Biochimica et Biophysica Acta, 570 (1979) 167—178

BBA 68811

THE ROLE OF ZINC WITH SPECIAL REFERENCE TO THE ESSENTIAL THIOL GROUPS IN δ-AMINOLEVULINIC ACID DEHYDRATASE OF BOVINE LIVER

IKUYO TSUKAMOTO, TAKEO YOSHINAGA and SEIYO SANO

Department of Public Health, Faculty of Medicine, Kyoto University, Kyoto 606 (Japan)

(Received March 5th, 1979)

Key words: δ-Aminolevulinic acid dehydratase; Porphobilinogen; Zn^{2+}; Thiol group; Histidyl residue

Summary

δ-Aminolevulinic acid dehydratase (5-aminolevulinic acid hydro-lyase (adding 5-aminolevulinic acid and cyclizing), EC 4.2.1.24 purified from bovine liver in the presence of both SH-reducing reagent and zinc during the purification contained one zinc atom and eight SH groups/subunit. This preparation showed the full enzymatic activity even in the absence of thiol activator.

It was found that two cysteine residues, one zinc atom and two histidine residues were involved in the active site. The enzyme was fully active as long as two SH groups in the active site remained in the reduced form even in the absence of zinc. However, the enzymatic activity was completely lost, with a concomitant loss of bound zinc, upon oxidation of the SH groups to a disulfide bond, modification of SH groups with chemical reagents, or mercaptide formation by heavy metals. Thus, it is apparent that the activity depends on the essential SH groups. The zinc is not absolutely essential for the activity but may be required to prevent the essential SH groups from autooxidation by coordination.

Binding experiments indicated that there was one binding site of zinc/subunit. Photooxidation of histidine residues diminished both enzymatic activity and bound zinc, suggesting that the histidine residues not only constituted the active site but also served as a possible ligand to zinc.

Abbreviation: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

Introduction

δ-Aminolevulinic acid dehydratase (5-aminolevulinic acid hydro-lyase (adding 5-aminolevulinic acid and cyclizing), EC 4.2.1.24) catalyzes the conversion of two molecules of δ-aminolevulinic acid into porphobilinogen and consists of eight subunits [1]. This enzyme is inhibited by sulfhydryl reagents such as *N*-ethylmaleimide, iodoacetamide, iodoacetic acid [2—5] and also by various heavy metals particularly including lead [6—8]. There are also several reports that eukaryotic δ-aminolevulinic acid dehydratase is a zinc enzyme [9,10], although neither the amount of zinc in the purified enzyme has been determined unequivocally, nor its role has yet been clarified.

In a previous paper [11], we reported that the enzyme was inactivated easily during purification with a concomitant disappearance of two SH groups presumably due to a disulfide bond formation. Moreover histidine residues were implicated to be essential for the activity. Recently we succeeded in obtaining the active enzyme in a stable form, thus more detailed studies on the active site of the enzyme being enabled. This report deals with the relationship between the zinc atom and the essential SH groups as well as the histidine residues in the active site.

Experimental

Materials

δ-Aminolevulinic acid dehydratase was purified from bovine liver according to the procedure reported previously [11], except that zinc (10 μM) and mercaptoethanol (10 mM) were added during the purification. Finally, the purified enzyme dissolved in 50 mM Tris/acetate buffer, pH 7.1, containing zinc and mercaptoethanol was precipitated by addition of ammonium sulfate to 55% saturation and stored at 4° C. Immediately before use, a portion of the enzyme precipitate was collected by centrifugation, dissolved in 50 mM Tris/acetate buffer, pH 7.1, and passed through a column (1.0 × 15.0 cm) of Sephadex G-50, which had been equilibrated with the same buffer. Unless otherwise stated, this buffer was used throughout a series of present experiments. The enzyme preparation with full complement of zinc will be called as holoenzyme. δ-Aminolevulinic acid hydrochloride was purchased from Nakarai Chemicals Ltd. We thank Dr. Shosuke Kojo of our laboratory for the synthesis of 1,3-dibromoacetone [12]. Other chemicals were of analytical grade. All glassware was washed extensively with HCl/HNO_3 and then rinsed thoroughly with metal-free distilled water. The glassware, buffer solutions and chemicals used were checked routinely for metal contamination by atomic absorption spectrophotometry (Shimadzu AA-650).

Methods

The enzymatic activity was determined by measuring the amount of porphobilinogen formed from δ-aminolevulinic acid by the method of Mauzerall and Granick [13]. A standard reaction mixture contained 4 mM δ-aminolevulinic acid hydrochloride (neutralized with Tris), the enzyme (5—10 μg protein), and 50 mM Tris/acetate buffer, pH 7.1, in a final volume of 0.5 ml.

