Erythrocyte factors concerned in the inhibition of ALA-D by lead

T SAKAI, S YANAGIHARA, AND K USHIO

From the Center of Occupational Medicine, Tokyo Labor Accident Hospital, Ota-Ku, Tokyo 143, Japan

ABSTRACT Erythrocyte factors are concerned in the inhibition of delta-aminolaevulinic acid dehydratase (ALA-D) by lead at 20 to 100 nM concentrations. The activity of the factors is detected in Hb fractions from Sephadex G-200 gel filtration of erythrocyte supernatant. After gel filtration of erythrocyte supernatant from a lead worker, 50% of lead is found in ALA-D fractions, although the fractions recover from ALA-D inhibition. The recovered activity is reinhibited if the enzyme fraction is preincubated with Hb fraction obtained from the same chromatography. Similarly obtained enzyme from a normal subject is also inhibited when it is preincubated with normal Hb fraction and lead acetate at 20 to 100 nM concentrations. The extent of the inhibition depends on the concentrations of Hb fraction and lead acetate preincubated. Reinhibition of lead worker enzyme with normal Hb fraction may be deleted not only by heating but also by zinc or DTT as well. Hb fraction heated at 60 °C for 5 min is also able to induce the lead-inhibition of the activity in ALA-D fraction. Half life of the factors is 26 min at 60 °C and 3 min at 80 °C.

Delta-aminolaevulinic acid dehydratase (ALA-D, EC 4.2.1.24) is an enzyme in the haem biosynthetic pathway that catalyses the dehydration and condensation of two moles of delta-aminolaevulinic acid into one mole of porphobilinogen. The enzyme, a homo-octamer with a molecular weight of 252 000, is activated by zinc ion at 0.1 and 0.02 mM concentrations and inhibited by lead at 1 and 5 μ M.¹ The ALA-D activity of human blood is inhibited at blood lead concentrations between 200 and 1000 μ g (1.0 and 4.8 μ moles)/l blood, and serves as an indicator of lead exposure.²⁻⁵

The inhibitory action of lead at low concentrations on ALA-D activity has been a subject of recent interest because of its characteristic properties. The depressed activity by lead exposure can be restored by the following agents or treatments *in vitro*: gel filtration, ammonium sulphate precipitation,⁶ or heating of haemolysate,⁶⁻⁹ or the addition of SH-compounds (such as dithiothreitol (DTT), reduced glutathione (GSH))¹⁰⁻¹² or zinc ion¹³⁻¹⁶ to the reaction mixture. Zinc ion introduced subcutaneously to an animal also reverses the lead-inhibition of ALA-D activity *in vivo*.¹⁷

Although several hypotheses have been raised on

the mechanism of ALA-D inhibition by lead,^{6 9 12 13 18} it has not yet been established. We have reported that the preincubation of lead salt with haemolysate intensifies ALA-D inhibition.¹⁹ Here we describe the involvement of protein factors in ALA-D inhibition by lead at 20 to 100 nM concentrations, which are 50-fold lower than the inhibition concentration of highly purified enzyme described previously.¹

Materials and methods

Heparinised venous blood was obtained from workers occupationally exposed to lead and from normal subjects without any history of lead exposure. All procedures for the preparation of ALA-D fractions and Hb fractions were carried out at 4 °C. Packed erythrocytes were washed three times in cooled saline (0.9%) and adjusted to the original haematocrit value with 0.025 M phosphate buffer pH 7.2. Erythrocytes were completely lysed by sonicating. The lysate was centrifuged at 24 000 g for 60 min. The obtained erythrocyte supernatant was applied to Sephadex G-25 or G-200 columns (2.6 × 90 cm) that had been equilibrated with 0.025 M phosphate buffer pH 7.2, and eluted with the same buffer. The ALA-D and Hb fractions thus obtained were pooled and used for the following experiments.

