Characterization of the two 5-aminolaevulinic acid binding sites, the A- and P-sites, of 5-aminolaevulinic acid dehydratase from *Escherichia coli*

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Experiments are described in which the individual properties of the two 5-aminolaevulinic acid (ALA) binding sites, the A-site and the P-site, of 5-aminolaevulinic acid dehydratase (ALAD) have been investigated. The ALA binding affinity at the A-site is greatly enhanced (at least 10-fold) on the binding of the catalytic metal ion (bound at the α -site). The nature of the catalytic metal ion, Mg^{2+} or Zn^{2+} , also gave major variations in the substrate K_m , P-site affinity for ALA, the effect of potassium and phosphate ions and the pH-dependence of substrate binding. Modification of the P-site by reaction of the enzyme–substrate Schiff base with $NaBH_4$ and analysis of the reduced adduct by electro-spray mass spectrometry indicated a maximum of 1 mol of substrate incorporated/mol of subunit, correlating with a linear loss of enzyme activity. The reduced Schiff-base adduct was used to investigate substrate binding at the A-site by using rate-of-

dialysis analysis. The affinity for ALA at the A-site of Mg_Zn_{β} ALAD was found to determine the K_m for the reaction and was pH-dependent, with its affinity increasing from 1 mM at pH 6 to 70 μ M at pH 8.5. The affinity of ALA at the P-site of $Zn_{\alpha}An_{\beta}$ ALAD is proposed to limit the K_m at pH values above 7, since the measured K_d for ALA at the A-site in 45 μ M Tris, pH 8, was well below the observed K_m (600 μ M) under the same conditions. The amino group of the ALA molecule bound at the P-site was identified as a critical binding component for the A-site, explaining why ALA binding to ALAD is ordered, with the P-site ALA binding first. Structural requirements for ALA binding at the A- and P-sites have been identified: the P-site requires the carbonyl and carboxylate groups, whereas the A-site requires the amino, carbonyl and carboxylate groups of the substrate.

INTRODUCTION

5-Aminolaevulinic acid dehydratase (ALAD; porphobilinogen synthase; EC 4.2.1.24) catalyses the condensation of two molecules of 5-aminolaevulinic acid (ALA), giving one molecule of porphobilinogen (PBG). All dehydratases to date have been shown to require a bivalent metal ion for activity, mammalian and some bacterial enzymes requiring Zn^{2+} ions (Gibbs et al., 1985; Spencer and Jordan, 1993), whereas plant and other bacterial dehydratases utilize Mg^{2+} (Liedgens et al., 1983; Nandi et al., 1968). The Zn^{2+} -dependent dehydratases are also oxygen-sensitive, due to disulphide-bond formation between the cysteine residues that act as metal ligands resulting in the loss of bound metal and enzyme activity (Spencer and Jordan, 1993, 1994). In contrast, those dehydratases utilizing Mg^{2+} are oxygen-insensitive (Liedgens et al., 1983). The availability of several ALAD gene sequences [human, Wetmur et al. (1986); *Escherichia coli*, Echelard et al. (1988), Li et al. (1989); pea, Boese et al. (1991); spinach, Schaumburg et al. (1991)] predict proteins with a high degree of similarity. The replacement of cysteine metal ligands found in the Zn^{2+} enzymes by aspartate residues in the Mg^{2+} enzymes explains the metal specificity, and also provides a molecular basis for the oxygen-stability of Mg^{2+} -dependent ALADs (Boese et al., 1991; Jordan, 1991).

The stoichiometry of Zn^{2+} binding for bovine and human ALADs is 1 mol/mol of subunit (Tsukamoto et al., 1979; Gibbs, 1984) and 2 mol/mol of subunit for *E. coli* ALAD (Spencer and Jordan, 1993; Mitchell and Jaffe, 1993). In bovine ALAD the metal ions appear to be bound at two distinct sites (Dent et al., 1990), an A metal-ion-binding site being present at four sites per octamer, with a single cysteine residue among its ligands, and a

B metal-ion-binding site, also present at four sites per octamer, using four cysteine ligands (Dent et al., 1990). *E. coli* ALAD shows no such half-site metal binding, with one α and one β metal-ion-binding site per subunit [the two metal-binding sites of *E. coli* ALAD have been designated α and β , rather than A and B (as used by Dent et al., 1990), to avoid potential confusion with the A and P substrate-binding sites]. The α -site contains one cysteine, whereas the β -site has three cysteine residues among the metal ligands when Co^{2+} is present at the β -site (Spencer and Jordan, 1994). Zn^{2+} binding at the A-site (in bovine ALAD; Dent et al., 1990) or the α -site (in *E. coli* ALAD; Spencer and Jordan, 1994) alone resulted in the restoration of full enzyme activity. The binding of Zn^{2+} at the β -site of *E. coli* ALAD does not result in the restoration of activity, but induces a protein conformational change allowing the subsequent binding of Mg^{2+} at the α -site with the restoration of activity (Spencer and Jordan, 1994).

Although much of the mechanistic detail of PBG formation is still unknown, several of the steps have been characterized. It has been shown for ALADs from several sources that a Schiff-base intermediate is formed between ALA and the enzyme that may be trapped with $NaBH_4$ borohydride (Nandi and Shemin, 1968b; Cheh and Neilands, 1976). Mechanistic studies using single-turnover reactions with labelled substrate ([5- ^{13}C]ALA, Jordan and Seehra, 1980; [5- ^{14}C]ALA, Jordan and Gibbs, 1985) have shown that the ALA molecule involved in the formation of the Schiff base gives rise to the propionic acid side chain of the PBG molecule, hence the Schiff-base ALA-binding site was termed the P-site. The active-site residue involved in Schiff-base formation has been identified as lysine-252 in human ALAD (Gibbs and Jordan, 1986), equivalent to lysine-247 in the *E. coli* sequence

(numbering of Echelard et al., 1988). The incorporation of ALA at the P-site of bovine ALAD, following reduction by NaBH_4 , was not prevented by the absence of bound metal ions, or alkylation of the metal cysteine ligands, giving a maximum of 0.5 mol/mol of subunit (Cheh and Neilands, 1976; Jaffe and Hanes, 1986). On the incorporation of 0.5 mol of ALA per subunit of bovine ALAD, all activity was lost, indicating one functional active site per two subunits (Cheh and Neilands, 1976; Jaffe and Hanes, 1986). This half-site reactivity of bovine ALAD also appears to be reflected in the nature of the metal ions binding, where maximum activity could be restored by addition of a half-molar equivalent of Zn^{2+} (Bevan et al., 1980; Jaffe et al., 1984) as discussed above.