Dithiothreitol was omitted, unless otherwise stated, from the reaction mixture throughout the course of this study. Incubation was carried out under N_2 at 37°C for 15 min. The reaction was stopped by adding an equal volume of a 20% solution of trichloroacetic acid containing 0.1 M $HgCl_2$. After centrifugation, an equal volume of Ehrlich's reagent was added to the supernatant and the mixture was allowed to stand at room temperature for 15 min. Porphobilinogen was estimated from the absorbance at 555 nm by using a molar extinction coefficient of $6.2 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$. The protein concentration was determined either by measuring absorbance at 280 nm ($E^{1\%}_{1cm} = 11.8$ was taken on the basis of dry weight) or by the method of Lowry et al. [14]. The amino acid compositions were determined on a Hitachi automatic amino acid analyzer (KLA-3B) after hydrolysis of the protein with 6 N HCl in the presence of 0.05% phenol at 110°C for 24 h. The absorption spectra in the ultraviolet and visible regions were measured with a Union Giken SM-401 spectrophotometer.

The quantitative analysis of the metals (zinc, silver, lead) in the enzyme preparation was carried out by three different methods. (1) After wet digestion of the enzyme with $HNO_3/HClO_4$ it was subjected to metal analysis by atomic absorption spectrophotometry (Shimadzu AA-650). (2) The protein moiety was precipitated by addition of trichloroacetic acid to 10% and the supernatant was treated as above. (3) The enzyme solution was applied directly to flame atomic absorption spectrophotometry (Shimadzu AA-650) [15] or flameless atomic absorption spectrophotometry (Shimadzu AA-640-13, GFA-2). The analytical results obtained with the three methods were found to be identical.

Determination of the number of SH groups in the enzyme

Titration of SH groups in the enzyme with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was carried out according to the method described by Ellman [16]. The enzyme in the Tris/acetate buffer was incubated with excess DTNB (125 μM) in a 1 ml cuvette at room temperature. The reaction was allowed to proceed for 60 min initially, and for another 30 min after denaturation of the enzyme with 0.4% (final) sodium dodecyl sulfate (SDS). During the reaction an absorbance increase was followed at 412 nm, and a molar extinction coefficient (412 nm) of 13 600 $M^{-1} \cdot cm^{-1}$ for the anion of thionitrobenzoic acid was used to determine the number of SH groups reacted [16]. The performic acid-oxidized enzyme was prepared according to the method of Hirs [17] at 0°C. Carboxymethylation of the enzyme was carried out according to the method of Cole et al. [18] at pH 8.5.

Preparation of apoenzyme

Purified holoenzyme (1.0—3.0 mg protein/ml) in the Tris/acetate buffer was incubated with 50 mM EDTA under N_2 for 30 min at 37°C, followed by filtration under N_2 through a column of Sephadex G-50 which had been equilibrated with the deoxygenated zinc-free buffer. The eluate was used immediately as a non-oxidized apoenzyme. On the contrary, the term oxidized apoenzyme refers to the apoenzyme that has been exposed to air for a while. Apoenzyme prepared by the EDTA treatment, either non-oxidized or oxidized, was found to contain less than 0.1 atom zinc/subunit.

Binding of Zn^{2+} to the apoenzyme

The oxidized apoenzyme (1.3 mg protein/ml; Zn, 0.09 atom/subunit) was incubated with various concentrations of zinc acetate (1-100 μM) in the presence of 5 mM dithiothreitol for 30 min at 37°C. The excess reagents were removed by chromatography on a column of Sephadex G-50 under N_2. Each eluate was used, either immediately or after 5 h standing under air, for the determinations of the zinc content, the number of SH groups and the enzymatic activity.

Oxidation of holoenzyme

The holoenzyme (430 μg protein/ml) was exposed to pure oxygen. At various time intervals aliquots were withdrawn and both the enzymatic activity and SH groups content were determined. The metal analysis of the oxidized sample was carried out after filtration through a column of Sephadex G-50.

Modification of the SH groups of the holoenzyme with various reagents

In the following experiments the enzyme preparation whose sulfhydryl groups had been modified chemically was freed of an excess of remaining reagent as well as its reaction product by chromatography on a column of Sephadex G-50 under N_2. The enzymatic activity, metal and sulfhydryl group contents were determined on the sample thus obtained.

DTNB. The holoenzyme (430 μg protein/ml) was incubated with various concentrations of DTNB (4.3—125 μM) at room temperature. The reaction was allowed to proceed until the absorbance increase at 412 nm became insignificant. Before the gel filtration, the number of modified SH groups was determined.

