active state requires the presence of a thiol (Batlle *et al.*, 1967). The isolated enzyme protein contains 2–6 g-atoms of zinc/mol of octamer and it binds a

maximum number of eight Zn^{2+} ions/octamer with high affinity (Tsukamoto *et al.*, 1979; Bevan *et al.*, 1980; Sommer & Beyersmann, 1984). The zinc chelators EDTA and 1,10-phenanthroline reversibly inhibit the enzyme, and full activity may be restored by binding four Zn^{2+} ions/octamer (Bevan *et al.*, 1980). Other metal ions re-activate the enzyme to a variable extent. Cd^{2+} restores full activity, Co^{2+} and Mn^{2+} yield about 20% activity, whereas the following are inactive: Ni^{2+} , Fe^{2+} , Fe^{3+} , Cu^{1+} , Cu^{2+} , Mg^{2+} , Ca^{2+} and Cr^{3+} (Cheh & Neilands, 1973). Pb^{2+} is an effective inhibitor of ALAD at micromolar concentrations, even in the presence of competing thiols (Wilson *et al.*, 1972).

The function of zinc in ALAD is not clear. In some enzymes, e.g. liver alcohol dehydrogenase, carboxypeptidase A, thermolysin and erythrocyte carbonic anhydrase, zinc has been shown to be part of the catalytic site and to interact directly with the substrate (Argos *et al.*, 1978; Dunn, 1975;

Abbreviations used: e.x.a.f.s., extended X-ray-absorption fine structure; ALAD, 5-aminolaevulinate dehydratase.

Chlebowski & Coleman, 1976). In other enzymes, zinc seems to have a predominantly structural function (Dunn, 1975; Chlebowski & Coleman, 1976). With ALAD, several findings imply a direct catalytic function for zinc, whereas other results support the assumption that zinc stabilizes the active structure of the enzyme protein, without directly interacting with the substrate. Comparisons of the kinetic data obtained for zinc-activated and zinc-deprived ALAD show that the presence of zinc strongly influences the Michaelis constant (which is decreased about 60-fold for the former as compared with the latter) but has only a very limited effect on the maximal velocity (which is increased about 2-fold for the former as compared with the latter) (Sommer & Beyersmann, 1984). Zinc in ALAD is accessible to external ligands, it is partially lost during the isolation of the protein, and it is extracted by EDTA (Cheh & Neilands, 1973). Also, this metal binds the affinity labelling agent 2-bromo-3-(imidazol-5-yl)propionic acid (Beyersmann & Cox, 1984), and the action of the latter is inhibited by the presence of substrate. Hence zinc seems to be part of the active site of ALAD. On the other hand, zinc in ALAD is hardly influenced by several agents that usually modify the properties of catalytically active zinc centres in enzymes. For example, ALAD activity is not influenced by cyanide and several other good anionic ligands; dipicolinic acid is ineffective, and 1,10-phenanthroline slowly inhibits ALAD and then only to a limited extent. Also, the concentration of EDTA required to inactivate ALAD completely is very high (i.e. 1 mM). Furthermore, a direct interaction between zinc in ALAD and the substrate is unlikely, since the substrate analogue laevulinic acid binds not only to zinc-activated ALAD but also to the zinc-deprived enzyme (Bevan *et al.*, 1980).

The ligands of zinc in ALAD have not been identified directly in any previous investigation; however, co-ordination by cysteine has been implied. Thus Bevan *et al.* (1980) showed that Zn^{2+} did not bind to the enzyme unless the essential thiol groups of the enzyme were first reduced, and that alkylation of thiol groups resulted in a decrease in the binding of Zn^{2+} to the enzyme. Jaffe *et al.* (1984) found that zinc was released from ALAD when three thiol groups/subunit were modified by reaction with methylmethanethiosulphonate. Attempts to determine the nature of the zinc sites in ALAD by using other metals as spectroscopic probes have been unsuccessful. Substitution of Zn^{2+} by Co^{2+} proved to be ineffective, since it yielded species with Co^{2+} bound non-specifically and the resultant u.v.-visible spectrum obscured possible absorption of the cobalt complex corresponding to the zinc site (D. Beyersmann & C.