The binding of the second molecule of ALA at the A-site (giving rise to the acetic acid side chain of PBG) is thought to be dependent on the presence of the metal ion, suggesting why the apo-ALAD is inactive. Inactivation of bovine ALAD by 5-chlorolaevulinic acid (5-CLA) did not affect the binding of ALA at the P-site, but did prevent PBG from binding, possibly indicating that the A-site had been modified (Jaffe et al., 1992). The stoichiometry of 5-CLA incorporation was 1 mol/mol of subunit, leading to complete inactivation, although extrapolation of the initial correlation between 5-CLA incorporation against activity revealed 50% inactivation on incorporation of 0.25 mol of 5-CLA, again suggesting that, like the P-site, the A-site may also be present at one site per two subunits (Seehra and Jordan, 1981).

The study on *E. coli* ALAD in the present paper focuses on the characterization of the A- and P-ALA binding sites using rate-of-dialysis analysis and substrate analogues. The reduced enzyme-ALA Schiff-base adduct was characterized by electro-spray mass spectrometry. In addition, the influence of the α -site metal and the effect of buffer composition on the V_{max} and K_m for ALA as well as substrate binding at the A- and P-sites were investigated.

MATERIALS AND METHODS

Chemicals

5-Amino[4- ^{14}C]laevulinic acid was purchased from New England Nuclear, DuPont Medical Products, Stevenage, Herts., U.K. ALA, laevulinic acid (LA), β -mercaptoethanol and Trizma base were purchased from Sigma Chemical Co., Poole, Dorset, U.K. 3-Oxo-1-hexylamine ester was a gift from D. Evans (Zeneca). Column-chromatography supplies were obtained from Pharmacia. All other chemicals were purchased from BDH (now Merck), Poole, Dorset, U.K.

Expression and purification of *E. coli* ALAD

E. coli ALAD was expressed in a recombinant strain containing the plasmid pUC18 harbouring the *hemB* gene. The strain was constructed by Dr. Charles Roessner (Texas A&M University) and was generously given. The enzyme was purified as described by Spencer and Jordan (1993).

Assay of *E. coli* ALAD

Portions of ALAD, pre-mixed with 50 mM potassium phosphate buffer containing 5 mM β -mercaptoethanol, were added to various amounts of ALA (final concn. 10 μM –5 mM) giving a known final pH. After incubation at 37 °C for 2 min, the reaction was terminated by 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 a portion was

removed and mixed with an equal volume of modified Ehrlich's reagent (Mauzerall and Granick, 1956). After 15 min for colour development, the A_{555} was determined ($\epsilon_{555} = 60200 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Mauzerall and Granick, 1956).

Atomic absorption studies

Enzyme samples for 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 into the linear response region of the standard curve: Zn^{2+} over the range of 5–30 μM and Mg^{2+} over the range 5–40 μM .

Preparation of *E. coli* apo-ALAD and mixed holo-ALAD metallo-species

E. coli apo-ALAD was prepared by overnight incubation of the holoenzyme with 10 mM EDTA and followed by gel filtration in 50 mM potassium phosphate buffer, pH 7, containing 5 mM β -mercaptoethanol to remove EDTA. The mixed metallo-species of *E. coli* ALAD, $\text{Mg}_x\text{Zn}_y\text{ALAD}$, was prepared as described by Spencer and Jordan (1994).

Covalent modification of the P-site of *E. coli* ALAD with ALA and NaBH_4

ALAD (0.2 ml; 2 mg/ml; 10 nmol) was added to 0.3 ml of 1 M potassium phosphate buffer, at pH values as stated in Figure legends for particular experiments, containing 50 μM Zn^{2+} and 10 mM β -mercaptoethanol at 20 °C. ALA (50 μl of 0.05–50 mM) was then added, followed immediately by 0.8 mg of NaBH_4 , and the sample was left for 1 h to react. Samples were then gel-filtered into 10 mM potassium phosphate buffer, pH 7, containing 10 mM β -mercaptoethanol and assayed for enzyme activity as above. Samples, modified as above, but with [5- ^{14}C]ALA [sp. radioactivity $(1\text{--}5) \times 10^{11}$ d.p.m./mol], were subsequently dialysed against 1×2 litres of 10 mM potassium phosphate buffer, pH 7, containing 10 mM β -mercaptoethanol and assayed for enzyme activity. The samples were then counted for radioactivity to determine radiolabel incorporation. For samples reductively modified at the P-site with ALA (or LA) before A-site binding studies, a similar procedure was performed using unlabelled ALA (or LA) on larger amounts of protein. This was followed by two further modification rounds, each after a dialysis step, to remove borate salts and excess reduced ALA (or LA). This protocol ensured maximum covalent modification of the P-site of the enzyme, which was then virtually inactive ($< 0.01\%$ of original specific activity). NaBH_4 treatment of the enzyme alone had no effect on activity. The reduced ALAD-ALA adduct was then concentrated to 6–8 mg/ml and dialysed against 10 mM Tris/HCl buffer, pH 7, containing 15 mM β -mercaptoethanol and 20 μM ZnCl_2 for the $\text{Zn}_x\text{Zn}_y\text{ALAD}$ species. Alternatively, the same buffer, but containing 1 mM Mg^{2+} instead of Zn^{2+} , was used for the $\text{Mg}_x\text{Zn}_y\text{ALAD}$ species. Apo-ALAD modified at the P-site was generated from P-site-labelled $\text{Zn}_x\text{Zn}_y\text{ALAD}$ by dialysis overnight against 10 mM Mes buffer, pH 6, containing 10 mM EDTA.

Preparation of samples for electro-spray mass spectroscopy

Samples of ALAD, modified at the P-site with ALA on reduction by NaBH_4 as above, were dialysed against 2×2 litres of 1 mM Tris/HCl buffer, pH 7, in the absence of β -mercaptoethanol. Activity and electro-spray mass-spectrometric determinations

were performed after prior incubation with dithiothreitol (5 mM) for 30 min. To samples (10 μ l) for electro-spray mass spectrometry, an equal volume of methanol (10 μ l) and acetic acid (1 μ l) were added before injection.

