Effect of lead ions on chick-embryo liver mitochondrial δ -aminolaevulinate synthase

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Pb^{2+} activated native chick-embryo liver mitochondrial δ -aminoalaevulinate synthase (EC 2.3.1.37). This result contradicted with the inhibitory effect observed by earlier workers who used degraded enzyme preparations. Enzyme activation was biphasic. An initial activation phase was observed with Pb^{2+} concentrations up to $200\ \mu\text{M}$, and a secondary phase with concentrations from $200\ \mu\text{M}$ to at least 2 mM . Maximum primary activation was 2.5-fold at $200\ \mu\text{M-Pb}^{2+}$, with a further 2-fold activation observed at 2 mM-Pb^{2+} . Primary activation was not affected by a 10-fold molar excess of dithioerythritol, but the secondary activation was abolished by dithioerythritol. Secondary-phase activation was lost upon increasing time of incubation of the enzyme with Pb^{2+} . The implications of these findings are discussed with reference to lead poisoning and the mechanism of δ -aminolaevulinate synthase.

The haem-biosynthetic pathway is particularly sensitive to lead poisoning (Goldberg, 1972). The biochemical features of lead poisoning resemble some of those in certain genetic porphyria diseases, one of these features being increased amounts of urinary δ -aminolaevulinate. In lead poisoning it is generally accepted that the increase in δ -aminoalaevulinate results from the inhibition of enzymes in the haem-biosynthetic pathway, notably inhibition of the second enzyme, δ -aminolaevulinate dehydratase (Goldberg, 1968). However, there is no consensus as to the effect of Pb^{2+} on δ -aminoalaevulinate synthase, the first and rate-limiting enzyme of the pathway. Whereas some workers (Morrow *et al.*, 1969; Whiting & Granick, 1976) have reported that δ -aminolaevulinate synthase is inhibited by Pb^{2+} , others (Scholnick *et al.*, 1969; Strand *et al.*, 1972) have observed that there is no effect. These studies are not readily interpreted, because they were all made with either crude cell extracts or partially purified, but degraded, δ -aminolaevulinate synthase (see Borthwick *et al.*, 1983).

Native chick-embryo liver mitochondrial δ -aminolaevulinate synthase of minimum M_r 68000 has been purified in this laboratory, and it has been shown that it can be proteolytically degraded to an enzymically active form of minimum M_r 50000 (Borthwick *et al.*, 1983).

In the present paper we report the effect of Pb^{2+} on the activity of both forms of the enzyme; their responses are quite different, and the possible implications of this finding on both lead poisoning

and the mechanism of action of δ -aminolaevulinate synthase are discussed.

Experimental

Materials

Succinyl-CoA, dithioerythritol, glycine, pyridoxal 5'-phosphate and Trizma base were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lead acetate was purchased from BDH Chemicals (Poole, Dorset, U.K.).

Enzyme purification

Chick embryos were induced with the porphyrinogenic drugs 2-allyl-2-isopropylacetamide and 1,4-dihydro-3,5-dicarbethoxycollidine. Native mitochondrial δ -aminolaevulinate synthase was purified to near-homogeneity by the method of Borthwick *et al.* (1983), except that the activity from the chromatofocusing chromatography step was collected over a narrow pH range and the final step of CoA affinity chromatography was omitted. This enzyme was judged to be at least 95% pure by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, with a single major protein band of minimum M_r 68000 and a specific activity of 32300 units/mg.

Degraded mitochondrial δ -aminolaevulinate synthase was isolated by a similar procedure to that described for the native enzyme, except that δ -aminolaevulinate synthase was released from mitochondria by sonication instead of by freeze-

drying a mitoplast preparation. Briefly, mitochondria were freeze-thawed once, followed by sonication for four 20 s periods on ice with a Branson sonicator at maximum output. The preparation was centrifuged at 100000g for 60 min, and the procedure for the native enzyme was followed. The final enzyme preparation (specific activity 31800 units/mg) was judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to be at least 90% pure, and the protein had a minimum M_r of 50000 (no native protein of M_r 68000 could be detected).