Weber, unpublished work). ^{113}Cd n.m.r. spectra were recorded after extraction of zinc from ALAD by EDTA and reconstitution with $^{113}\text{Cd}^{2+}$ (Sommer & Beyersmann, 1984). These authors, however, have been unable to obtain ^{113}Cd n.m.r. spectra in recent experiments where zinc was extracted from the enzyme by a different technique, i.e. by treatment with Chelex. Furthermore, 'foreign' metal ions do not necessarily reflect the true chemical environment in the zinc enzyme; thus Co^{2+} yields only about 20% ALAD activity, and Cd^{2+} causes a 20-fold increase in the K_m of the enzyme in spite of giving full maximal activity (Sommer & Beyersmann, 1984).

Hence it was considered to be highly desirable to investigate the nature of the zinc site(s) in various forms of ALAD by the direct procedure of measuring and interpreting the extended X-ray-absorption fine structure (e.x.a.f.s.) associated with the zinc *K*-edge. In the present paper we report the results accomplished for four forms of ALAD: native, native plus the inhibitor 2-bromo-3-(imidazol-5-yl)propionate, and air-oxidized, alone and with substrate.

Materials and methods

Materials

Water was bidistilled over quartz. All salts were of analytical grade (p.a.). 2-Mercaptoethanol and spherical hydroxyapatite were obtained from Merck (Darmstadt, Germany), 5-aminolaevulinic acid, Pipes and DEAE-cellulose were purchased from Serva (Heidelberg, Germany) and activated CH-Sepharose 4B was acquired from Pharmacia (Uppsala, Sweden). The SM 14549 ultrafilter, which retained proteins larger than 20000 Da, was supplied by Sartorius (Göttingen, Germany).

Enzyme purification

The enzyme was purified by a modification of the affinity-chromatography methods of Stella & Batlle (1977). All operations were performed at approx. 5°C, unless specified otherwise. The standard buffer was 10 mM-potassium phosphate, pH 6.8, containing 40 mM-2-mercaptoethanol and 0.01 mM-zinc acetate. A 800 g portion of bovine liver was homogenized in an Omni-Mix (Sorvall) with standard buffer and centrifuged at 20000g. The supernatant was fractionated by heat denaturation: it was heated to 80°C in a boiling-water bath, kept for 1 min at this temperature and chilled quickly to 5°C. The precipitate formed was removed by centrifugation at 20000g. The supernatant was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (at 65% saturation), redissolution in and dialysis against the standard buffer. The sample was further purified by chromatography on

DEAE-cellulose, which was eluted with 0.5M-potassium phosphate buffer, pH6.8, containing 40mM-2-mercaptoethanol and 0.01mM-zinc acetate. The active fractions were concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$, redissolution in and dialysis against 10mM-potassium phosphate buffer, pH6.8, containing 40mM-2-mercaptoethanol and 0.01mM-zinc acetate. Further purification was achieved by chromatography on hydroxyapatite, the elution being accomplished by a linear gradient of 10–500mM-potassium phosphate buffer, pH6.8, containing 40mM-2-mercaptoethanol and 0.01mM-zinc acetate. The active fractions were concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$, redissolution and dialysis against standard buffer. The final step was the affinity chromatography on 5-carboxamidolaevulinate-3-carboxypropionamidoethyl-Sepharose, which was eluted with a linear gradient of 0–100mM-KCl in standard buffer. The active fractions were concentrated and dialysed by diafiltration under N_2 through an SM14549 ultrafilter in an Amicon 8MC diafiltration apparatus, with 10mM-Pipes buffer, pH6.8, containing 10mM-2-mercaptoethanol and 0.01mM-zinc acetate. The properties of the purified enzyme were: purification 725-fold; specific activity $0.02\mu\text{mol}/\text{min}$ per mg; protein concentration 78.3mg/ml, corresponding to 0.28mM of octameric protein or 2.24mM of subunits. The zinc concentration was determined by flame atomic absorption with a Perkin-Elmer model 2380 spectrophotometer. The macromolecular fraction was 2.70mM and the diafiltrate was 0.01mM in zinc, i.e. 0.37% of the zinc was present as low- M_r compounds. Solutions of the concentrated enzyme were very stable, and no significant decrease in activity occurred on exposure of the enzyme, maintained at room temperature (approx. 23°C), to synchrotron radiation for 24h.

Determination of enzyme activity

The enzyme was assayed by the method of Mauzerall & Granick (1956).