1,3-Dibromoacetone. 1,3-Dibromoacetone (final concen. 14—84 μM) [19,20] dissolved in 10 μl of acetone was added to the holoenzyme solution (1.05 mg protein/ml) and allowed to react for 10 min at room temperature. The number of modified histidine residues was determined on an amino acid analyzer after gel filtration.

Ag^+. The holoenzyme (730 μg protein/ml) was incubated with various concentrations of silver nitrate (6—80 μM) for 10 min at 37°C.

Pb^{2+}. The holoenzyme (570 μg protein/ml) was incubated with various concentrations of lead acetate (6—100 μM) for 30 min at 37°C under N_2.

Histidine modification by photooxidation

Photooxidation of the enzyme was carried out according to the procedure described previously [11]. At various time intervals of photoillumination the enzymatic activity was determined. The zinc content and the number of modified histidine residues were estimated after gel filtration.

Results

Properties of purified enzymes

A purified and stable holoenzyme preparation was obtained when zinc and a SH-reducing agent were supplemented during the purification procedures. The specific activity was found to be 16 μmol of porphobilinogen formed/mg

protein per h, and it remained almost unchanged for more than a week even when kept under air. The zinc content was eight atoms zinc/molecule (one atom/subunit) of the enzyme. The total number of SH groups as determined by reaction with DTNB, carboxymethylation or performic acid oxidation was 8 mol/subunit of 35 000 daltons and no disulfide bond was observed in the purified holoenzyme. The SH groups were classified into three types on the basis of reactivity toward DTNB. As shown in Fig. 1 (curve b), treatment of the holoenzyme with DTNB revealed the presence of two rapidly reacting SH groups (2.0 ± 0.1) which were modified within 1 min (type I). Subsequently three additional SH groups (2.6 ± 0.2) were modified taking 1 h for completion (type II). SDS treatment of the enzyme further liberated three SH groups (3.1 ± 0.1) (type III).

Changes in enzymatic activity and SH group content by zinc removal from the enzyme

Non-oxidized apoenzyme Freshly prepared non-oxidized apoenzyme (zinc, 0.09 atom/subunit) under N_2 gave almost a full activity. When kept under N_2 about 90% of the original activity was maintained even after 12 h and the total number of SH groups reactive with DTNB was 8 mol/subunit of 35 000 daltons, in good agreement with that of the holoenzyme (Fig. 1). These experiments clearly indicate that a zinc atom is not necessarily essential for the enzymatic activity.

By comparison of a reaction profile of the non-oxidized apoenzyme with DTNB (Fig. 1a) with that of the holoenzyme (Fig. 1b), it is apparent that two of the type II SH groups in the latter were converted into type I in the apoenzyme. An increase in the number of type I SH groups by 1.5 upon dissocia-

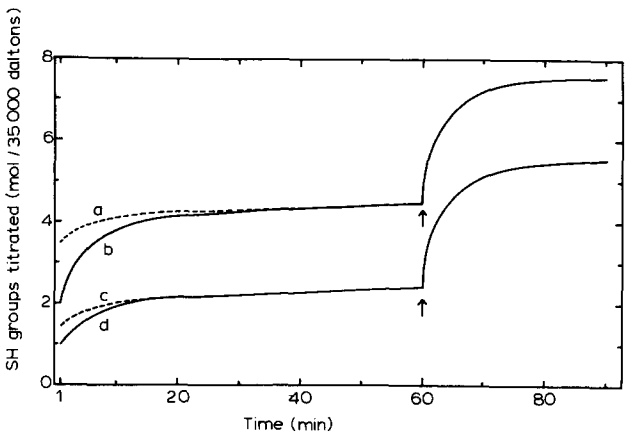

Fig. 1. Titration of SH groups of δ-aminolevulinic acid dehydratase with DTNB. The enzyme (130 μg protein/ml) in 50 mM Tris/acetate buffer, pH 7.1, was incubated with excess DTNB (125 μM) for 60 min initially, and for another 30 min after addition of 0.4% (final concentration) SDS at room temperature. The oxidized enzyme showed about 10% of the original activity. The addition of SDS is indicated by an arrow. Absorbance values at 412 nm were corrected for the absorption by the enzyme and DTNB and also corrected for a volume change due to the addition of SDS. These values were converted into mol of SH groups using a molar extinction coefficient of $1.36 \cdot 10^4\ M^{-1} \cdot cm^{-1}$. a, Non-oxidized apoenzyme; b, holoenzyme; c, oxidized apoenzyme; d, oxidized enzyme obtained by exposure of the holoenzyme to oxygen.

tion of zinc from the holoenzyme indicates a possible involvement of two SH groups in the coordination of one zinc atom. No change was observed in the number of SH groups of type III.