Measurement of ligand binding at the A-site by 'rate-of-dialysis' investigations

ALAD modified at the P-site with ALA (or LA) on reduction with NaBH_4 as above (120 μ l, 130–170 μ M) was buffered by addition of 12 μ l of 1 M Tris buffer/HCl, at pH 7 or 8, in a rapid-equilibrium-dialysis chamber (Womack and Colowick, 1973). External buffer, of identical composition, was circulated at 3 ml/min beneath the chamber. Labelled ALA (10 nmol, sp. radioactivity 1.015×10^{14} d.p.m./mol) was added either alone or with unlabelled ALA (or LA) (maximum of 10 μ l of 4–100 mM). A constant rate of efflux was reached after 1.5 min. After 2 min, four 30 s samples (each 0.5 ml) were collected and counted for radioactivity in 3 ml of Optiphase Hisafe III (Lablogic, Sheffield, U.K.) on a Philips PW4700 liquid-scintillation counter with quench correction to give d.p.m. The total d.p.m. eluted during the course of an experiment represented a maximum of 6% of the initial label added.

RESULTS AND DISCUSSION

Correlation between [^{14}C]ALA incorporated by NaBH_4 reduction into *E. coli* ALAD with enzyme activity

On reduction of *E. coli* ALAD with NaBH_4 in the presence of [^{14}C]ALA (see the Materials and methods section), a loss of enzyme activity occurred that paralleled incorporation of ^{14}C label, with 1 mol of ALA/mol of subunit leading to complete inactivation (Figure 1). These observations indicate that *E. coli* ALAD, like other ALADs [*Rhodopseudomonas sphaeroides*, Nandi and Shemin (1968b); bovine liver, Cheh and Neilands (1976); human, Gibbs and Jordan (1986)] also forms a Schiff base with ALA at the P-site. No evidence for half-site reactivity was found in *E. coli* ALAD, as has been shown for bovine ALAD (Cheh and Neilands, 1976; Jaffe and Hanes, 1986). As

Figure 1 Correlation of [^{14}C]ALA incorporation into ALAD on reduction by NaBH_4 against the percentage remaining of maximum activity

[^{14}C]ALA was incorporated into $\text{Zn}_2\text{Zn}_\beta\text{ALAD}$ as described in the Materials and methods section. After removal of excess [^{14}C]ALA, as described in the Materials and methods section, samples were assayed for enzyme activity and their content of [^{14}C]ALA was determined.

noted for bovine ALAD (Jaffe and Hanes, 1986), removal of the bound metal ions from *E. coli* ALAD did not prevent Schiff-base formation with ALA.

The extent of Schiff-base formation at different pH values was determined by monitoring the incorporation of [^{14}C]ALA at saturating ALA concentrations (see the Materials and methods section). The pK_a for [^{14}C]ALA incorporation was approx. 6.2 for $\text{Zn}_2\text{Zn}_\beta\text{ALAD}$ and 6.0 for $\text{Mg}_2\text{Zn}_\beta\text{ALAD}$ (results not shown). This pK_a reflects most likely the protonation state of the active-site lysine at the P-site, and is apparently little affected by the nature of the bivalent metal ion bound at the α -site. Although a pK_a of 6 for the lysine at the P-site of the *E. coli* ALAD is a remarkable perturbation from the pK_a of 10.5 found in free solution, such perturbations are not unknown. For instance, that of the active-site lysine of acetoacetate decarboxylase was determined to be 5.9 (Schmidt and Westheimer, 1971). An accurate determination of the K_d at the P-site, by plotting the extent of ALA incorporated at the P-site after reduction of the Schiff base, is precluded, as the relative speed of subsequent catalytic steps will also influence the ability to trap the Schiff-base intermediate.

Electro-spray mass-spectrometric analysis of *E. coli* ALAD and its reduced ALA adduct

Analysis of native $\text{Zn}_2\text{Zn}_\beta\text{ALAD}$ by electro-spray mass spectrometry (see the Materials and methods section) gave an M_r of 35500 ± 3 (Figure 2a). The M_r was unaffected by prior exposure of the enzyme to NaBH_4 in the absence of substrate (results not shown). ApoALAD and $\text{Co}_2\text{Co}_\beta\text{ALAD}$ (Spencer and Jordan, 1993) also had an M_r of 35500 ± 3 (results not shown), indicating that the M_r observed was not that of the metalloprotein but that of the polypeptide chain alone.

Electro-spray mass spectrometry has proved useful for the direct observation of enzymes with covalently bound substrate and intermediates (Aplin et al., 1990, 1991). The predicted M_r of the ALAD with its reduced ALA Schiff-base would be 114 higher, since the M_r of ALA is 132, minus 18 for the loss of H_2O on formation of the Schiff base, and adding 2 hydrogen atoms on reduction to the Schiff base with NaBH_4 . Reduction of the $\text{Zn}_2\text{Zn}_\beta\text{ALAD}$ species with NaBH_4 in the presence of 0.5 mM [^{14}C]ALA resulted in the incorporation of 0.4 mol of ALA/mol of subunit, with 60% of the original activity remaining. On analysis by electro-spray mass spectrometry, two major peaks of M_r 35497 ± 4 and 35608 ± 8 , were observed (Figure 2b), representing unmodified ALAD and the reduced ALA–ALAD adduct respectively, a difference of 111 ± 12 , in close agreement with the predicted value of 114. Reduction of the $\text{Zn}_2\text{Zn}_\beta\text{ALAD}$ species with NaBH_4 in the presence of 10 mM [^{14}C]ALA resulted in the incorporation of 1.1 mol of ALA/mol of subunit, with less than 5% of the original activity remaining. On analysis by electro-spray mass spectrometry, a major peak of 35614 ± 5 was observed, representing an increase of 114 ± 8 corresponding to the reduced ALA–ALAD adduct (Figure 2c), and there was no evidence of ALAD subunits with two ALA molecules or any significant unmodified species, again indicating no half-site reactivity. A small ($\approx 10\%$) 'shadow' peak $98 \pm 8 M_r$ units greater than the preceding major peak was observed in all samples. This satellite peak appeared to be an ALAD species, since it moves with NaBH_4 reduction in the presence of ALA. Whether it represents some minor heterogeneity of ALAD not observable on SDS/PAGE or an ionic adduct of sulphate or phosphate (despite exhaustive dialysis against Tris/HCl) is unclear, but does not affect interpretation of the data. Similarly, no significance is given to a small peak observed at M_r 36000 in Figure 2(c).