Preparation of the inhibitor-bound form of ALAD

A 0.2ml portion of the solution of the native enzyme (2.24mM in subunits) was mixed with 0.05ml of 0.2M-2-bromo-3-(imidazol-5-yl)propionic acid in 10mM-Pipes buffer, pH6.8, and allowed to react for 3h at 23°C .

Preparation of the air-oxidized enzyme

A 0.5ml portion of the active reduced enzyme (78.3mg/ml) was dialysed for 5h at 0°C against two changes of 100ml of Pipes buffer, pH6.8, containing 0.01mM-zinc acetate but no 2-mercaptoethanol. The product, which is called the air-

oxidized enzyme, had lost all detectable activity owing to the oxidation of essential thiol groups. Hence the residual mercaptoethanol, which had not been dialysed away, was oxidized too.

Preparation of the substrate-bound form of ALAD

A 0.3ml portion of the solution of the air-oxidized enzyme (see above) was mixed with 0.02ml of 85mM neutralized 5-aminolaevulinic acid hydrochloride.

Determination of the enzyme protein concentration

A sample of the enzyme solution was dialysed against a 1000-fold excess of bidistilled water at 5°C overnight, to remove low- M_r substances. Four portions of the dialysed enzyme solution, containing 2.1mg each, were freeze-dried and weighed. An $A_{280}^{1\text{cm}}$ value of 1 corresponded to a concentration of 0.69mg/ml.

Measurement and interpretation of e.x.a.f.s.

X-ray-absorption spectra were recorded in the fluorescence mode as fluorescence excitation spectra by using the fluorescence-detection system (Hasnain *et al.*, 1984) at the Daresbury Synchrotron Radiation Source operating at an energy of 1.8GeV with an average current of 90mA. An Si-111 channel-cut monochromator was used, and a number of spectra were recorded and averaged for each state of the enzyme. For the inhibitor-bound form only four scans were recorded, thus giving only a limited statistical quality. Data analysis utilized the single-scattering spherical-wave method for calculating e.x.a.f.s. with phase shifts derived from 'ab-initio' calculations as described previously (Lee & Pendry, 1975; Perutz *et al.*, 1982; Blackburn *et al.*, 1983).

Results

We investigated the zinc enzyme ALAD by e.x.a.f.s. spectroscopy. We recorded spectra of four different forms of the enzyme: (a) the native enzyme with the O_2 -sensitive thiol groups reduced by 2-mercaptoethanol; (b) the air-oxidized enzyme with its essential, O_2 -sensitive, thiol groups reversibly oxidized to disulphides; (c) the air-oxidized enzyme with the substrate, 5-aminolaevulinic acid, bound; (d) the native enzyme after reaction with the inhibitor 2-bromo-3-(imidazol-5-yl)propionic acid. The relationship between these four forms is depicted in Scheme 1.

Fig. 1 shows the near-edge and e.x.a.f.s. spectra of the four forms studied. The spectra of native, air-oxidized and substrate-bound forms of the enzyme are similar in their overall appearance, suggesting that each involves a similar environment for the zinc. The overall e.x.a.f.s. profile is

Scheme 1. Relationship between forms of ALAD studied by *e.x.a.f.s.*

Table 1. Parameters used to simulate the *e.x.a.f.s.* associated with the zinc K-edge of native ALAD, inhibitor-bound ALAD, air-oxidized ALAD and air-oxidized ALAD plus substrate
N is the number of atoms.

Atom	Native (reduced) form			Inhibitor-bound form			Air-oxidized form			Air-oxidized form + substrate		
	<i>N</i>	σ^2 (nm ²)	<i>R</i> (nm)	<i>N</i>	σ^2 (nm ²)	<i>R</i> (nm)	<i>N</i>	σ^2 (nm ²)	<i>R</i> (nm)	<i>N</i>	σ^2 (nm ²)	<i>R</i> (nm)
N†	1	0.00007	0.192*	1	0.00002	0.193	1	0.00004	0.188	1	0.00006	0.185
N†	—	—	—	1	0.00007	0.214	—	—	—	—	—	—
O†	—	—	—	—	—	—	1	0.00008	0.205	1	0.00009	0.202
S‡	3	0.00006	0.228	3	0.00010	0.222	3	0.00009	0.225	3	0.00009	0.227
O§	—	—	—	—	—	—	—	—	—	1	0.00008	0.236
Cl	—	—	—	2	0.00005	0.269	—	—	—	—	—	—
C/N	—	—	—	2	0.00008	0.341	—	—	—	—	—	—
C/N	—	—	—	5	0.00005	0.424	—	—	—	—	—	—

* If oxygen, a distance of 0.189 nm would be more optimum.