Oxidized apoenzyme. Storage of the apoenzyme under air led not only to a complete loss of the enzymatic activity within 5 h but also to a simultaneous disappearance of two SH groups/subunit as determined by the DTNB method (Fig. 1c). The latter finding was in accord with a decrease of two carboxymethylcysteine residues/subunit in the carboxymethylated apoenzyme, indicating that the loss of two SH groups was due to a disulfide bond formation which occurred easily after removal of the intrinsic zinc.

SDS disc electrophoresis of the oxidized apoenzyme revealed one band which was identical in the mobility with that of the holoenzyme, eliminating the possibility of an intersubunit disulfide bond formation. Aggregation of the apoenzyme was less likely, since the results of gel filtration on Ultrogel AcA 34 were the same for the both holo- and apoenzyme.

The titration study of the oxidized apoenzyme with DTNB gave two (1.5 ± 0.1) type I SH groups, one (1.0 ± 0.1) type II and three (3.1 ± 0.1) type III. This result suggested that an intrasubunit disulfide bond was formed between two SH groups of type I in the non-oxidized apoenzyme (Fig. 1a and c).

Binding experiments of Zn^{2+} to the apoenzyme

Zinc was incorporated into the oxidized apoenzyme only after the apoenzyme had been reduced with dithiothreitol (5 mM). This result indicates that for the zinc incorporation the reduction of the disulfide bond is prerequisite (Table I). By adding zinc in small portions to a solution of the apoenzyme, the zinc was incorporated stoichiometrically into the apoprotein until each subunit was saturated with one zinc atom, thus one binding site for zinc in each subunit being indicated strongly.

The zinc-binding studies were carried out as described in Experimental. Various amounts of zinc were incorporated into the apoenzyme, and the effects of the amount of incorporated zinc on the enzymatic activity and the sulfhydryl content were examined as illustrated in Fig. 2. Even when the zinc to subunit ratio was 0.1, more than 50% of the original activity was recovered

TABLE I

EFFECT OF DITHIOTHREITOL ON BINDING OF ZINC TO THE OXIDIZED APOENZYME

The oxidized apoenzyme (1.3 mg protein/ml, two SH groups decreased) prepared as described in 0.5 ml of 50 mM Tris/acetate buffer, pH 7.1, was incubated with zinc acetate (0.1 mM) dithiothreitol (5 mM), or both of them for 30 min at 37°C. The reaction mixture was passed through a column of Sephadex G-50 under N_2, and subjected to assays of the protein and zinc contents and the enzymatic activity.

Addition to apoenzyme	Zinc bound (atom/subunit)	Specific activity (μmol of porphobilinogen formed/mg protein per h)
None	0.1	1.4
0.1 mM Zn^{2+}	0.2	1.3
5 mM dithiothreitol	0.1	10.1
0.1 mM Zn^{2+} + 5 mM dithiothreitol	1.1	16.1

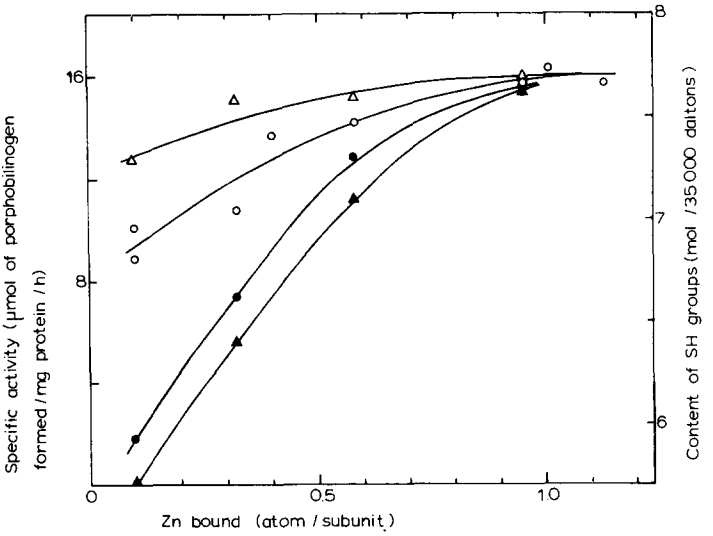

Fig. 2. Effect of the amount of incorporated zinc on the enzymatic activity and SH group content. The oxidized apoenzyme (1.3 mg protein/ml) in 0.5 ml of 50 mM Tris/acetate buffer, pH 7.1, was incubated with various concentrations of zinc acetate (1—100 μM) in the presence of 5 mM dithiothreitol for 30 min at 37°C, followed by passing through a column of Sephadex G-50 under N_2. The eluate was used for determination of protein and divided into two portions. One portion was used immediately (○, △) for determinations of the enzymatic activity (○) and the content of SH groups (△) and zinc according to the procedures described in 'Experimental'. The other was kept under air for 5 h, and then passed through a column of Sephadex G-50. The eluate was used for the same kinds of assays (●, ▲).