Figure 2 Determination of the M_r of *E. coli* ALAD and its ALA adduct formed on reduction with NaBH_4 by electron-spray mass spectrometry

[^{14}C]ALA was incorporated into Zn_2ZnALAD as described in the Materials and methods section. After removal of unbound [^{14}C]radioactivity, samples were prepared by electro-spray mass spectrometry as described in the Materials and methods section. (a) Mass transformation of the spectrum (shown in the inset) of unlabelled Zn_2ZnALAD , yielding a major species of $35\,500 \pm 3$. A minor species, of M_r 98 higher, is discussed in the text. (b) Mass transformation of the spectrum (shown in the inset) of Zn_2ZnALAD with 0.4 mol of [^{14}C]ALA incorporated per mol of subunit, as determined by radiolabelled incorporation. Two major species were found, of M_r $35\,497 \pm 4$ (unlabelled ALAD) and $35\,608 \pm 8$ (ALAD-ALA adduct). A minor species, of M_r 98 higher than the major peak, is discussed in the text. (c) Mass transformation of the spectrum (shown in the inset) of labelled Zn_2ZnALAD (0.9 mol of [^{14}C]ALA incorporated per mol of subunit, as determined by radiolabel incorporation) yielding a major species of M_r $35\,614 \pm 5$ (reduced ALAD-ALA adduct). A minor species of M_r 98 higher than the major peak, is discussed in the text.

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MPLDSTNIRQMTM·PRMFEETTLSLNDLVLPIFVEEID·ER·P·RAEGIAEY·P·N·
MT·RVCLKRQHLALTTCRSLKKKLT·SD·R·PREAIRES·A·D·
T·PRMFEETTLSLNDLVLPIFVEEID·ER·R·RAEGIAEY·A·D·
2 18                                42 9091 104 226    233 241 254 325

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Scheme 1 Resolution of the gene-derived protein sequence differences of *E. coli* ALAD by comparison of protein sequence with data obtained by electro-spray mass spectrometry

The differences in the two published gene sequences are shown (upper, Li et al., 1989; middle, Echelard et al., 1988) along with the resolved ALAD sequence (lower) determined by protein sequencing (N-terminal, residues 104, 241 and 254; Spencer and Jordan 1993) and electro-spray mass spectrometry (resolving residues 18–42, 90–91, 226–233). Residue numbering is that of Echelard et al. (1988).

Resolution of sequence discrepancies in *E. coli* ALAD by electro-spray mass spectrometry

Discrepancies between the two gene sequences published by Li et al. (1989) and Echelard et al. (1988) are shown in Scheme 1. Previous protein sequencing has resolved some of these differences (Spencer and Jordan, 1993). Other discrepancies can now be resolved by electro-spray mass spectrometry determination of the M_r of ALAD. The determined M_r of $35\,499 \pm 3$ (after subtraction of 1, as the M_r of the 1+ species is determined) was found to be consistent with the predicted M_r (including isotopes) of 35494 if the sequence given by Li et al. (1989), between residues 18–42 and 226–233, is used and residues 90 and 91 are E and R. These two amino acids are predicted by the gene sequence but not found in the derived protein sequence, where S and D are shown in Li et al. (1989).

Effect of buffer composition on the $V_{\max}$ and K_m for ALA of *E. coli* Zn_2ZnALAD and Mg_2ZnALAD

Under the conditions employed for substrate labelling, it was apparent that potassium phosphate was affecting enzymic

activity. Both K^+ and phosphate ions were shown to affect activity, whereas Na_2SO_4 and NaCl were without effect. K^+ ions were found to increase the $V_{\max}$ of the Zn_2ZnALAD species at pH values from 6 to 9 (Figure 3a), with a pH-independent K_s of $8(\pm 2)$ mM (Figure 3b) over the range pH 6–9. The K_m for ALA was unaffected over this pH range. K^+ ions had no effect on either the $V_{\max}$ or the K_m for ALA with the Mg_2ZnALAD species. K^+ ions have been shown to increase the activity of ALAD from *R. sphaeroides* by stimulating the aggregation of the original oligomers, giving oligomeric dimers and trimers (Nandi and Shemin, 1968a). However, there was no evidence for the formation of multiple oligomer forms of *E. coli* ALAD in the presence of K^+ ions (results not shown). K^+ ions have also been observed to increase the activity of type II metallo-aldolases (Horecker et al., 1972), which share some similarities with the reaction catalysed by ALAD. However, the mechanism of the K^+ stimulation of type II aldolases, as with the case of *E. coli* ALAD, is still unknown.

Phosphate ions were also found to increase the $V_{\max}$ of the Zn_2ZnALAD species over the pH range 6–9 (Figure 3a), together with the K_m for ALA. The virtually pH-independent K_m for ALA

Figure 3 Effect of buffer composition on the kinetic parameters K_m and V_{max} of $Zn_2ZnALAD$

(a) Effect of buffer composition on the V_{max} of $Zn_2ZnALAD$. Samples of $Zn_2ZnALAD$ were assayed with increasing concentrations of ALA (0.25–3 mM) at the pH and buffer compositions shown. The V_{max} values were then determined from double-reciprocal plots and are plotted against pH as shown: ●, 100 mM Tris buffer alone, or Mes buffer alone, pH 6; ■, 100 mM Tris buffer, or Mes buffer, pH 6, both containing 100 mM KCl; ▲, 100 mM Tris buffer, or Mes buffer, pH 6, both containing 300 mM sodium phosphate. (b) K_s for KCl stimulation of the V_{max} of $Zn_2ZnALAD$. Samples of $Zn_2ZnALAD$ were assayed with increasing concentrations of ALA (0.25–3 mM) in Tris buffer containing various amounts of KCl as shown. The V_{max} was then determined from a double-reciprocal plot, and is plotted as a percentage of the value in the absence of KCl against KCl concentration. K_s for KCl was determined as 8 ± 2 mM. (c) Effect of buffer composition on the K_m of $Zn_2ZnALAD$ for ALA. Samples of $Zn_2ZnALAD$ were assayed with increasing concentrations of ALA (0.25–3 mM) at the pH and buffer compositions shown. The K_m values were then determined from double-reciprocal plots and are plotted against pH as shown: ●, 100 mM Tris buffer alone, or Mes buffer alone, pH 6; ■, 100 mM Tris buffer, or Mes buffer, pH 6, both containing 50 mM sodium phosphate; ▲, 100 mM Tris buffer, or Mes buffer, pH 6, both containing 100 mM sodium phosphate; ◆, 100 mM Tris buffer, or Mes buffer, pH 6, both containing 200 mM sodium phosphate; ▼, 100 mM Tris buffer, or Mes buffer, pH 6, both containing 300 mM sodium phosphate; ▽, 100 mM Tris buffer, or Mes buffer, pH 6, both containing 400 mM sodium phosphate. (d) K_s for phosphate effect on the V_{max} and K_m for ALA of $Zn_2ZnALAD$. Samples of $Zn_2ZnALAD$ were assayed with increasing concentrations of ALA (0.25–3 mM) in Tris buffer containing various amounts of sodium phosphate (0–400 mM). The binding constants for the phosphate ion effect on V_{max} (■) and K_m (●) (both determined from double-reciprocal plots) are plotted against pH.