† These distances could be in error by as much as 0.005 nm. Fitting error is less than 0.001 nm.

‡ This is the primary contribution in all cases. An error of less than 0.002 nm is expected. Fitting error is 0.0001 nm.

§ See the text.

|| In view of the poor statistical quality of *e.x.a.f.s.* data for the inhibitor-bound form, error for these distances is difficult to assess.

typical of back-scattering from sulphur atoms. However, the near-edge and *e.x.a.f.s.* profiles observed for the inhibitor-bound form are markedly different from the corresponding profiles for the other forms, clearly indicating that the binding of the inhibitor produces major changes in the zinc environment. Detailed analyses of the *e.x.a.f.s.* have confirmed these preliminary conclusions, and the parameters used for successful simulations of these data are summarized in Table 1.

Native enzyme

Fig. 2(a) provides a comparison between the experimental *e.x.a.f.s.* and that calculated from the parameters given in Table 1; the corresponding Fourier transforms are compared in Fig. 2(b). The phase and the back-scattering amplitude observed in the experimental *e.x.a.f.s.* spectrum is dominated by the sulphur atoms. This is illustrated in Fig. 2(c), where a theoretical simulation of the three sulphur atoms at 0.228 nm (2.28 Å) is compared with that of the three nitrogen atoms at the same distance. The inset in Fig. 2(c) shows the respective Fourier transforms. The best agreement between

the experimental and calculated *e.x.a.f.s.* was obtained with zinc co-ordinated by four ligands. Three of these are sulphur atoms at a distance of 0.228 nm (2.28 Å) and one is a lower-Z atom at the substantially shorter distance of approx. 0.192 nm (1.92 Å) (if it is nitrogen) or approx. 0.189 nm (1.89 Å) (if it is oxygen). On the basis of the *e.x.a.f.s.* simulations, we favour nitrogen as the fourth ligand, but cannot be definitive. The error associated with the Zn-S distance is considered to be less than 0.002 nm (0.02 Å) whereas the Zn-N(O) distance could be in error by 0.005 nm (0.05 Å), since the latter makes a much weaker contribution to the *e.x.a.f.s.* than that from the three sulphur atoms. The Debye-Waller constant, σ^2 , gives a measure of correlated disorder of the distances and thus can be used as an indicator for assessing the homogeneity of the metal site. The low values needed to simulate the observed *e.x.a.f.s.* spectra are typical for a homogeneous environment. This is consistent with the observation that only one affinity constant is found for all eight zinc atoms per octameric enzyme (Gibbs & Jordan, 1981; Sommer & Beyersmann, 1984).

Fig. 1. Zn *K*-edge X-ray-absorption near-edge structure and e.x.a.f.s. of (a) native (reduced) ALAD, (b) air-oxidized ALAD, (c) air-oxidized ALAD plus substrate and (d) native ALAD plus inhibitor [2-bromo-3-(imidazol-5-yl)propionic acid]

E_0 is defined as the mid-point of the absorption edge. $\chi(E)$ is the normalized e.x.a.f.s.

However, a complete molecular-structure determination is necessary for a direct proof that all zinc atoms have identical co-ordination.

Inhibitor-bound form

After the enzyme had reacted with 2-bromo-3-(imidazol-5-yl)propionate, the e.x.a.f.s. spectrum shown in Fig. 3(a) was obtained. The experimentally obtained data are of a poorer statistical quality than those obtained for the other forms in view of the limited number of scans completed for this form; nevertheless, the change in the e.x.a.f.s.

profile from that of the native form is quite distinct.

