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Investigation of the effect of metal ions on the reactivity of thiol groups in human 5-aminolaevulinate dehydratase

Philip N. B. GIBBS, Michael G. GORE and Peter M. JORDAN
Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

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1. The reaction of human 5-aminolaevulinate dehydratase with 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2) results in the release of 4 molar equivalents of 5-mercapto-2-nitrobenzoic acid (Nbs) per subunit. 2. Two of the thiol groups reacted very rapidly (groups I and II), and their rate constants were determined by stopped-flow spectrophotometry; the other two thiol groups (groups III and IV) were observed by conventional spectroscopy. 3. Titration of the enzyme with a 1 molar equivalent concentration of Nbs_2 resulted in the release of 2 molar equivalents of Nbs and the concomitant formation of an intramolecular disulphide bond between groups I and II. 4. Removal of zinc from the holoenzyme increased the reactivity of groups I and II without significantly affecting the rate of reaction of the other groups. 5. The reactions of the thiol groups in both the holoenzyme and apoenzyme were little affected by the presence of Pb^{2+} ions at concentrations that strongly inhibit the enzyme, suggesting that Zn^{2+} and Pb^{2+} ions may have independent binding sites. Protein fluorescence studies with Pb^{2+} and Zn^{2+} have shown that the binding of both metal ions results in perturbation of the protein fluorescence.

5-Aminolaevulinate dehydratase (EC 4.2.1.24), the second enzyme of the haem-biosynthetic pathway, catalyses the condensation between two molecules of 5-aminolaevulinic acid to form the pyrrole porphobilinogen. The human enzyme has been purified to homogeneity (Anderson & Desnick, 1979; Gibbs, 1984) and has been shown to consist of eight identical subunits of M_r 31000–35000.

Human 5-aminolaevulinate dehydratase, in common with all mammalian dehydratases, is sensitive to oxygen and requires a high exogeneous thiol concentration for the maintenance of full catalytic activity. This requirement is related to the presence of 32 reactive thiol groups per octamer, four per subunit (Gibbs & Jordan, 1981). Previous studies have provided detailed evidence for the close interrelationship between the status of thiol groups and the catalytic activity of the bovine liver enzyme (see Seehra *et al.*, 1981).

Detailed studies with $^{65}Zn^{2+}$ ions have shown that 8 mol of Zn^{2+} is bound per mol of octamer and that the amount of ^{65}Zn bound to the enzyme closely parallels enzyme activity. Treatment of the enzyme with oxygen or Nbs_2 causes the release of ^{65}Zn label and inactivation of the enzyme, highlighting the importance of the availability of free thiol groups for the binding of the Zn^{2+} ion (Gibbs & Jordan, 1981).

The activity of 5-aminolaevulinate dehydratase in human erythrocytes has been used as a highly specific and sensitive indication in lead poisoning, since the enzyme is strikingly sensitive *in vivo* to low concentrations of blood lead (Hernberg & Nikkanen, 1970; Haeger-Aronsen *et al.*, 1971). Zn^{2+} completely reverses the lead-induced inhibition both *in vivo* and *in vitro* (Finelli *et al.*, 1975; Haeger-Aronsen *et al.*, 1976).

In view of the importance of the integrity of thiol groups for the catalytic activity of the dehydratase, we decided to investigate the nature of any interactions between these groups and the metal ions Zn^{2+} and Pb^{2+} and to establish the relationship between the binding of these metal ions and the activity of the human erythrocyte enzyme.

Abbreviations used: Nbs, 5-mercapto-2-nitrobenzoic acid; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

Materials and methods

Chemicals

Dithioerythritol, 5-aminolaevulinic acid hydrochloride and 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs_2) were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. Sephadex G-50 was obtained from Pharmacia, Uppsala, Sweden. All other laboratory reagents were obtained from BDH Laboratories, Poole, Dorset, U.K.

5-Aminolaevulinate dehydratase

The enzyme was purified from 2.4 litres of human blood by the method of Gibbs (1984). The purified enzyme had a specific activity of 24.0 units/mg in the presence of 100 μM-$ZnCl_2$ (15–18 units/mg in the absence of the metal ion) and was stored as a 60%-satn.-$(NH_4)_2SO_4$ precipitate at 0°C. One unit of enzyme catalyses the formation of 1 μmol of porphobilinogen/h at 37°C under the conditions described below.