and more than seven sulfhydryl residues per subunit were detected in a freshly prepared sample. However, as these samples were allowed to stand under air for 5 h, both the activity and the number of sulfhydryl groups decreased appreciably especially when the amount of the incorporated zinc was low. In other words, as shown in Fig. 2, both the activity and the sulfhydryl content apparently changed depending on the zinc content. The fully reconstituted enzyme was stable at least for 5 h when allowed to stand under air. These results indicate that the sulfhydryl groups in the reconstituted enzyme in which less than one zinc atom/subunit was incorporated tend to be oxidized easily accompanying a decrease in activity.

Oxidation of holoenzyme with oxygen

As already pointed out, the holoenzyme (one atom of zinc/subunit) was stable at least for 1 week even when kept under air. However, when it was exposed to pure oxygen for 12 h at 37°C, two SH groups were oxidized resulting in a nearly complete loss (90%) of the enzymatic activity with a concomitant loss of bound zinc by 60% (Fig. 3).

One (1.0 ± 0.2) type I SH group, two (1.5 ± 0.2) type II and three (3.1 ± 0.1) type III were detected for the oxidized holoenzyme (Fig. 1d). This result suggests that a disulfide bond was formed between each SH group of types I and II of the holoenzyme or between two out of three type I SH groups. The latter case is conceivable to occur when one of the type II SH

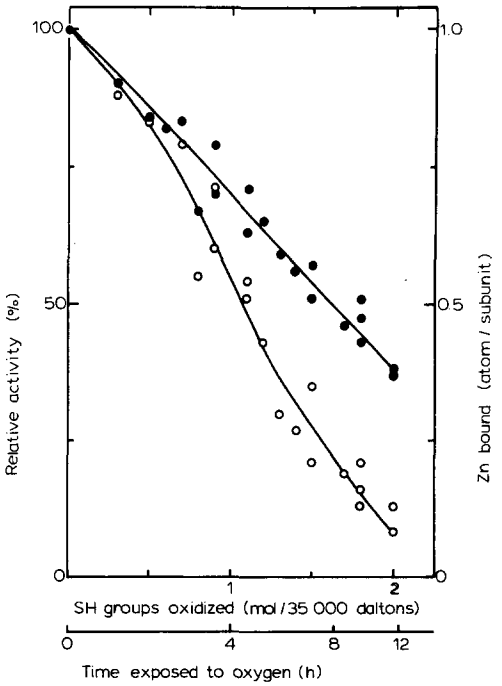

Fig. 3. Effect of oxidation of δ-aminolevulinic acid dehydratase with oxygen on the enzymatic activity and zinc content. The holoenzyme (430 μg protein/ml) in 50 mM Tris/acetate buffer, pH 7.1, was exposed to pure oxygen for 0—12 h at 37°C. At various time intervals, 20-μl and 0.35 ml aliquots were withdrawn and used for determination of the enzymatic activity (○) and the content of SH groups, respectively. Another 0.5 ml aliquots were also withdrawn and passed through a column of Sephadex G-50. After the determination of protein the zinc content was estimated (●).

groups in holoenzyme is transformed into type I in advance of the disulfide bond formation.

Evidence for a vicinal pair of the SH groups of the enzyme

The addition of one equivalent of DTNB/subunit of the holoenzyme liberated about two equivalents of thionitrobenzoic acid (Table II). This result indicates that the loss of two SH groups is due to a disulfide bond formation and supports the presence of a vicinal pair of SH groups. This pair must be identical with the two SH groups that formed a disulfide bond on air oxidation. The existence of a vicinal SH pair was already reported in the case of transglutaminase of guinea pig liver [21] or glyceraldehyde-3-phosphate dehydrogenase of lobster muscle [22].