ALA-D activity was determined by the method by

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Ushio *et al.*⁷ with the exception that ALA-D fractions pooled were diluted 2- to 5-fold with the elution buffer and used as the enzyme solution in the present experiments. The standard mixture contained 0.5 ml of enzyme solution, 0.5 ml of 0.2 M phosphate buffer pH 7.0, 0.5 ml of 20 mM ALA (delta-aminolaevulinic acid) solution, and 1 ml of distilled water. To inhibit the recovered activity of ALA-D through Sephadex G-200 gel filtration, the pooled Hb fraction and lead acetate, or both, were added to the mixture without ALA solution and preincubated at 4°C for 20 hours. For the activation of ALA-D, zinc chloride or DTT was added to the mixture instead of distilled water. Another activation procedure was the heat treatment of enzyme solution in the presence of phosphate buffer at 60°C for 5 min as reported by Ushio *et al.*⁷ After the activation procedures, ALA-solution was added, and the reaction was carried out at 37°C for 60 min. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid solution containing 0.1 M HgCl₂. The amount of formed porphobilinogen was measured at 555 nm by adding 1 ml of Ehrlich's reagent to 1 ml of supernatant.

The protein concentration was measured by the absorbance at 280 nm, and the Hb concentration was determined using a haemoglobin counter (TOA Medical Electronic Co, Ltd, Japan), which directly measures Hb concentration spectrophotometrically as cyanmethaemoglobin. The lead concentration in the pooled fractions was determined as reported by Ushio *et al.*⁷

Results

Figure 1 shows the pH optimum and heat activation of ALA-D prepared from Sephadex G-25 (a) and G-200 (b) gel filtrations. Results with the enzyme from Sephadex G-25 (a) is similar to those obtained with whole blood⁷: the pH optimum of ALA-D from a lead worker is slightly lower than that from a normal subject. After heat treatment, the depressed enzyme activity in the lead workers is appreciably restored in the high pH range. These findings indicate that erythrocyte components of low molecular weight have no effect on the inhibition of ALA-D by lead and on its restoration by heat treatment. On the contrary, the inhibition due to lead apparently disappears after Sephadex G-200 gel filtration (b). The effect of heat treatment on the activity of the enzyme from lead workers, however, is different from that of the normal enzyme. The activity of the normal enzyme is decreased by heat treatment in the high pH range, while that from lead workers is rather stable. These results indicate that the effect of lead on the enzyme remains even after

Sephadex G-200 gel filtration, and also suggests that certain components with a high molecular weight are concerned in the disappearance of ALA-D inhibition after gel filtration.

Figures 2(a) and 3(a) show Sephadex G-200 chromatography of erythrocyte supernatant from a lead worker and from a normal subject, respectively. Lead concentrations were determined in four fractions (I-IV) pooled as shown in figs 2(a) and 3(a)

Fig 1 pH optimum and heat activation of ALA-D obtained by Sephadex G-25 (a) or G-200 (b) gel filtration of erythrocyte supernatant. Erythrocyte supernatant from a lead worker (64 µg/100 g, —●—, —○—) or from a normal subject (10 µg/100 g, —▲—, —△—) was gel filtrated. Pooled ALA-D fraction was used as enzyme solution and heated at 60°C for 5 min (---○---, ---△---). Control (—●—, —▲—) was not heated.

Fig 2 Sephadex G-200 gel filtration of erythrocyte supernatant from a lead worker (a), and the factors concerned in ALA-D inhibition by lead (b). (a): Ten millilitre of whole blood from a lead worker (51 $\mu\text{g}/100\text{ g}$) was used to prepare erythrocyte supernatant. (b): Pooled ALA-D fraction was preincubated with each fraction of 22 to 36 at 4°C for 20 hr. After preincubation, the mixture was further heated at 60°C for 5 min (—○—). Control (—●—) was not heated.