Activation of enzyme

5-Aminolaevulinate dehydratase was activated before use by incubation in 0.1 M-potassium phosphate buffer, pH 6.8, containing 10 mM-dithioerythritol and 100 μM-$ZnCl_2$ at 37°C for 10 min. The enzyme, thus activated, was then desalted under an atmosphere of N_2 on a column of Sephadex G-50 equilibrated at 0°C in 0.1 M-potassium phosphate buffer and stored at 0°C under N_2 until required.

Assay for enzymic activity

The incubation mixtures contained 100 μmol of potassium phosphate buffer, pH 6.8, 10 μmol of dithioerythritol, 100 μmol of $ZnCl_2$, activated enzyme (maximum of 0.015 unit) and 5 μmol of 5-aminolaevulinate, neutralized with 0.1 M-NaOH before use, in a final volume of 1 ml. The incubations were carried out for 10 min at 37°C and were terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid containing 0.1 M-$HgCl_2$. The solution was centrifuged and the supernatant was added to an equal volume of freshly prepared modified Ehrlich's reagent [4-dimethylaminobenzaldehyde (1 g) in acetic acid (40 ml) and 60–62% (w/v) $HClO_4$ (10 ml)]. The coloured complex formed with porphobilinogen was measured spectrophotometrically ($\lambda_{max.} = 555$ nm; $\varepsilon_{555} = 60200\ M^{-1} \cdot cm^{-1}$) (Mauzerall & Granick, 1956).

When the enzyme activity was assayed in the absence of thiol, the incubations were carried out as described above except that all buffers were saturated with N_2 before use. Incubations were carried out in stoppered tubes for 2 min at 37°C under an atmosphere of N_2 with up to 0.05 unit of enzyme.

Preparation of the apoenzyme

At all stages precautions were taken to ensure minimal contamination by trace elements (see Thiers, 1957). The human 5-aminolaevulinate dehydratase apoenzyme was prepared by a method similar to that described by Tsukamoto *et al.* (1979). Purified holoenzyme (2–3 mg) was incubated in 0.1 M-Tris/HCl buffer, pH 7.1 (200 μl), containing 10 μmol of EDTA and 2 μmol of dithioerythritol for 30 min at 37°C. Column chromatography of the incubation mixture under N_2 on Sephadex G-50 equilibrated in 0.1 M-Tris/HCl buffer, pH 7.1, yielded the apoenzyme, which contained between 0.07 and 0.09 Zn atoms per subunit of the enzyme (as determined by atomic absorption spectroscopy). The holoenzyme was obtained when 100 μM-$ZnCl_2$ (final concentration) was added to the apoenzyme and incubated at 25°C for 2 min before use.

Spectrophotometric determinations

Reaction of 5-aminolaevulinate dehydratase with Nbs_2, with the use of conventional spectrophotometry. The human dehydratase apoenzyme (10 nmol), prepared as described above, was mixed in a final volume of 1 ml with Nbs_2 (5–500 nmol) in 100 μmol of Tris/HCl buffer, pH 7.1, at 25°C in the presence and in the absence of 100 nmol of $ZnCl_2$. The absorbance of the liberated Nbs was measured at 412 nm with a Pye–Unicam SP. 8400 spectrophotometer ($\varepsilon_{412} = 12800\ M^{-1} \cdot cm^{-1}$) and monitored until the reaction was complete (approx. 30 min). Similar reactions were performed in the presence and in the absence of 50 nmol of $PbCl_2$ (see the Results section and Fig. 1). This method was used for time courses of greater than 30 s.