δ -Aminolaevulinate synthase assay

δ -Aminolaevulinate synthase activity was measured colorimetrically (Poland & Glover, 1983) in an assay mixture containing 20 units (nmol of δ -laevulinate/h at 37°C) of δ -aminolaevulinate synthase, succinyl-CoA (100 μ M), glycine (100 mM), pyridoxal 5'-phosphate (250 μ M) and Tris/HCl buffer (100 mM), pH 7.6, in a total volume of 150 μ l. The mixture was incubated at 37°C, and under these conditions the assay was linear for at least 15 min. Separate experiments established that there was no degradation of either form of δ -aminolaevulinate synthase during the assay procedure.

To examine the effect of lead, Pb^{2+} was added to the assay mixture containing enzyme (but not succinyl-CoA) and incubated for various times before the assay was commenced by addition of succinyl-CoA.

Results

Effect of Pb^{2+} on δ -aminolaevulinate synthase activity

Pb^{2+} shows quite different effects on the activity of the two forms of chick-embryo liver mitochondrial δ -aminolaevulinate synthase. The native form of M_r 68000 is markedly activated by Pb^{2+} (when added at zero time to the assay mixture), and the effect is biphasic (Fig. 1). There is a primary phase of activation with Pb^{2+} up to 200 μ M, at which concentration the enzyme is activated 2.5-fold. At concentrations of Pb^{2+} greater than 500 μ M a secondary phase of activation occurs that is linear up to the highest concentration tested (2000 μ M). At 2000 μ M- Pb^{2+} the activity is 5 times that of enzyme in the absence of Pb^{2+} . In contrast, the degraded form of the enzyme (M_r 50000) shows no initial activation by Pb^{2+} , and even at 2000 μ M there is only a 75% activation.

In a series of similar experiments, enzyme was pre-incubated at 37°C in the presence of Pb^{2+} for 30 min before the commencement of the enzyme assay (Fig. 2). Pre-incubation of the degraded form of the enzyme with Pb^{2+} at concentrations greater than 100 μ M resulted in a significant inhibition of

Fig. 1. Effect on δ -aminolaevulinate synthase activity of adding Pb^{2+} at zero time

Native (●) or degraded (▲) δ -aminolaevulinate synthase (20 units) was assayed in the presence of Pb^{2+} added at zero time as described in the Experimental section. Control samples were assayed in the absence of Pb^{2+} .

Fig. 2. Effect on δ -aminolaevulinate synthase activity of pre-incubating with Pb^{2+} for 30 min

Native (●) or degraded (▲) synthase (20 units) was assayed in the presence of Pb^{2+} after a 30 min incubation with Pb^{2+} as described in the Experimental section. Control samples were assayed in the absence of Pb^{2+} after a 30 min pre-incubation.

activity. When the native form was pre-incubated with Pb^{2+} the previously observed secondary activation phase was abolished, whereas the primary activation phase remained unchanged (com-

pare Figs. 1 and 2). As lead acetate was used as a source of Pb^{2+} throughout this study, the effect of sodium acetate was examined; with both forms of the enzyme it was found to have no effect on the range 0–4 mM when tested at zero time or after preincubation (results not shown).

Time-dependency of Pb^{2+} inactivation of δ -aminolaevulinate synthase

The loss of the secondary activation phase after 30 min pre-incubation of native δ -aminolaevulinate synthase with Pb^{2+} suggested a time-dependent inactivation. To examine this, native enzyme was pre-incubated for different times with 200 μM - or 1000 μM - Pb^{2+} (Fig. 3). It can be seen that at 200 μM - Pb^{2+} the primary 2.5-fold activation of δ -aminolaevulinate synthase observed in Fig. 1 was not affected by pre-incubation. This contrasted dramatically with the observed time-dependent loss of activity with 1000 μM - Pb^{2+} . At this concentration of Pb^{2+} the enzyme was initially activated, but then lost activity, so that the activity approached that of enzyme not exposed to Pb^{2+} .