(table 1). Fifty per cent of the lead in erythrocyte supernatant from a lead worker is bound to proteins in ALA-D fractions (II). The lead found in ALA-D fractions may be responsible for the different effect of heating on the enzyme activity as seen in fig 1(b).

To detect the factors possibly involved in ALA-D inhibition by lead, we assayed the inhibitory action of some fractions obtained by Sephadex G-200 gel filtration (figs 2(b) and 3(b, c)). The enzyme from a lead worker with a blood lead concentration of 51 $\mu\text{g}/100\text{ g}$ is extremely inhibited in the presence of Hb fractions, and the inhibition is partially restored by heat treatment (fig 2(b)). By contrast, in a normal subject, ALA-D inhibition with Hb fractions is less than that in a lead worker. The slight inhibition is probably the effect of Hb itself²⁰ (fig 3(b)). The normal enzyme is appreciably inhibited by lead acetate at a concentration of 100 nM in the presence of Hb

Fig 3 Sephadex G-200 gel filtration of erythrocyte supernatant from a normal subject (a), and the factors concerned in ALA-D inhibition by lead (b and c). (a): Ten millilitre of whole blood from a normal subject (9 $\mu\text{g}/100\text{ g}$) was used to prepare erythrocyte supernatant. (b) and (c): Pooled ALA-D fraction was preincubated with each fraction of 4 to 9 and 14 to 30 in presence of lead acetate (100 nM) (c) or in the absence (b). After preincubation, mixture was further heated at 60°C for 5 min (—○—). Control (—●—) was not heated.

fractions, and the inhibition is also partially restored by heat treatment (fig 3(c)). These findings indicate that in Hb fractions factors induce or intensify ALA-D inhibition by lead at low concentrations. In view of the fact that Hb further purified by ion

Table 1 Distribution of lead in the erythrocyte supernatant

Lead in erythrocyte supernatant (ng/10 ml)	Lead in pooled fractions (ng)				
	I	II	III	IV	Total
900 ^a	96	330	330	96	862
4900 ^b	374	2499	1661	434	4968

Erythrocyte supernatant from a lead worker (51 $\mu\text{g}/100\text{ g}$)^b and from a normal subject (9 $\mu\text{g}/100\text{ g}$)^a was gel filtrated on a Sephadex G-200 column. Lead contents were determined in four pooled fractions (I-IV as shown in figs 2 and 3).

Fig 4 Effect of increasing amount of pooled Hb fraction on ALA-D inhibition and on its heat-restoration. Pooled ALA-D fraction and Hb fraction were prepared from normal erythrocytes. ALA-D fraction was preincubated with increasing amount of Hb fraction in presence (—●—, —○—) or absence (—▲—, —△—) of lead acetate (50 nM). After preincubation, enzyme was heated (—○—, —△—) or not heated (—●—, —▲—).

exchange chromatography has a similar effect on ALA-D inhibition by lead, Hb itself seems to have such an effect (data not shown).

Figure 4 shows the effect of increasing the amount of proteins in the pooled Hb fraction on the inhibition of ALA-D by lead acetate and also on its restoration by heat treatment. Lead acetate at a concentration of 50 nM causes little inhibition (under 3%) in the absence of pooled Hb fraction. As the amount of Hb increases (100 to 2500 μg/ml), ALA-D inhibition by lead acetate progresses appreciably. In the absence of lead these concentrations of Hb produce slight inhibition, an effect due solely to Hb.²⁰ The inhibition of ALA-D activity by lead, which is intensified by the Hb fraction, may be restored by heat treatment. Under our assay conditions, a concentration of 2500 μg/ml is derived from blood with 12.5 gHb/dl.