Reaction of 5-aminolaevulinate dehydratase with Nbs_2, with the use of stopped-flow spectrophotometry. For time courses of the reaction between 5-aminolaevulinate dehydratase and Nbs_2 from 0.25 to 20 s, a Hi-Tech Instruments (Salisbury, Wilts., U.K.) stopped-flow spectrophotometer was used. The apparatus had a dead-time of approx. 1.8 ms with a pathlength of 2 mm. The change in absorbance of each reaction was monitored simultaneously on an oscilloscope and on a Biomac 1000 (Data Handling Services, Mitcham, Surrey, U.K.), which stored and averaged four consecutive traces before displaying the resultant trace on to a chart recorder. The stopped-flow apparatus was fitted with two 4 ml syringes, one containing 10 μM-enzyme in 0.1 M-Tris/HCl buffer, pH 7.1, at 25°C and the other containing 1 mM-Nbs_2 in same buffer. The reaction of 5-aminolaevulinate dehydratase with Nbs_2 was performed in the presence and in the absence of either $ZnCl_2$ (100 μM) or $PbCl_2$ (50 μM) (final concentrations).

Protein fluorescence spectrophotometry. The human dehydratase apoenzyme (180μg, 5.14nmol) was dissolved in 200μmol of potassium phosphate buffer, pH6.8 (2ml), containing 20μmol of dithioerythritol and placed in a fluorimeter cell (1cm × 1cm). The fluorescence maximum of the apoenzyme was determined in a Perkin–Elmer MPF3 fluorimeter thermostatically maintained at 21°C by excitation of the endogenous aromatic amino acids at 280nm and scanning the emission wavelength from 290nm to 370nm at a scan rate of 15nm/min. Small volumes (10μl) of the bivalent metal ions, $ZnCl_2$ (4–800nmol) and $PbCl_2$ (10–300nmol), were mixed with the apoenzyme, and the fluorescence intensity and the wavelength of maximum emission were measured. The effect of the chelating agent EDTA (2–18μmol) on the fluorescence of both the apoenzyme and holoenzyme was also investigated.

Oxidation of the enzyme

Exogenous thiol was removed from the activated enzyme on a column of Sephadex G-50 equilibrated at 0°C in 0.1M-potassium phosphate buffer, pH6.8, and the enzyme was exposed to air oxidation at 37°C in the presence and in the absence of 100μM-$ZnCl_2$. Samples were removed at timed intervals and assayed for both enzyme activity (under N_2) and protein concentration.

Protein measurements

Protein concentrations were determined by the method of Lowry *et al.* (1951) in the absence of thiol or by the measurement of the absorbance at 280nm against the appropriate blank ($\varepsilon_{280} = 28570 M^{-1} \cdot cm^{-1}$, assuming a subunit M_r of 35000).

Results

Reaction of 5-aminolaevulinate dehydratase with Nbs_2

When the native human apoenzyme was treated with a 50-fold molar excess of Nbs_2 at 25°C in the presence and in the absence of metal ions, 4mol of Nbs was released into solution per mol of enzyme subunit (M_r 35000) over a period of 30min (Fig. 1). The progress curve for the reaction of the apoenzyme in the absence of added $ZnCl_2$ shows the rapid liberation of 2mol of Nbs per mol of enzyme subunit (within 10s), implying the presence of two highly reactive thiol groups (groups I and II). A third group titrated over a period of 4–5min (group III), followed by the slow reaction of the final group (group IV), which took about 30min to react completely with the Nbs_2. Semilogarithmic analysis of the data in Fig. 1 yielded a curve with two linear slopes, attributable to groups III and IV, which intersected the ordinate at 25% and 50%. The true second or rate constants for groups III and IV obtained by using a range of Nbs_2 concentrations were $1.63 \times 10^3 M^{-1} \cdot min^{-1}$ and $2.76 \times 10^2 M^{-1} \cdot min^{-1}$ respectively. The initial half of the curve attributable to the reaction of groups I and II with Nbs_2 could not be analysed from these data owing to the extreme speed of reaction.

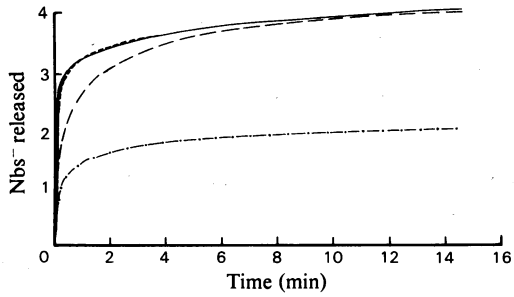

Fig. 1. *Reaction of 5-aminolaevulinate dehydratase with Nbs_2*

The Figure shows the reaction progress curves obtained when 0.5mM-Nbs_2 reacted with: ——, reduced apoenzyme; – – – –, reduced apoenzyme in the presence of 50μM-Pb^{2+}; ——, reduced enzyme in the presence of 100μM-Zn^{2+}; —·—, oxidized enzyme in the presence or in the absence of 50μM-Pb^{2+} or 100μM-Zn^{2+}. The reactions were carried out at 25°C in 0.1M-Tris/HCl buffer, pH7.1.