in Tris/HCl buffer became markedly pH-dependent in 200 mM sodium phosphate buffer (Figure 3c). The K_s for the phosphate ion binding affecting the K_m and V_{max} was found to be pH-dependent, falling from around 150 mM at pH 6 to 50 mM at pH 8 (Figure 3d). The marked-pH dependence of the K_m for ALA for the $Mg_2ZnALAD$ species in Tris buffer was further emphasized by the presence of phosphate ions, the K_m falling from 1 mM at pH 6 to 70 μ M at pH 8 (Figure 4a). However, in contrast with the $Zn_2ZnALAD$ species, the V_{max} for the $Mg_2ZnALAD$ species was decreased on addition of high concentrations of phosphate ions (Figure 4b). The binding constant for phosphate ions to the $Mg_2ZnALAD$ species affecting the K_m and V_{max} was pH-independent from 6 to 9 at 100 mM.

By plotting $1/K_m$ against pH, the pK_a of ionizing groups in the free enzyme and free substrate can be determined. Similarly, a plot of V_{max}/K_m also gives a value for the ionization of a group required for activity in the free enzyme and free substrate. Different values in the two plots would indicate a change in the rate-determining step with pH. For the $Mg_2ZnALAD$ species a plot of both $1/K_m$ and V_{max}/K_m against pH, in both Tris and phosphate buffer, indicated a group ionizing in the free enzyme (or free substrate) with a pK_a of 7.3 (Figures 5a and 5b). As the free substrate has groups with pK_a 4 (carboxyl group) and 8.9 (amino group) (Schlösser and Beyersmann, 1987), the pK_a of 7.3

can be assigned to the free enzyme (Table 1). A similar pK_a value of around 7.1 (in both Tris and phosphate buffers) of a group ionizing in the free $Zn_2ZnALAD$ species was obtained from a plot of V_{max}/K_m against pH (Figure 5c); however, the plot of $1/K_m$ against pH indicated values of 6.2 and 6.5 in Tris and phosphate buffers respectively, possibly indicating a change in the rate-determining step with pH (Figure 5d). The activity/pH profile indicates the pK_a of a group required for activity in the ES complex. For the $Zn_2ZnALAD$ species this was determined to be approx. 6.2 in phosphate buffer and 6.8 in Tris buffer (Figure 3a). A similar activity/pH profile was obtained for the $Mg_2ZnALAD$ species, with a pK_a of approx. 6 in both phosphate and Tris buffer (Figure 4b). Table 1 summarizes the pK_a values of catalytically important groups in the E and ES forms of $Zn_2ZnALAD$ and $Mg_2ZnALAD$.

A detailed kinetic study on the comparison of bovine Zn^{2+} and Cd^{2+} ALADs (Schlösser and Beyersmann, 1987) revealed a change in the pK_a of a group in the free enzyme from 6.5 for Zn^{2+} -ALAD to 7.0 for Cd^{2+} -ALAD; the group requiring deprotonation for activity was determined to be a histidine residue. On comparing these results with this study of *E. coli* ALAD, a group of similar pK_a , 7.2, in the free $Mg_2ZnALAD$ enzyme species was also found, although whether this pK_a was influenced by the nature of the α -site metal ion is unclear, due to an

Figure 4 Effect of buffer composition on the K_m of $MgZnALAD$ for ALA (a) and on the V_{max} of $MgZnALAD$

Samples of $MgZnALAD$ were assayed with increasing concentrations of ALA (25 μM –4 mM) at the pH and buffer compositions shown. (a) The K_m values were then determined from double-reciprocal plots and are plotted against pH as shown: $\bullet$, 100 mM Tris buffer alone, or Mes buffer alone, pH 6; $\blacksquare$, 100 mM Tris buffer, or Mes buffer, pH 6, both containing 100 mM sodium phosphate; $\blacktriangle$, 100 mM Tris buffer, or Mes buffer, pH 6, both containing 200 mM sodium phosphate. (b) The V_{max} values were then determined from double-reciprocal plots and are plotted against pH as shown: $\bullet$, 100 mM Tris buffer alone, or Mes buffer alone, pH 6; $\blacksquare$, 100 mM Tris buffer, or Mes buffer, pH 6, both containing 300 mM sodium phosphate.

apparent change in the rate-determining step with pH for $Zn_2ZnALAD$ compared with the $MgZnALAD$ species. On substrate binding, the pK_a of a group requiring deprotonation for activity in the ES complex of $MgZnALAD$ and $Zn_2ZnALAD$ was 6.0 and 6.2 respectively, implying a pH-dependence of substrate binding (Figures 3b and 3a). Both bovine Zn^{2+} - and Cd^{2+} -ALAD-substrate complexes were shown to have altered pK_a values of 6 and 6.4, respectively, compared with free enzyme, implying a pH-dependence of substrate binding. This group was proposed to reflect the ionization of an active-site cysteine (Schlösser and Beyersmann, 1987), although the pK_a of the group required for activity in the ES complex of *E. coli* ALAD might represent the pK_a of the 5-amino group of the ALA bound at the P-site.

Although no firm mechanistic conclusions can be drawn from the above kinetic findings, they do allow the selection of the best conditions under which to study particular properties, for example the optimum binding of ALA to the A-site.