Figure 5 shows the inhibition of ALA-D by increasing amounts of lead acetate in the presence of pooled Hb fraction, and its restoration by three agents or manoeuvres. In the presence of the Hb fraction 80 and 100 nM of lead acetate causes more than 90% inhibition, and 10 mM DTT or 100 μM zinc can restore the activity completely; restoration is incomplete with heating. In the absence of the Hb fraction lead acetate at concentration between 0 and 100 nM does not inhibit the activity of the enzyme (data not shown). Based on these observations, it is clear that factors in the Hb fractions are concerned

Fig 5 ALA-D inhibition by increasing amount of lead acetate, and its restoration by each of three agents. Pooled ALA-D fraction and Hb fraction were prepared from normal erythrocytes. Pooled ALA-D fraction was preincubated with Hb fraction and varying amount of lead acetate (0-100 nM). After preincubation, the inhibited activity (—●—) was restored by heating (—○—), 100 μM zinc (—△—), or 10 mM DTT (—▲—).

in the inhibition of ALA-D by lead at low concentrations.

Table 2 shows the effect of various treatments on

Table 2 Effect of various treatments on the activity of lead-bound ALA-D in the presence or absence of pooled Hb fraction

Treatments	ALA-D activity (A ₅₅₅)	
	— Hb fr	+ Hb fr
Control	0.423	0.039
Heating	0.390	0.181
Zinc (20 μM)	0.320	0.120
Zinc (100 μM)	0.081	0.296
DTT (10 mM)	0.447	0.351

Enzyme from Sephadex G-200 gel filtration of erythrocyte supernatant of a lead worker (57 μg/100 g) was preincubated in presence or absence of Hb fraction from Sephadex G-200 gel filtration of normal erythrocyte supernatant (10 μg/100 g).

Fig 6 Heat stability of the factors. Pooled Hb fraction from normal erythrocytes was heated at 60°C (○) or 80°C (△) for 0-30 min. Heated Hb fraction was then preincubated with enzyme fraction from normal erythrocytes and lead acetate (50 nM). Enzyme activity was determined with (H) or without (NH) 2nd heat treatment (60°C, 5 min). Ability of factors in Hb fraction was expressed as ratio of ALA-D activity (H/NH).

the activity of lead-bound enzyme from a lead worker. In the absence of pooled Hb fraction the activity of the lead-bound enzyme, which has been reversed by gel filtration, is not more activated by heating, zinc, or by DTT. The reversed lead-bound enzyme is reinhibited if it is preincubated with the Hb fraction from a normal subject. The reinhibition is partially removed again by heating, or by the addition of zinc or DTT. The optimum concentration of zinc is 20 μ M in the absence of the Hb fraction, and 100 μ M in its presence. An optimum concentration of zinc is dependent on the concentration of Hb (data not shown).

Figure 6 indicates the heat stability of the factors found in the Hb fractions. The ability of the factors to restore the ALA-D inhibition is decreased by preheating at 60°C or 80°C. The half life is about 26 min at 60°C and 3 min at 80°C. The ability is not influenced by preheating for 5 min at 60°C. This indicates that the restoration of depressed activity by heat treatment is not due to the inactivation of the inhibiting factors.

Discussion

In the present study we have shown the involvement of erythrocyte factors in the inhibition of ALA-D at low lead concentrations. The ability of these factors to intensify the inhibition of ALA-D by lead was detected in the Hb fractions obtained by Sephadex

G-200 gel filtration of erythrocyte supernatant.

Highly purified ALA-D is inhibited by lead at 1 and 5 μ M concentrations ($K_i = 1.7 \mu$ M),¹ and the crude enzyme is also inhibited at concentrations exceeding 1 μ M.¹⁶ Both are final concentrations in the assay mixture. Under our routine conditions, lead at toxic concentrations (50-100 μ g/100 g of blood) are finally between 50 and 100 nM in the mixture, because the whole blood is diluted 50-fold. These concentrations are 10 to 50-fold lower than those in the purified or crude enzyme *in vitro*.