The reaction rate of the reduced apoenzyme in the presence of 100μM-$ZnCl_2$ (Fig. 1) was significantly different. This difference appeared to be confined to the reaction of the two fastest-reacting thiol groups (groups I and II), whereas the rates of reaction of groups III ($k = 1.43 \times 10^3 M^{-1} \cdot min^{-1}$) and IV ($k = 2.5 \times 10^2 M^{-1} \cdot min^{-1}$) were largely unaffected by the presence of the Zn^{2+} ions. The interaction of Zn^{2+} with the enzyme appeared to be almost instantaneous, since the protection afforded by Zn^{2+} was virtually identical either when the apoenzyme was preincubated with Zn^{2+} before the reaction with Nbs_2 or when the Zn^{2+} was added to the apoenzyme together with the Nbs_2 reagent itself.

When the reduced apoenzyme was treated with Nbs_2 in the presence of 50μM-$PbCl_2$ (Fig. 1) the reaction did not appear to be significantly affected, although this concentration of Pb^{2+} is known to inhibit enzyme activity by approx. 70% (Gibbs, 1984).

Oxidation of the apoenzyme resulted in the loss of catalytic activity and was associated with the loss of the ability of groups I and II to react with Nbs_2 (Fig. 1).

Effect of Zn^{2+} ions on the oxidation of 5-aminolaevulinate dehydratase

Inactivation of the human enzyme by oxygen ($t_{\frac{1}{2}}$ = 135 min) led to the loss of two Nbs_2-reactive equivalents (groups I and II; Fig. 1). The inactivation of the human apoenzyme was almost completely blocked by Zn^{2+} (100 μm), suggesting that in the Zn^{2+}–enzyme complex the sensitive thiol groups were no longer readily available for reaction.

Porphobilinogen (5 mM) did not prevent the reaction of Nbs_2 with groups I and II (as is seen with the bovine enzyme) (Seehra *et al.*, 1981). Neither the substrate 5-aminolaevulinate (5 mM) nor the substrate analogue laevulinate (5 mM) had any effect on the reaction progress curves.

Stopped-flow spectrophotometry

When either the apoenzyme or holoenzyme was mixed with a 50-fold molar excess of Nbs_2 (without or with 100 μM-Zn^{2+} respectively) in a stopped-flow spectrophotometer, reaction progress curves were obtained (Fig. 2), which when subjected to semi-logarithmic analysis revealed two first-order rates, due to the reaction of groups I and II. When apoenzyme was treated with Nbs_2, apparent first-order rate constants of 135 min^{-1} and 5.3 min^{-1} were obtained for the two site reactions (curve A− in Fig. 2). However, in the presence of Zn^{2+} ions (holoenzyme) the reactivity of group I decreased to about 12–16% of that of the apoenzyme with an apparent rate constant of 18 min^{-1} (curve A+ in Fig. 2). The reactivity of group II was less influenced by the presence of Zn^{2+}, with a rate constant of 2.1 min^{-1}.

The presence of Pb^{2+} ions appeared to have little effect on the reactivies of the thiol groups with Nbs_2 (Fig. 1), although detailed examination by the stopped-flow technique showed a decrease in reactivity to Nbs_2 by approx. 25%. Other studies (unpublished work) have shown, interestingly, that this concentration of Pb^{2+} ions will displace approx. 20% of enzyme-bound zinc (with the use of $^{65}Zn^{2+}$ ions).