Modification of SH groups in relation to the enzymatic activity and bound zinc

Fig. 4 illustrates the effects of modification of SH groups by either DTNB, 1,3-dibromoacetone, or Ag^+ on the activity and the intrinsic zinc content of the enzyme. It was confirmed that no histidine residue was affected by 1,3-dibromoacetone under the condition as described above, and the amount of bound Ag^+ coincided with a decrease in the number of DTNB-detectable SH

TABLE II

MODIFICATION OF δ-AMINOLEVULINIC ACID DEHYDRATASE WITH DTNB

The holoenzyme (430 μg protein/ml) in 50 mM Tris/acetate buffer, pH 7.1, was incubated with various concentrations of DTNB (5.4—17.2 μM) at room temperature. The reaction was terminated when no further increase in absorbance at 412 nm was observed. The reaction mixture was passed through a column of Sephadex G-50, followed by determination of the enzymatic activities using 50-μl aliquots of the eluates.

	DTNB used for modification (mol/35 000 daltons)	SH groups modified (mol/35 000 daltons)	Relative activity (%)
Expt. 1	0	0	100
	0.47	0.91	66
	0.92	1.67	37
	1.40	2.44	12
Expt. 2	0.44	1.00	64
	0.88	1.60	49
	1.31	2.25	21

groups. It is to be noted that in these three cases the activity always diminished in advance to the liberation of the metal. Therefore, although it is conceivable that two of these SH groups are involved in the coordination of zinc, it is a matter of further studies to determine how this structure controls the activity.

Table III demonstrates that one equivalent of lead could replace one

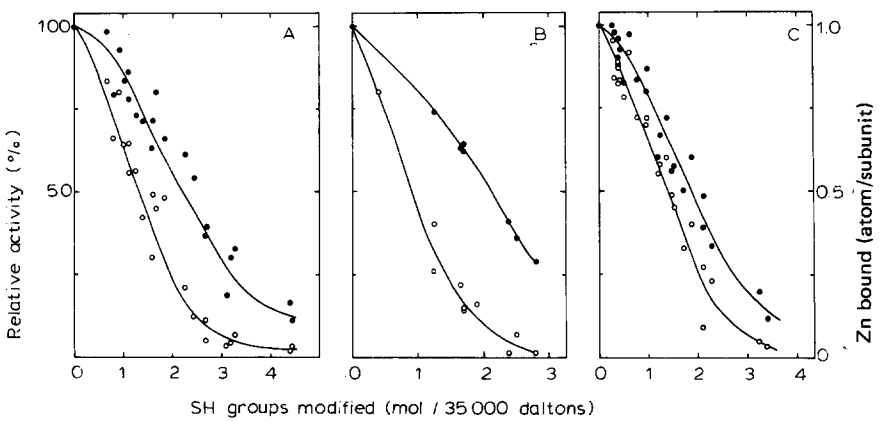

Fig. 4. Effect of modification of δ-aminolevulinic acid dehydratase with DTNB, 1,3-dibromoacetone, or Ag^+ on the enzymatic activity and zinc content. (A) DTNB. The holoenzyme (430 μg protein/ml) in 50 mM Tris/acetate buffer, pH 7.1, was incubated with various concentrations of DTNB (4.3—125 μM) at room temperature. The experimental procedures were the same as described in Table II and 'Experimental'. ○, ●, the enzymatic activity and zinc content, respectively. (B) 1,3-Dibromoacetone. The holoenzyme (1.05 mg protein/ml) in 0.5 ml of the Tris/acetate buffer was incubated with various concentrations of 1,3-dibromoacetone (14—84 μM) dissolved in 10 μl of acetone at room temperature, followed by passing through a column of Sephadex G-50 under N_2. 20-μl, 0.3 ml aliquots and the remaining solution of each eluate were used for determinations of the enzymatic activity (○), the number of SH groups modified, and the zinc content (●), respectively. (C) Ag^+. The holoenzyme (730 μg protein/ml) in 0.5 ml of the Tris/acetate buffer was incubated with various concentrations of silver nitrate (6—80 μM) for 10 min at 37°C, followed by gel filtration of Sephadex G-50. 30-μl, 0.4 ml aliquots and the remaining solution of each eluate were used for determinations of the enzymatic activity (○), the number of SH groups modified, and the contents of zinc (●) and silver, respectively.

TABLE III

COMPLEMENTARY REPLACEMENT OF ZINC BY LEAD IN δ-AMINOLEVULINIC ACID DEHYDRATASE AND LOSS OF THE ENZYMATIC ACTIVITY

The holoenzyme (570 μg protein/ml) in 0.5 ml of 50 mM Tris/acetate buffer, pH 7.1, was incubated with various concentrations of lead acetate (6, 10, 40 and 100 μM) under N_2 for 30 min at 37°C, followed by passing through a column of Sephadex G-50 under N_2.