Structural components of the A-site for ALA-binding in *E. coli* ALAD determined from ligand-binding analysis by rate-of-dialysis

As the two substrates of ALAD are identical, it has been difficult to determine independently the individual properties of the two binding sites. Some characteristics of the P-site have been

Figure 5 Determination of the pK_a of a group required for enzyme activity in the free ALAD enzyme from plots of $1/K_m$ and V_{max}/K_m

(a) Plot of $1/K_m$ against pH for $MgZnALAD$: $\bullet$, in 300 mM Tris buffer; $\blacksquare$, in 300 mM sodium phosphate buffer. The plot indicates a group with a pK_a of 7.2 required for activity in the free enzyme $MgZnALAD$ species. (b) Plot of V_{max}/K_m against pH for $MgZnALAD$: $\bullet$, in 300 mM Tris buffer; $\blacksquare$, in 300 mM sodium phosphate buffer. The plot indicates a group with a pK_a of 7.2 required for activity in the free enzyme $MgZnALAD$ species. (c) Plot of V_{max}/K_m against pH for $Zn_2ZnALAD$: $\bullet$, in 300 mM Tris buffer; $\blacksquare$, in 300 mM sodium phosphate buffer. The plot indicates a group with a pK_a of 7.1 required for activity in the free enzyme $Zn_2ZnALAD$ species. (d) Plot of $1/K_m$ against pH for $Zn_2ZnALAD$: $\bullet$, in 300 mM Tris buffer; $\blacksquare$, in 300 mM sodium phosphate buffer. Values determined at pH 5.2 contained in addition 100 mM sodium acetate to buffer at this pH. The plot indicates a group with a pK_a of 6.2 (in phosphate) and 6.5 (in Tris) required for activity in the free enzyme $Zn_2ZnALAD$ species.

Table 1 Summary of the pK_a of ionizing groups in the free enzyme and ES complexes of $Zn_2ZnALAD$ and $MgZnALAD$ in 300 mM phosphate and Tris buffer

For comparison, the pK_a values determined for Zn^{2+} - and Cd^{2+} -(bovine ALAD) (Schlösser and Beyersmann, 1987) are also shown.

	<i>E. coli</i> ALAD		Bovine ALAD	
	$Zn_2Zn\beta$	$MgZn\beta$	Zn^{2+}	Cd^{2+}
pK_a of free enzyme in Tris	7.2/6.2*	7.2	6.5	7.0
pK_a of ES complex in Tris	6.8	6.0	6.0	6.4
pK_a of free enzyme in phosphate	7.1/6.5*	7.2		
pK_a of ES complex in phosphate	6.2	6.0		

* Plots of $1/K_m$ and V_{max}/K_m gave different values for the pK_a of the free enzyme, indicating a change in the rate-limiting step with pH.

determined by analysis of the ability to form the enzyme-ALA Schiff base, the identity of the residue involved (Nandi and Shemin, 1968b) and its sequence position (Gibbs and Jordan, 1986). However, the A-site has remained uncharacterized, owing to the difficulty of loading the P-site quantitatively with ALA by reduction with $NaBH_4$. Thus any residual activity due to unmodified P-site would preclude equilibrium-dialysis experi-

Figure 6 Determination of the affinity of ALA binding to the A-site of P-site-modified ALADs with different metal ions at the α -site

The concentration of free and bound enzyme species could be calculated from the percentage of maximum label eluted after addition of [^{14}C]ALA alone or with various amounts of unlabelled ALA. Klotz analysis of this data (where Q is the fractional saturation and n the ligand concn.) can then determine the number of sites per subunit and the affinity of binding. (a) $\text{Mg}_\alpha\text{Zn}_\beta\text{ALAD}$. Klotz analysis of data is shown in the text. One binding site per subunit was present, with a dissociation constant of $69 \pm 20 \mu\text{M}$. (b) $\text{Zn}_\alpha\text{Zn}_\beta\text{ALAD}$. A $5 \mu\text{l}$ portion of [^{14}C]ALA (10 nmol) was added to a $132 \mu\text{l}$ sample of $\text{Zn}_\alpha\text{Zn}_\beta\text{ALAD}$ (final concn. $148 \mu\text{M}$) in 100 mM Tris buffer, pH 8, alone, or with various amounts of unlabelled ALA ($3 \mu\text{l}$ of 4 mM; $6 \mu\text{l}$ of 4 mM; $3.5 \mu\text{l}$ of 10 mM). Klotz analysis indicated one binding site per subunit, with a dissociation constant of $45 \pm 15 \mu\text{M}$.

ments to determine ALA binding to the A-site, as some of the ALA present would give rise to product. To overcome these difficulties, ALAD was exhaustively modified at the P-site by ALA (or LA) by three courses of reduction (see the Materials and methods section). The resulting modified enzyme species was virtually inactive ($< 0.01\%$ activity). Electro-spray mass-spectrometric analysis of ALAD after multiple rounds of reduction in the presence of ALA indicated the presence of a single species, corresponding to the reduced ALAD-ALA adduct. ALA binding exclusively to the A-site of the reduced ALAD-ALA adduct was then investigated by the rapid technique of rate-of-dialysis analysis (see the Materials and methods section).

On addition of [^{14}C]ALA ($5 \mu\text{l}$; 10 nmol; 1×10^6 d.p.m.) to buffer alone (see the Materials and methods section), a constant rate of ALA efflux from the rate of dialysis apparatus was observed, of 16400 d.p.m./min. Addition of [^{14}C]ALA to the $\text{Mg}_\alpha\text{Zn}_\beta\text{ALAD}$ species (final enzyme concn. $143 \mu\text{M}$), modified at the P-site with ALA, resulted in a decrease in the rate of efflux to 6200 d.p.m./min, indicating that ALA was no longer free in solution, but was binding to the modified enzyme. Repeating the experiment with addition of [^{14}C]ALA together with known

amounts of unlabelled ALA (40 nmol and 100 nmol) resulted in a progressive displacement of [^{14}C]ALA from the enzyme, observed as an increase in the rate of [^{14}C]ALA efflux (11400 and 14000 d.p.m./min respectively). The percentage of maximum radioactivity eluted indicates the percentage of [^{14}C]ALA added that is unbound, thus allowing the determination of both substrate-bound and free enzyme, since ALA and enzyme concentrations are known. From this information, Klotz analysis (Stinson and Holbrook, 1973) of the data indicated that one binding site per subunit was present in the $\text{Mg}_\alpha\text{Zn}_\beta\text{ALAD}$ species, with a K_d of $69 \pm 20 \mu\text{M}$ (Figure 6a). Similar experiments with the $\text{Zn}_\alpha\text{Zn}_\beta\text{ALAD}$ species also indicated one binding site per subunit, with a broadly similar K_d of $45 \pm 15 \mu\text{M}$ (Figure 6b). As the $\text{Zn}_\alpha\text{Zn}_\beta\text{ALAD}$ and $\text{Mg}_\alpha\text{Zn}_\beta\text{ALAD}$ species in Tris, pH 8, both have a similar K_d for ALA at the A-site, and yet have different K_m values (in Tris, pH 8; $600 \mu\text{M}$ and $70 \mu\text{M}$ respectively), this would imply that the α -site metal is affecting the binding affinity at the P-site. The ability of ALAD, in which the P-site is reductively modified with ALA, to bind ALA at the A-site with considerable affinity implies that the slight alteration of the substrate conformation bound at the P-site (sp_2 to sp_3 on reduction of the Schiff base) may not have appreciably altered the nature of the A-binding site.