Titration of the human 5-aminolaevulinate dehydratase apoenzyme with Nbs_2

When human apoenzyme (10 nmol in 1 ml) was titrated with an equimolar concentration of Nbs_2 (10 nmol) at 25°C, approx. 2 molar equivalents of Nbs (19.2 nmol) were liberated (Fig. 3). Similarly, when 1 molar equivalent of enzyme was titrated with 0.5 molar equivalents of Nbs_2 (5 nmol), approx. 1 molar equivalent of Nbs (10.6 mol) was released. The end points of these two titrations were essentially unaffected by the presence of the bivalent metal ions Zn^{2+} and Pb^{2+}.

On analysis of the reaction progress curves (Fig. 4), again it becomes clear that the presence of Zn^{2+} ions decreases the reactivity of the thiol group I. The second-order plot of the data from Fig. 3 (curve A−) is composed of two sections: an initial curved section, the rate of which decreases to reveal a second, slower, reaction.

Correction of the initial section of the curve by subtraction of this lower-rate reaction made it

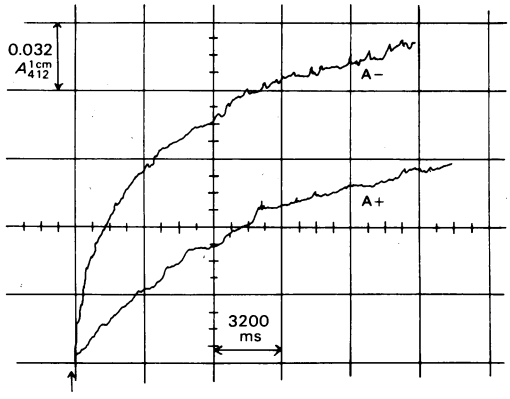

Fig. 2. *Stopped-flow spectrophotometric observation of the reaction between Nbs_2 and 5-aminolaevulinate dehydratase*
The enzyme (10 μM initial concentration) was mixed 1:1 with Nbs_2 at 25°C in 0.1 M-Tris/HCl buffer, pH 7.1. The curve A− and A+ are reaction progress curves of apoenzyme alone or holoenzyme in the presence of 100 μM-Zn^{2+}; the time scale was 3200 ms/division. The arrow indicates the point of mixing. The vertical scales represent 0.5 molar equivalent of Nbs^- released. Molarity is calculated in terms of the monomer.

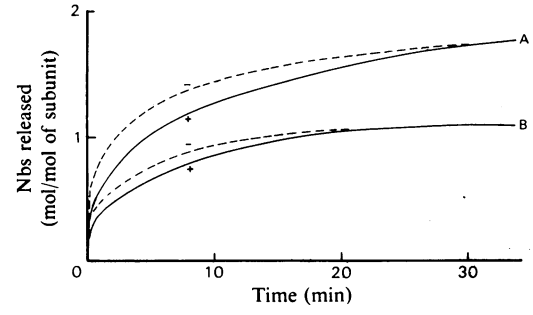

Fig. 3. *Titration of 5-aminolaevulinate dehydratase with Nbs_2*
Nbs_2 and enzyme (10 μM) were mixed as described in the Materials and methods section. Curves A, reaction of the enzyme with 1 mol of Nbs_2/mol of subunit in the absence (−) and in the presence (+) of 100 μM-Zn^{2+}. Curve B, reaction of the enzyme with 0.5 mol of Nbs_2/mol of subunit in the absence of (−) and in the presence (+) of 100 μM-Zn^{2+}.

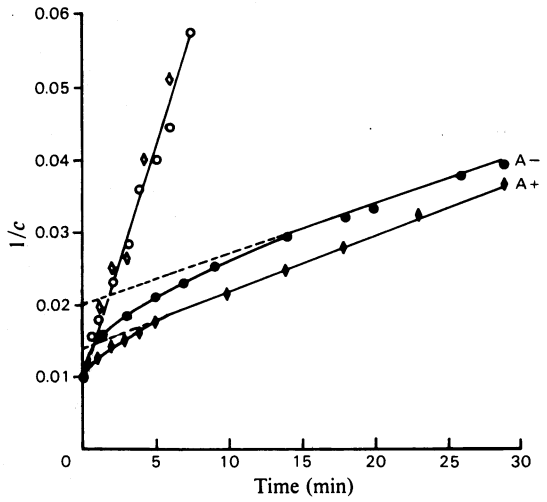

Fig. 4. *Second-order rate analysis of the reaction progress curves from Fig. 3*

The curves A− and A+ from Fig. 3 were subjected to second-order analysis. The reactant concentrations were equal at time = 0, and thus in the plot above $1/c$ is the reciprocal of the concentration (at time t) of one of the reactants (Nbs_2). ●, Data from curve A−; ◆, data from curve A+. ○ and ◇ represent derived rates from data taken from the difference between the initial part of each rate curve and the extrapolated portions (broken lines) of those respective curves.