When an attempt was made to simulate the e.x.a.f.s. of the inhibitor-bound form, the environment obtained for the reduced form was used as the starting point. This immediately suggested that the primary shell distance (i.e. that for the sulphur atoms) should be decreased. Fig. 3(a) includes a comparison of the experimentally obtained data with the e.x.a.f.s. calculated with three Zn-S distance of 0.222 nm (2.22 Å) and one Zn-N distance of 0.193 nm (1.93 Å). It is clear, from this comparison, that significant additional contributions need to be included in order to achieve a successful simulation of the experimentally obtained data. This was further confirmed by a refinement of the Debye-Waller term (σ^2) for the nitrogen atom, which gave a negative value, suggesting a higher occupancy for this shell. Thus another nitrogen atom was added and this new Zn-N distance was refined to 0.214 nm (2.14 Å). A similar refinement for the sulphur shell resulted in an increase in the associated σ^2 , perhaps indicating a lower occupancy of this shell. However, this was not compelling, and the number of sulphur atoms was maintained at three, although we note that a decrease in this co-ordination number would be consistent with the shorter Zn-S distance.

The strong beat pattern observed in the e.x.a.f.s. of the inhibitor-bound form is typical of back-scattering from multi-shell systems, as observed for the copper (and zinc)-imidazole co-ordination in superoxide dismutase (Blackburn *et al.*, 1984). Thus the carbon and nitrogen atoms of an imidazole group associated with the Zn-N distance of 0.214 nm (2.14 Å) were included. However, further back-scattering contributions were required, and the final e.x.a.f.s. simulation (Fig. 3b) involves about five carbon atoms at 0.424 nm (4.24 Å); these additional atoms are taken to represent portions of the inhibitor (Fig. 4) other than the imidazole moiety.

The results obtained for the inhibitor-bound form of ALAD clearly demonstrate that the inhibitor profoundly changes the zinc environment of the native enzyme and most probably binds directly to the metal via its imidazole group.

Air-oxidized form

Fig. 5 shows the e.x.a.f.s. associated with the zinc *K*-edge of ALAD after the oxidation of the essential thiol groups by air. Simulations of these data commenced with the parameters used to interpret the e.x.a.f.s. of the native form. A fit index of 0.031 was obtained, and the nature of the discrepancies between the experimental and calculated profiles suggested that the distance of the primary co-ordination shell should be decreased.

Fig. 2. (a) Zn K-edge e.x.a.f.s. and (b) Fourier transforms of native ALAD

Continuous lines represent the experimental spectra, broken lines the simulated spectra. The experimental e.x.a.f.s. spectra were shifted by $\Delta E_0 = 19 \text{ eV}$, as was required for the theoretical simulation. A value of 0.015 was obtained for the fit-index parameter. The data range was $3.3\text{--}11.6 \text{ (\AA}^{-1}\text{)}$. E.x.a.f.s. is presented as $\chi(k) \times k$ plotted versus k , where k is in $\text{\AA}^{-1}$ (equivalent to 10 nm^{-1}). (c) Theoretical simulations with three sulphur atoms (----) and three nitrogen atoms (-.-) are shown, together with the experimentally obtained data (—). The inset shows the corresponding Fourier transforms.

Therefore, in air-oxidized ALAD, three sulphur atoms are considered to be co-ordinated to the zinc at a distance of approx. 0.225 nm ($2.25 \text{ \AA}$). However, a slightly higher value of the Debye-Waller parameter is required, compared with that for the native form, consistent with an increase in the spread of the Zn-S bond distances upon air-oxidation. This resulted in an improved fit index of 0.024. In addition to these sulphur atoms, successful simulations of the e.x.a.f.s. required a nitrogen atom, as for the native form, but at a slightly shorter distance [approx. $0.188(5) \text{ nm}$ [$1.88(5) \text{ \AA}$]], and a further low-Z (nitrogen) atom at approx. $0.205(5) \text{ nm}$ [$2.05(5) \text{ \AA}$]. A fit index of 0.017 was

obtained with these parameters (Table 1), and the agreement between the experimental and calculated e.x.a.f.s. is shown in Fig. 5.

Therefore these e.x.a.f.s. studies have demonstrated that, as in the native form of ALAD, the air-oxidized form involves zinc co-ordinated to three sulphur atoms. Hence the oxidation of cysteine residues by air involves residues that are not co-ordinated to zinc.