The K_d of ALA binding to the A-site of the modified $\text{Mg}_\alpha\text{Zn}_\beta\text{ALAD}$ species increased from $69 \pm 20 \mu\text{M}$ at pH 8.2 to $214 \pm 40 \mu\text{M}$ at pH 7, and no significant binding of ALA to the A-site of modified $\text{Zn}_\alpha\text{Zn}_\beta\text{ALAD}$ was observed at pH 6 ($K_d > 800 \mu\text{M}$), indicating that the protonation of a group below pH 8 was decreasing the affinity of the A-site for ALA. However, a precise pK_a could not be determined, as only K_d values below approx. $700 \mu\text{M}$ could be determined under the conditions chosen. The values measured for the A-site affinity of the modified $\text{Mg}_\alpha\text{Zn}_\beta\text{ALAD}$ species with pH were similar to the K_m measured under the same conditions, indicating that, for this species, the A-site affinity was limiting the K_m . It is possible that the protonation of the 5-amino group of ALA bound at the P-site in the ES complex may be responsible for decreasing the affinity of ALA at the A-site, possibly by electrostatic repulsion of the two charged amino groups. N.m.r. studies of bovine Zn^{2+} -ALAD, with ALA bound as a Schiff base at the P-site, have indicated that the 5-amino group of P-site ALA is indeed partially deprotonated at pH 7 (Jaffe et al., 1990), although its precise pK_a remains to be determined.

The A-site affinity of the modified $\text{Zn}_\alpha\text{Zn}_\beta\text{ALAD}$ species at pH 8 was determined as $45 \pm 15 \mu\text{M}$ in Tris buffer and this increased to $150 \pm 35 \mu\text{M}$ in 200 mM sodium phosphate. Neither of the K_d values determined for A-site affinity of the modified $\text{Zn}_\alpha\text{Zn}_\beta\text{ALAD}$ species, in Tris or phosphate buffer, could account for the observed K_m values for ALA under the same conditions of $400 \mu\text{M}$ and 1.2 mM respectively, suggesting that the affinity for ALA at the P-site for this species is limiting the K_m . Therefore the increase in K_m for ALA in the modified $\text{Zn}_\alpha\text{Zn}_\beta\text{ALAD}$ species, when in phosphate buffer, is likely to result from a decrease in affinity for ALA at the P-site.

Addition of [^{14}C]ALA to modified apo-ALAD (prepared by the removal of the metal ions from the $\text{Mg}_\alpha\text{Zn}_\beta\text{ALAD}$ species modified at the P-site by reduction by NaBH_4 in the presence of ALA) indicated a small amount of possible ALA binding (maximum 15%), but a K_d could not be determined, as the magnitude of signal difference was variable. It is likely that the presence of a small amount of holo-ALAD could account for this observation. As apo-ALAD appeared to be unable to bind ALA at the A-site, a role of one of the bound metal ions in binding ALA at this site is implicated. Since species of ALAD with a single metal ion bound at the α -site are active (e.g.

Co_{0.8}ALAD; Spencer and Jordan, 1994), the ability of ALA to bind at the A-site can be assigned to the α -site metal.

Addition of [¹⁴C]ALA to the Mg₂Zn₂ALAD species, modified reductively at the P-site with LA, showed no significant binding of ALA, indicating a $K_d > 800 \mu\text{M}$. Therefore, the 5-amino group of ALA bound at the P-site appears to be a critical component in the formation of the ALA A-binding site, and could explain why ALAD has an ordered kinetic mechanism, with ALA first binding at the P-site, as determined by Jordan and Seehra (1980).

Structural requirements of ligands binding at the A- and P-site of ALAD

Previous studies have identified the relative importance of the three functional groups of ALA in its binding to *R. sphaeroides* ALAD (Lüönd et al., 1992) and bovine ALAD (Ha et al., 1993), but could not distinguish whether the same functional group was equally important in the binding to both the A- and P- substrate-binding sites. To investigate further the structural requirements for both the A- and P-sites, a range of compounds related to ALA was investigated to determine whether they would interact specifically with one or other site. 5-Aminovaleric acid (20 mM) did not inhibit ALAD, and failed to compete with [¹⁴C]ALA for the A-site, indicating a critical requirement for the carbonyl group of ALA in binding to both the A- and P-sites of ALAD. The α -aminoketone 3-oxo-1-hexylamine (2 mM) did not result in any inhibition of the Mg₂Zn₂ALAD enzyme when assayed with 0.5 mM ALA in Tris buffer, pH 8. This implied a critical role for the carboxylic acid group for ALA binding at both the A- and P-sites.

LA is a competitive inhibitor of ALAD from *E. coli*, and was shown to be able to form a Schiff base at the P-site (results not shown). However, it has not been possible previously to determine whether LA inhibition also occurs at the A-site. Addition of LA to modified ALAD failed to displace [¹⁴C]ALA bound at the A-site. The inability of LA to displace ALA from the A-site indicates that LA is acting as a specific P-site inhibitor of ALAD. This finding also implies a critical role for the 5-amino group of ALA in substrate binding at the A-site, in contrast with the redundancy of this group as a binding requirement at the P-site. Therefore *E. coli* ALAD would not be expected to be able to form mixed pyrroles between ALA and LA, as proposed by Nandi and Shemin (1968b) for the *R. sphaeroides* ALAD. The earlier report by Nandi and Shemin (1968b) of heterologous pyrrole formation of ALA with LA has not been confirmed (Jordan and Seehra, 1980) and may have arisen from non-enzymic condensation of ALA and LA at the high pH and long incubation times employed.