possible to estimate the rate of the fast phase in isolation. It can be noted that both reactions are of equal size, with the line of the extrapolated slow phase intercepting the axis (time = 0) at the position equivalent to 50% of the total reaction (release of Nbs). Also shown in Fig. 4 are the data from the same reaction performed in the additional presence of 100 μM-Zn^{2+} ions. It can be noted that the intercept obtained in this case by back-extrapolation of the low rate is no longer at the 50% reaction point but is now around the 80% of reaction point. The fast phase of this reaction, although similar in amplitude, proceeds at approximately the same rate as that in the absence of Zn^{2+} (Fig. 4). The slower phase appears to be similar in rate to that obtained in the absence of Zn^{2+} ions, although larger in amplitude.

Protein fluorescence

The human apoenzyme exhibited fluorescence emission of 332nm when the endogenous tryptophan was excited by light at 280nm. Zn^{2+} ions increased the fluorescence intensity of the apoenzyme in a concentration-dependent manner up to a maximum of between 4.5 and 5% (Table 1).

Table 1. *Effects of Zn^{2+} and Pb^{2+} ions on the fluorescence intensity of the human 5-aminolaevulinate dehydratase apoenzyme*

The protein fluorescence of human 5-aminolaevulinic acid dehydratase apoenzyme (180 μg; 5.14 nmole) was determined at 335nm (excitation at 280nm) either in the presence of increasing amounts of zinc or lead in a solution of 200 μmole potassium phosphate buffer (pH 6.8; 2ml) containing 20 μmole dithioerythritol. For experimental details see the text.

Metal ion	Concn. (μM)	Change in fluorescence (%)
Zn^{2+}	2	+0.19
	10	+1.69
	57	+3.31
	108	+4.19
	203	+4.33
Pb^{2+}	5	−2.25
	10	−4.42
	29	−7.45
	48	−9.99
	99	−11.62
	124	−11.93

This effect was completely reversed on addition of a 40-fold excess of EDTA.

The addition of increasing concentrations of Pb^{2+} to the apoenzyme, however, caused a concentration-dependent decrease in the intensity of fluorescence (Table 1); this decrease in fluorescence intensity was proportional to the loss of enzyme activity. Addition of EDTA to the Pb^{2+}-treated enzyme did not cause any further decrease in protein fluorescence. When the Pb^{2+}-inhibited apoenzyme was treated with Zn^{2+} ions the fluoresence intensity was largely restored to that of the holoenzyme. On addition of excess EDTA to the enzyme that had been treated with Pb^{2+} and Zn^{2+} there was a decrease in the fluorescence intensity to values similar to that of the Pb^{2+}-inhibited apoenzyme, this presumably being due to preferential removal of Zn^{2+} by the EDTA.

When the apoenzyme was oxidized with Nbs_2, the resulting protein (after isolation by gel filtration) exhibited no enzyme activity and failed to show any changes in fluorescence on addition of either Zn^{2+} or Pb^{2+}.

The changes in the protein fluorescence on addition of various amounts of the metal ions Zn^{2+} and Pb^{2+} were used to calculate dissociation constants (K_d) for the two metal ions from their respective binary complexes. A second estimate of these dissociation constants was made by steady-state kinetics (Dixon, 1953). The values obtained were compared with those measured by fluorescence (Table 2).

Table 2. *Dissociation constants of Zn^{2+} and Pb^{2+} from human 5-aminolaevulinate dehydratase*

	Dissociation constant (K_D) (μM)	
	Fluorescence	Steady-state kinetics
Zn^{2+}	19.9±4.5	≃20
Pb^{2+}	26.5±2.6	32.6±8.6

Discussion

Mammalian 5-aminolaevulinate dehydratases are zinc-containing octameric enzymes that require the presence of reduced thiol groups for full catalytic activity. The human 5-aminolaevulinate dehydratase is notorious for its extreme sensitivity to Pb^{2+} ions, the activity being lowered dramatically in individuals suffering from only slight lead poisoning. Since inhibition by Pb^{2+} can be reversed by the addition of Zn^{2+} ions and an exogenous thiol, it has always been considered likely that a close relationship exists between the thiol status of the enzyme and the binding of the two metal ions.