Substrate-bound form

The close resemblance between the zinc environment in the air-oxidized and substrate-bound forms of ALAD is clear from the correspondence

Fig. 3. Zn K-edge e.x.a.f.s. of inhibitor-bound ALAD. Continuous lines represent the experimental spectra, broken lines the simulated spectra. (a) Best simulation obtained when the simulation for native enzyme was used as the starting model; (b) best simulation obtained when inhibitor was assumed to be directly co-ordinated to zinc. ΔE_0 for (a) was 19 eV and for (b) was 22 eV. Values for the fit-index parameter were (a) 0.167 and (b) 0.028. The data range was 3.6–10.5 ($\text{\AA}^{-1}$).

Fig. 4. 2-Bromo-3-(imidazol-3-yl)propionic acid, inhibitor of ALAD

of their near-edge and e.x.a.f.s. profiles (Fig. 1). However, closer inspection reveals subtle differences, suggesting slight changes in the zinc environment from one form of the enzyme to the other. This view received confirmation from

Fig. 5. Zn K-edge e.x.a.f.s. and simulated spectrum of air-oxidized ALAD. Continuous line represents the experimental spectrum, broken line the simulated spectrum. ΔE_0 was 23 eV.

Fig. 6. Zn K-edge e.x.a.f.s. of air-oxidized ALAD plus substrate. Continuous line represents the experimental spectrum, broken line the simulated spectrum. The theoretical best fit shown was obtained with parameters given in Table 1. ΔE_0 was 23 eV.

detailed analyses of the e.x.a.f.s., made by commencing the interpretations with the parameters (Table 1) used to simulate the e.x.a.f.s. of the oxidized enzyme (Fig. 6). A fit index of 0.032 was

obtained and disagreement between the calculated and observed spectra was evident in the range $K = 3\text{--}5$ ($\text{\AA}^{-1}$), and this suggested that the simulation lacked a weak contribution from a low- Z atom at a relatively long distance. Further analyses were initiated with an additional oxygen atom placed at approx 0.21 nm (2.1 $\text{\AA}$) and, to decrease the number of parameters that were varied, only the distance of this additional oxygen atom and that of the sulphur shell were refined. These distances refined to 0.236 and 0.227 nm (2.36 and 2.27 $\text{\AA}$) respectively, giving an improved fit index of 0.027. We note that the determination of an oxygen (or nitrogen) atom at such a long distance, in the presence of a relatively much stronger back-scattering contribution (from three sulphur atoms) at a similar distance, is at the limits of the e.x.a.f.s. technique, even when an 'ab-initio' approach and a spherical-wave treatment (Lee & Pendry, 1975; Perutz *et al.*, 1982) are used. However, the subtle changes, from that of the oxidized form, observed in the e.x.a.f.s. when the substrate is added are consistent with the inclusion of this long distance. The presence of this additional oxygen atom was further assessed by taking a Fourier-filtered spectrum, so that uncertainties in improvement of the fit index due to a limited signal-to-noise ratio in the experimentally obtained data can be eliminated. Thus a simulation with the model of the oxidized form gave a fit index of 0.0021, whereas with the additional atom, which refined to 0.234 nm (2.34 $\text{\AA}$), a fit index of 0.0017 was obtained. We note that an attempt to introduce such an atom for the oxidized form resulted in a deterioration of the fit index. Of course, it cannot be deduced from these data whether this additional atom derives from the substrate itself or originates from the enzyme protein as a consequence of a substrate-induced conformational change.

Discussion

Herein we have presented evidence, based upon zinc K -edge e.x.a.f.s., that zinc in native ALAD is four-co-ordinate, ligation being by three sulphur atoms and one nitrogen or oxygen atom. The number of three sulphur ligands of zinc in ALAD is in accordance with the finding by Jaffe *et al.* (1984) that zinc is released from this enzyme when three thiol groups per subunit are modified by reaction with methylmethanethiosulphonate. The availability of low- M_r thiols in the biological source of the enzyme and the presence of low- M_r thiols during the course of isolation of the enzyme may raise the question whether one or more of the sulphur ligands to zinc in ALAD are from low- M_r sulphur compounds. However, the enzyme binds the thiol compound [^{14}C]cysteine only weakly,