Pb^{2+} added (μM)	Zn^{2+}	Metal-bound Pb^{2+} (atom/subunit)	Zn^{2+} + Pb^{2+}	Relative activity (%)
0	0.91	0.06	0.97	100
6	0.78	0.21	0.99	75
10	0.66	0.34	1.00	37
40	0.30	1.78	1.08	18
100	0.15	0.93	1.08	7

equivalent of zinc/subunit resulting in a complete loss of the enzymatic activity. Such an inhibitory effect by lead occurred even under anaerobic condition, suggesting that the inhibition was not due to the disulfide bond formation but most probably to the mercaptide formation. However, when we titrated the lead enzyme with DTNB, all of the SH groups were recovered as in the zinc enzyme. This result suggests that a lead mercaptide in the enzyme, if any, has a stability comparable to that of a zinc mercaptide. The inhibition of the activity by lead was restored completely by the addition of zinc and dithiothreitol.

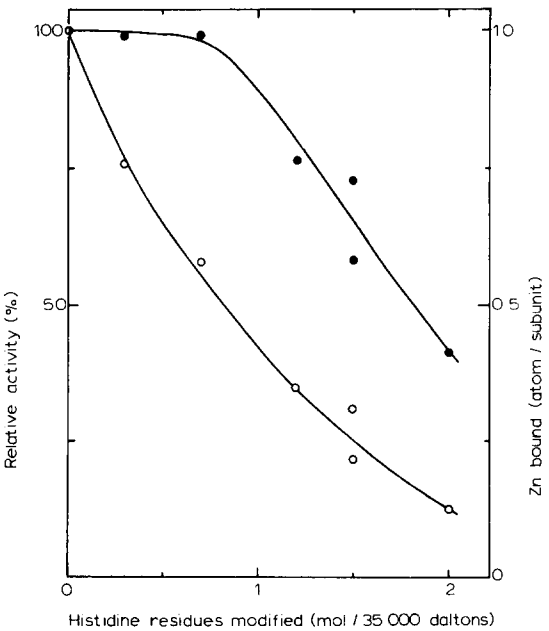

Fig. 5. Effect of photooxidation of histidine residues on the enzymatic activity and zinc content. The holoenzyme (1.3 mg protein/ml) in 8.0 ml of 50 mM Tris/acetate buffer, pH 7.1, was photooxidized in the presence of 0.001% methylene blue. At various time intervals, 5-μl aliquots were withdrawn and incubated in a standard assay mixture containing 5 mM dithiothreitol to determine the enzymatic activity (○). Another 1.0 ml aliquot was passed through a column of Sephadex G-50, and the eluate was divided into two portions. One portion was used for determination of the zinc content (●) and the other for amino acid analysis to determine the number of histidine residues modified.

Modification of histidine residue by photooxidation

Fig. 5 showed that modification of one histidine residue decreased the enzymatic activity to approximately 40% of the original one without accompanying a significant release of bound zinc. The subsequent oxidation of one more histidine residue resulted in an almost complete loss of the enzymatic activity and 60% loss of bound zinc. As previously reported [11], the photooxidized SH groups, but not histidine residues, were found to be fully reduced on treatment with dithiothreitol. However, the incorporation of zinc to the photoinactivated enzyme in the presence of dithiothreitol increased the amount of bound zinc only to 50% of the initial level and failed to restore the activity. This result strongly indicates the significance of histidine residues in the zinc binding.

Discussion

δ-Aminolevulinic acid dehydratase, as prepared by the methods previously reported [2,7,9,11], was usually very low in enzymatic activity and for elicitation of the maximal activity the addition of a thiol compound was required. It is also known that mammalian δ-aminolevulinic acid dehydratase is a zinc-containing enzyme, first reported by Gurba et al. [9], and later Cheh and Neilands [10] reported that δ-aminolevulinic acid dehydratase was a somewhat atypical metalloenzyme because the zinc was lost often in the course of isolation. Thus various zinc contents have been reported for the purified enzyme preparations so far. The role of zinc in structure-function relationship of this enzyme has been unknown.

The enzyme prepared according to the present method was found to be stable. It contained one atom of zinc/subunit and the maximum enzymatic activity was observed even without the addition of a thiol activator. Quantitative binding experiment of zinc showed the existence of one binding site having a strong affinity for zinc in each subunit. Determination of SH groups by three different methods unequivocally gave the number of 8/subunit. No disulfide bond was detected in the enzyme containing one atom of zinc/subunit.