Our initial experiments showed that the reaction of native human enzyme with Nbs_2 followed an overall pattern quite similar to that of the bovine liver 5-aminolaevulinate dehydratase (Seehra *et al.*, 1981). There was a rapid initial release of 2 mol of Nbs per mol of subunit (groups I and II) followed by a slower release of a further 2 mol of Nbs per mol of subunit (groups III and IV). Thus 32 thiol groups titrated per octameric unit overall. From the spectroscopic data shown in Fig. 1 it was clear that Zn^{2+} ions had a quite striking effect on the rate of reaction of one of the two faster-reacting groups. An investigation by stopped-flow analysis on this initial part of the progress curve revealed that the effect of Zn^{2+} ions on the reactivity of enzyme thiol groups with Nbs_2 was largely confined to the decrease in reactivity of group I; the apparent rate of reaction of group II was only marginally decreased by the presence of the metal ion.

The reaction of the bovine liver enzyme with Nbs_2 has been shown to result in the formation of an intramolecular disulphide bond (Tsukamoto *et al.*, 1979; Seehra *et al.*, 1981). That a similar mechanism was operative in the human enzyme was concluded from the reaction of enzyme with limiting amounts of Nbs_2. The data in Fig. 3 demonstrate conclusively that the human enzyme must also contain two thiol residues that are close enough to form a disulphide bridge. Two molar equivalents of Nbs were released from the reaction with 1 mol of Nbs_2, suggesting that, after reaction of thiol group 1, an intramolecular rearrangement takes place involving displacement of the enzyme-bound Nbs by attack from an adjacent unmodified cysteine residue (group II). In Fig. 4, the faster phase of the reaction (curve A−) represents the initial attack (second-order) of the enzyme thiol group I on Nbs_2, with resultant liberation of 1 molar equivalent of Nbs and formation of an enzyme–Nbs intermediate. The second, slower, phase of this curve is attributable to the pseudo-first-order intramolecular re-arrangement, which displaces the second molar equivalent of Nbs to form the disulphide bond. Significantly, each phase approximated to 50% of the total release of the Nbs. The presence of Zn^{2+} ions resulted in the reaction proceeding with only a small fast component. This suggests that the presence of Zn^{2+} slows down the rate of the initial attack on Nbs_2 to a rate approximately equal to, or less than, that of the intramolecular re-arrangement. The small amount of fast phase is probably due to unliganded enzyme still present in solution.

Further evidence for the formation of an intramolecular disulphide bridge during the reaction of thiol groups I and II with Nbs_2 was afforded by the observation that oxidation of the native enzyme before reaction with Nbs_2 resulted in the loss of both of the two faster-reacting components (I and II), and only two groups remained for titration, with rate constants similar to those calculated for thiol groups III and IV in the non-oxidized enzyme.

The formation of an intramolecular disulphide bond on reaction with Nbs_2 has been observed in several other enzymes, notably pyruvate kinase (Flashner *et al.*, 1972), phosphoenolpyruvate carboxykinase (Carlson *et al.*, 1978) and monoamine oxidase (Gornes *et al.*, 1976). It appears that two thiol groups are also present in the structure of human 5-aminolaevulinate dehydratase, as is the case in the bovine liver enzyme (Barnard *et al.*, 1977; Tsukamoto *et al.*, 1979; Seehra *et al.*, 1981), which are highly reactive and adjacent to one another. One of the groups binds with, or is in close proximity to, the Zn^{2+} ion, and the other group is close enough to permit intramolecular disulphide-bond formation either with oxygen or the modified group I–Nbs intermediate.