We have previously suggested that there are two types of ALA-D inhibition *in vitro*, using whole blood as the enzyme source.¹⁹ If the enzyme is preincubated with lead at 50-500 nM concentrations, pronounced inhibition can be induced and then reversed by heating. By contrast, the inhibition by lead acetate at concentrations exceeding 1 μ M cannot be restored by heating. The difference between the two types of ALA-D inhibition by lead at low or high concentrations suggests the presence of two different binding sites for lead ions on the enzyme molecules. Lead ion at the low concentrations probably binds to certain site on the enzyme molecules during preincubation, and results in the inhibition that can be restored by heating. This site should have a high affinity for lead ions. At the high concentrations, lead would bind to sites other than the high affinity site, and this results in the type of inhibition that cannot be restored by heating. Thus we postulate two types of site on the enzyme molecules. The enzyme has many SH-groups²¹ that seem to have individual sensitivity to lead.

After Sephadex G-200 gel filtration of the erythrocyte supernatant from lead workers, 50% of the lead was actually found in the ALA-D fraction. The activity of the ALA-D fraction was inhibited if preincubated with the pooled Hb fraction from a normal subject. Enzyme obtained in a similar fashion from normal erythrocyte supernatant was also considerably inhibited if it had been preincubated with lead acetate (100 nM) in the presence of the Hb fraction. This inhibition could be partially reversed by heat treatment. In the absence of the Hb fraction enzyme from both a lead worker and a normal subject was not inhibited even if preincubated. Thus factors found in the Hb fraction may mediate between the lead ion and the high affinity site on the molecules, resulting in the inhibition of ALA-D by low concentrations of lead.

The inhibition of ALA-D by low concentrations of lead is non-competitive,⁷ while the inhibition of the highly purified enzyme by lead ion at high concentrations is complex in type.¹ The type of inhibition mediated by the factors *in vitro* seems to be similar to the former, and to be non-competitive because of

the inhibition by lead at low concentrations and of the restoration of activity by heat. We may speculate on the non-competitive action of lead (bound to the high affinity site) at low concentrations as follows:

Although lead bound to the high affinity site(s) does not inhibit enzyme activity without the factor (F), the factor might affect the conformation of enzyme-lead complex (E-Pb), resulting in the failure of dissociation of the intermediate complex (E-Pb-F-S) into the product (P).²²

The inhibition by lead bound to the high affinity site that was mediated by the factors was restored by heating, zinc, or DTT. The restoration of activity by heat may be due to the following, reversible process:¹⁹

where the forward reaction is promoted by heat treatment at 60°C for 5 min, and the backward reaction by preincubation at 4°C for 20 hr or 37°C for 3 h. The factors mentioned above may be the same as those suggested by Vergnano *et al.*,⁶ although ours are not inactivated by heating at 60°C for 5 min, and their action is reversible.¹⁹ The nature of the factors remains to be resolved.

The action of zinc or DTT in restoring the activity of the enzyme seems to differ from that of heating. Zinc is most effective and may be essential for the activity of the enzyme since zinc ion restores the inhibition by both low and high lead concentrations. Relatively high concentrations of zinc have an antagonistic effect on the inhibition of ALA-D by lead in vitro.¹³ Zinc added at a high concentration could replace lead on the enzyme molecule, although lead has a greater affinity for binding with SH-group than zinc.²³ DTT has dual effects on the activity through SH-groups on the molecules;²⁴ it not only restores the activity of the enzyme when inhibited by lead but also when inactivated by the oxidation of SH-groups. While the inhibition by low lead concentrations was reversed by DTT, increasing amounts of lead reduced the degree to which DTT restored activity. This suggests that DTT only restores the inhibition due to lead bound to high affinity sites. The recent finding that the addition of zinc to the apoenzyme completely restores the activity if the essential SH-groups are first reduced with SH-reagents is of considerable interest.²¹ Further study is necessary to clarify the mode of action of zinc, DTT, or the factors in the restoration of lead-inhibited activity or all.

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