When the holoenzyme was oxidized with pure oxygen, the loss of the SH groups resulted in the decrease of the enzymatic activity with a concomitant liberation of zinc (Fig. 3). Similar results were obtained when the SH groups were modified by DTNB, 1,3-dibromoacetone, or metals (Fig. 4 and Table III). Conversely, when zinc was removed by the treatment with EDTA, sulfhydryl groups in the enzyme easily formed a disulfide bond by air oxidation and the catalytic activity was lost. The oxidized apoenzyme thus obtained never incorporated zinc in the absence of a thiol activator (Table I). These results suggest that one zinc atom and at least two cysteine residues are closely interacting at the active site. The role of zinc would be to protect the two essential SH groups from autooxidation presumably by coordination with them, although the strength of each bond may not be equivalent. This possibility is further supported by the facts that on dissociation of zinc from the holoenzyme two SH groups became more reactive toward DTNB (Fig. 1a and b) and that the treatment of the enzyme with 0.5 M dithiothreitol resulted in liberation of bound zinc (50%).

Barnard et al. [5] reported that there were two sulfhydryl groups essential for the activity. Modification of either one of them with iodoacetate or iodoacetamide completely abolished the activity. However, since this inhibition was observed only when the enzyme had been regenerated by treating the inactivated one with sulfhydryl-reducing reagents, it is highly possible that the present two essential SH groups coincide with the two residues as indicated by Barnard et al., although the final identification must await further studies.

It is worthwhile to note that zinc was not absolutely essential for the catalytic function of the enzyme. This was supported by the evidence that the zinc-depleted enzyme maintained almost a full activity if the apoenzyme was prepared and kept under strictly anaerobic condition. This result also suggests that a gross conformational change by zinc removal at the active site is unlikely to occur. However, the possible occurrence of a fine conformational change which facilitates the disulfide bond formation after the zinc dissociation is not still excluded.

From the photooxidation study histidine residues are shown to be present in the active site, and perhaps one of them would participate in the binding of zinc (Fig. 5). This may account for the observation that the extent of the zinc liberation is smaller than that of the loss of enzymatic activity in the experiments of oxidation or modification of SH groups.

Acknowledgements

This work was supported in part by Research Grant of Fujiwara Foundation of Kyoto University and Scientific Research Fund of the Ministry of Education of Japan. Our appreciation goes to Dr. Y. Orii for his reading and correcting of the manuscript as well as for his kind comments. This work forms part of a thesis submitted for the degree of Ph.D. to Kyoto University by I.T.

References

1 Wu, W.H., Shemin, D., Richards, K.E. and Williams, R.C. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1767—1770
2 Batlle, A.M. del C., Ferramola, A.M. and Grinstein, M. (1967) Biochem. J. 104, 244—249
3 Chaudhry, A.G., Gore, M.G. and Jordan, P.M. (1976) Biochem. Soc. Trans. 4, 301—303
4 Jordan, P.M., Gore, M.G. and Chaudhry, A.G. (1976) Biochem. Soc. Trans. 4, 762—763
5 Barnard, G.F., Itoh, R., Hohberger, L.H. and Shemin, D. (1977) J. Biol. Chem. 252, 8965—8974
6 Gibson, K.D., Neuberger, A. and Scott, J.J. (1955) Biochem. J. 61, 618—629
7 Wilson, E.L., Burger, P.E. and Dowdle, E.B. (1972) Eur. J. Biochem. 29, 563—571
8 Finelli, V.N., Klauder, D.S., Karaffa, M.A. and Petering, H.G. (1975) Biochem. Biophys. Res. Commun. 65, 303—311
9 Gurba, P.E., Sennett, R.E. and Kobes, R.D. (1972) Arch. Biochem. Biophys. 150, 130—136
10 Cheh, A. and Neilands, J.B. (1973) Biochem. Biophys. Res. Commun. 55, 1060—1063
11 Tsukamoto, I., Yoshinaga, T. and Sano, S. (1975) Biochem. Biophys. Res. Commun. 67, 294—300
12 Husain, S.S. and Lowe, G. (1968) Biochem. J. 108, 855—859
13 Mauzerall, D. and Granick, S. (1956) J. Biol. Chem. 219, 435—446
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Fuwa, K., Pulido, P., McKay, R. and Vallee, B.L. (1964) Anal. Chem. 36, 2407—2411
16 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70—77
17 Hirs, C.H.W. (1967) Methods Enzymol. 2, 197—199
18 Cole, R.D., Stein, W.H. and Moore, S. (1958) J. Biol. Chem. 233, 1359—1363
19 Husain, S.S. and Lowe, G. (1970) Biochem. J. 117, 333—340
20 Husain, S.S. and Lowe, G. (1970) Biochem. J. 117, 341—346
21 Connellan, J.M. and Folk, J.E. (1969) J. Biol. Chem. 244, 3173—3181
22 Wassarman, P.M. and Major, J.P. (1969) Biochemistry 8, 1076—1082