In concord with these findings, we have observed that Zn^{2+} protects the enzyme from oxygen inactivation in the absence of exogenous thiols, suggesting that Zn^{2+} may play a crucial stabilizing role in the maintenance of the reduced thiol configuration. This observation suggested several possibilities: (*a*) that Zn^{2+} binds directly to thiol group I; (*b*) that Zn^{2+} is chelated in close proximity

to thiol group I, leading to de-activation of the group by steric hindrance; (*c*) that Zn^{2+} elicits a conformational change that alters the reactivity of the thiol group I. It is noteworthy that the effect of Zn^{2+} was virtually instantaneous in inhibiting the rate of reaction of group I, suggesting that, if Zn^{2+} was inducing any conformational change in the enzyme, the effect was extremely rapid.

The possible role of Zn^{2+} in the catalytic mechanism has been discussed previously (Cheh & Neilands, 1976). Although we can envisage such a role for the metal ion, our current studies did not reveal any effect of substrate, product or substrate analogues on the reaction of Nbs_2 with group I or any of the other three reactive thiol groups, suggesting that the metal ion may be participating in a structural rather than a catalytic capacity.

One of the most interesting properties of the human erythrocyte 5-aminolaevulinate dehydratase relates to the reaction with, and inactivation by, relatively low (70–100 μM) concentrations of Pb^{2+}, and not surprisingly it has been suggested that Pb^{2+} may bind to one or more of the sensitive thiol groups. Accordingly, we investigated the reaction of thiol groups I–IV with Nbs_2 in the presence of a concentration of Pb^{2+} (50 μM) that inhibits the human enzyme almost completely. The data shown in Fig. 1 demonstrate that Pb^{2+} had only a marginal inhibitory effect on the reactivity of the thiol groups compared with the Zn^{2+}. Stopped-flow analysis revealed, however, that Pb^{2+} did have a measurable effect (approx. 25%) on the rate of reaction of thiol group I with Nbs_2. It is noteworthy that at this Pb^{2+} concentration, which inhibits the enzyme by about 80%, we have also observed that approx. 20% of $^{65}Zn^{2+}$ is displaced from the $^{65}Zn^{2+}$-labelled holoenzyme (Gibbs, 1984). It is therefore tempting to suggest that only at high concentrations of Pb^{2+} is a significant proportion of the Zn^{2+}-binding site occupied by the heavy-metal ion.

Further evidence for the differences in the effects of the metal ion Zn^{2+} and Pb^{2+} on the enzyme were deduced from the tryptophan fluorescence data shown in Table 1.

When Zn^{2+} was added to Pb^{2+}-inhibited enzyme in such a concentration (200 μM) that the enzyme activity was recovered, there was a return towards the fluorescence intensity of the zinc holoenzyme. Interestingly, when both Zn^{2+} and Pb^{2+} were present with the enzyme, addition of EDTA, which chelates the Zn^{2+} preferentially, caused a decrease in the fluorescence to that of the Pb^{2+}-inhibited enzyme, together with a loss of catalytic activity.

The close agreement of the values of K_d by two techniques (steady-state kinetics and fluorescence titration; Table 2) suggests that the perturbation of the fluorescence of the protein by each metal ion is caused by the same interactions that cause inhibition (by Pb^{2+}) or activation (by Zn^{2+}). Furthermore, the fact that both metal ions participate in binding reactions with the enzyme with similar affinities demonstrates that the metal ions are probably interacting at two different sites on the enzyme. A two-site hypothesis would explain the ability of Zn^{2+} (100 μM) to activate the enzyme over 90% even in the presence of high concentrations of Pb^{2+} (300 μM). This degree of activation could also be explained if the enzyme behaved in an asymmetric manner towards the two metal ions.

A two-site model is further corroborated by other data; activity of the enzyme increases with the concentration of Zn^{2+} up to 200 μM but then decreases with higher concentrations of the metal ion, suggesting that the Zn^{2+} first occupies its own binding site but then proceeds to occupy a second site, possibly the one that binds Pb^{2+} (Gibbs, 1984). Whether the two metal ions are interacting at different sites or at the same site, but with different ligands and co-ordination geometry, cannot be unambiguously resolved owing to the non-availability of a suitable radioactive lead isotope. However, the cumulative findings from the above experiments point to the fact that Zn^{2+} and Pb^{2+} interact with the human 5-aminolaevulinate dehydratase by different mechanisms.

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