Until now it has not been possible to determine whether inhibitors of ALAD have been acting at the A- and/or P-sites (Lüönd et al., 1992; Ha et al., 1993). From experiments described above, the interaction of inhibitors at the A-site can now be determined by competition with ALA for the A-site of ALAD reductively modified with ALA at the P-site. In addition, the interaction of inhibitors at the P-site may be determined by prevention of Schiff-base formation between ALAD and [¹⁴C]ALA determined on reduction with NaBH₄. 5-CLA inactivation of ALAD is not prevented by addition of LA (Seehra and Jordan, 1981), and, with the knowledge that LA can only bind to the P-site, this would indicate that the site of alkylation of

5-CLA is not at the P-site. However, protection by LA against 3-chlorolaevulinic acid (3-CLA) inactivation of ALAD is evident, indicating that this inhibitor is inactivating at the P-site, although it does not form a Schiff base (Seehra and Jordan, 1981). The site of action of 5-CLA in bovine ALAD has recently been identified as a cysteine residue (equivalent to Ser-229 in the *E. coli* sequence), which results in the loss of the bound A-site metal ion (Jaffe et al., 1992) and would therefore result in loss of the A-binding site.

The greater understanding of the interaction of ALA with its A- and P-binding sites on ALAD as gained from the above studies should allow a more informed design of specific inhibitors for both sites of ALAD.

The S.E.R.C. and A.F.R.C. are gratefully acknowledged for financial support. Thanks are due to Dr. C. Roessner, Texas A&M University, TX, U.S.A., for providing the *E. coli hemB* expression system, to Mr D. Barton and Mr. P. Skelton (Cambridge) for electro-spray mass-spectrometry determinations, and to Dr. J. Cheung, Queen Mary and Westfield College, London, for deprotecting the 3-oxo-1-hexylamine.

REFERENCES

- Aplin, R. T., Baldwin, J. E., Fyishima, Y., Schofield, C. J. and Waley, S. G. (1990) FEBS Lett. **277**, 212–215
- Aplin, R. T., Baldwin, J. E., Pichon, C., Roessner, C. A., Scott, A. I., Schofield, C. J., Stolorow, N. J. and Warren, M. J. (1991) Bioorg. Med. Chem. Lett. **1**, 503–507
- Bevan, D. R., Bodlaender, P. and Shemin, D. (1980) J. Biol. Chem. **255**, 2030–2035
- Boese, Q. F., Spano, A. J., Li, J. and Timko, M. P. (1991) J. Biol. Chem. **266**, 17060–17066
- Chen, A. M. and Neilands, J. B. (1976) Struct. Bonding (Berlin) **29**, 123–169
- Dent, A. J., Beyersmann, D., Block, C. and Hasnain, S. S. (1990) Biochemistry **29**, 7822–7828
- Echelard, Y., Dymetysyn, J., Drolet, M. and Sasarman, A. (1988) Mol. Gen. Genet. **214**, 503–508
- Gibbs, P. N. B. (1984) Ph.D. Dissertation, University of Southampton
- Gibbs, P. N. B. and Jordan, P. M. (1986) Biochem. J. **236**, 447–451
- Gibbs, P. N. B., Gore, M. G. and Jordan, P. M. (1985) Biochem. J. **225**, 573–850
- Ha, H.-J., Park, J.-W., Oh, S.-J., Lee, J.-C. and Song, C. (1993) Bull. Korean Chem. Soc. **14**, 652–654
- Horecker, B. L., Tsolas, O. and Lai, C. Y. (1972) Enzymes 3rd Ed. **7**, 213–258
- Jaffe, E. K. and Hanes, D. (1986) J. Biol. Chem. **261**, 9348–9353
- Jaffe, E. K., Salowe, S. P., Chen, N. T. and DeHaven, P. A. (1984) J. Biol. Chem. **259**, 5032–5036
- Jaffe, E. K., Markham, G. D. and Rajagoplan, J. S. (1990) Biochemistry **29**, 8345–8350
- Jaffe, E. K., Abrams, W. R., Kaempfen, H. X. and Harris, K. A., Jr. (1992) Biochemistry **31**, 2113–2123
- Jordan, P. M. (1991) Biosynthesis of Tetrapyrroles: New Compr. Biochem. **19**, 1–66
- Jordan, P. M. and Gibbs, P. N. B. (1985) Biochem. J. **227**, 1015–1020
- Jordan, P. M. and Seehra, J. S. (1980) FEBS Lett. **114**, 283–286
- Li, J. M., Russell, C. S. and Cosloy, S. D. (1989) Gene **75**, 177–184
- Liedgens, W., Lutz, C. and Schneider, H. A. W. (1983) Eur. J. Biochem. **135**, 75–79
- Lüönd, R. M., Walker, J. and Neier, R. W. (1992) J. Org. Chem. **57**, 5005–5013
- Mitchell, L. W. and Jaffe, E. K. (1993) Arch. Biochem. Biophys. **300**, 169–177
- Mauzerall, D. and Granick, S. (1956) J. Biol. Chem. **219**, 435–446
- Nandi, D. L. and Shemin, D. (1968a) J. Biol. Chem. **243**, 1231–1235
- Nandi, D. L. and Shemin, D. (1968b) J. Biol. Chem. **243**, 1236–1242
- Nandi, D. L., Baker-Cohen, K. F. and Shemin, D. (1968) J. Biol. Chem. **243**, 1224–1230
- Schaumburg, A., Schneider-Poetsch, A. A. W. and Eckerskorn, C. (1991) Z. Naturforsch. **47C**, 77–84
- Schlösser, M. and Beyersmann, D. (1987) Biol. Chem. Hoppe-Seyler **368**, 1469–1477
- Schmidt, D. E. and Westheimer, F. H. (1971) Biochemistry **10**, 1249–1253
- Seehra, J. S. and Jordan, P. M. (1981) Eur. J. Biochem. **113**, 435–446
- Spencer, P. and Jordan, P. M. (1993) Biochem. J. **290**, 279–287
- Spencer, P. and Jordan, P. M. (1994) Biochem. J. **300**, 373–381
- Stinson, R. A. and Holbrook, J. J. (1973) Biochem. J. **131**, 719–728
- Tsukamoto, I., Yoshinaga, T. and Sano, S. (1979) Biochim. Biophys. Acta. **570**, 167–178
- Wetmur, J. G., Bishop, D. F., Cantelmo, C. and Desnick, R. J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7703–7707
- Womack, F. C. and Colowick, S. P. (1973) Methods Enzymol. **27**, 464–471