with an affinity constant of about $5 \times 10^2 \text{M}^{-1}$. Furthermore, the enzyme deprived of zinc by EDTA and the reconstituted zinc enzyme bind [^{14}C]cysteine to the same extent (M. Schlösser & D. Beyersmann, unpublished work). Hence a significant contribution of low- M_r sulphur ligands to the e.x.a.f.s. spectrum of the zinc enzyme is excluded. Our results are consistent with the observation that, so far, only three types of amino acid residues have been identified as zinc ligands in zinc metalloproteins, i.e. the imidazole nitrogen atom of histidine, the γ -carboxy oxygen atom of glutamic acid or aspartic acid, and the thiol sulphur atom of cysteine (Dunn, 1975; Chlebowski & Coleman, 1976). If the low- Z atom is nitrogen, it could belong to a histidine imidazole group, since this amino acid residue has been demonstrated to be essential for the enzyme activity (Tsukamoto *et al.*, 1979). However, as demonstrated for the copper and zinc sites of bovine superoxide dismutase (Blackburn *et al.*, 1984) and considered above for the inhibitor-bound form of ALAD, imidazole groups typically produce a strong and characteristic e.x.a.f.s. profile. The absence of such effects from the e.x.a.f.s. of native (and air-oxidized and substrate-bound) form(s) prevents any positive conclusion concerning the co-ordination of imidazole in these forms of ALAD. However, we note that a minimum contribution is expected if the histidine ring is twisted relative to the $\text{Zn-N}_{(\alpha)}$ bond. If the low- Z ligand is oxygen it could be a γ -carboxy oxygen atom of glutamic acid, and we note that the suggested Zn-O distance [approx. 0.19 nm (1.9 $\text{\AA}$)] is rather short for a co-ordinated water molecule. However, we note that the zinc site of native ALAD does appear to be accessible to other ligands. Especially important is the binding of the inhibitor 2-bromo-3-(imidazol-5-yl)propionic acid to the native enzyme, which markedly changes the zinc co-ordination. The e.x.a.f.s. is fitted best with the three sulphur atoms and a low- Z atom, as found in the native enzyme, plus an imidazole group. This identification of an imidazole group fits well with the evidence from kinetic studies that the binding of the inhibitor is a function of the zinc content of the enzyme (Beyersmann & Cox, 1984).

The third important result of the e.x.a.f.s. investigation is that the oxidation of ALAD by air does not involve one of the three cysteine ligands of zinc, and therefore affects other thiol groups. Nevertheless, oxidation changes the co-ordination at the zinc, and this has been interpreted as involving the ligation of an additional oxygen or nitrogen atom. This change is attributed to a conformational shift of the protein, induced by oxidation of the thiol groups essential for the activity of the enzyme, which are probably located at or near the active centre. This result demon-

strates that zinc protects its thiol ligands, but not the other essential thiol groups, from oxidation by air. The binding of the substrate to the oxidized (inactive) enzyme induces a slight change in the zinc environment. In addition to the five atoms surrounding the zinc in the oxidized enzyme, a further low-Z atom may be present at approx. 0.237 nm (2.37 Å). Unfortunately, the crucial question as to whether or not zinc is in direct contact with an oxygen atom of the substrate cannot be answered unambiguously by the method employed. However, the minor perturbation in the zinc environment upon addition of the substrate and the very long Zn–O distance, which would be involved if an oxygen atom of the substrate were responsible for the approx. 0.237 nm (2.37 Å) distance, may suggest that the substrate does not bind to zinc, at least in the oxidized form of the enzyme. Rather, the slight change in the environment at the zinc is more probably due to a substrate-induced shift in the protein conformation. This latter interpretation is in accordance with other findings on the role of zinc in ALAD: the substrate analogue laevulinic acid binds to the zinc-deprived enzyme; Zn^{2+} ions have a very limited influence on the substrate saturation, and the enzyme is not inhibited by cyanide or dipicolinic acid (Sommer & Beyersmann, 1984). On the other hand, the substrate does induce a slight change in the zinc environment in ALAD. This result is compatible with the idea that the zinc site and the binding site, for at least one of the two substrate molecules reacting on the enzyme, are within the same domain of the protein structure. This view is further supported by the previous finding that the inhibition of ALAD by 2-bromo-3-(imidazol-5-yl)propionic acid is a function of the zinc content of the enzyme and that this inhibition is prevented by the binding of the substrate (Beyersmann & Cox, 1984).

Therefore we suggest that zinc plays a structural role in ALAD, enabling the enzyme to adopt the conformation required for activity, without binding and activating the substrate directly.

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