Fig. 3. Effect on δ -aminolaevulinate synthase activity of pre-incubating with Pb^{2+} for different times

Native δ -aminolaevulinate synthase (20 units) was pre-incubated with 0 (●), 200 μM (□) and 1000 μM - (■) Pb^{2+} for up to 120 min before assay as described in the Experimental section. Control samples were assayed in the absence of Pb^{2+} without a period of pre-incubation.

Effect of dithioerythritol on Pb^{2+} activation of δ -aminolaevulinate synthase

The effect of dithioerythritol on the activity of the native enzyme was examined in the presence of Pb^{2+} . Dithioerythritol at a 10-fold molar excess and added at zero time together with Pb^{2+} did not affect the primary 2.5-fold activation observed with 200 μM - Pb^{2+} alone. However, dithioerythritol almost completely abolished the secondary activation of Pb^{2+} at 2000 μM , decreasing the activation by Pb^{2+} back to a 2.5-fold stimulation. When native enzyme was pre-incubated for 30 min with Pb^{2+} , followed by a further 15 min incubation with dithioerythritol, it was found that dithioerythritol had no effect on δ -aminolaevulinate synthase in the presence of either 200 μM - or 2000 μM - Pb^{2+} .

Discussion

The present study shows that native chick-embryo liver mitochondrial δ -aminolaevulinate synthase is activated by Pb^{2+} at concentrations of up to 2000 μM and that the activation is complex, occurring in two distinct phases. An initial primary activation of the enzyme was observed at Pb^{2+} concentrations up to 200 μM , with the enzyme being stimulated maximally about 2.5-fold. This primary activation was not affected by dithioerythritol, which suggests either that interaction of enzyme thiol groups with Pb^{2+} is not responsible or that the interaction with such groups is too strong to be disrupted by dithioerythritol. We have shown (Pirola *et al.*, 1984) that the enzyme can be also activated by Mg^{2+} and Na^{+} ions. However, this stimulation appears to be different from that observed with Pb^{2+} . There is no biphasic response, and the concentrations of Mg^{2+} and Na^{+} ions required for a similar degree of activation are two to three orders of magnitude greater than that of Pb^{2+} . In the light of these observations it would seem unlikely that the effect of Pb^{2+} on enzyme activity is due to a general metal-ion effect.

A secondary activation of native δ -aminolaevulinate synthase by concentrations of Pb^{2+} greater than 500 μM could be reversed by dithioerythritol, suggesting that this additional activation was possibly due to an interaction of Pb^{2+} with the protein via thiol groups. The observation that, unlike the primary activation, this secondary effect decreased with the time of exposure of the enzyme to Pb^{2+} implied that a number of complex interactions were occurring, and further studies will be required to account for this observation.

In marked contrast with the native enzyme, a proteolytically degraded form of the enzyme of M_r 50000 was significantly inhibited by prolonged exposure to Pb^{2+} at concentrations greater than

100 μM . This result agreed with the earlier report by Whiting & Granick (1976), who carried out studies with what has now been shown to be a degraded form of δ -aminolaevulinate synthase (Borthwick *et al.*, 1983).

Despite many studies into the effects of Pb^{2+} on the enzymes of haem biosynthesis, the precise mechanism of lead poisoning is still unclear. Elevated amounts of urinary δ -aminolaevulinate are a primary symptom and are thought to be due to two major effects of Pb^{2+} , namely the inhibition of δ -aminolaevulinate dehydratase activity and the induction of hepatic δ -aminolaevulinate synthase (Goldberg, 1968). It is not known whether the present findings of a 2.5-fold enhancement of δ -aminolaevulinate synthase activity by Pb^{2+} at 200 μM is applicable to the situation *in vivo*. However, if it occurs *in vivo* the activation of δ -aminolaevulinate synthase by Pb^{2+} during lead poisoning could clearly contribute to increased urinary excretion of δ -aminolaevulinate. It is noteworthy that in lead-poisoned animals concentrations of Pb^{2+} in the mitochondria are of the order of 1 mM (Miani & Viterbo, 1958).

The present work has a direct bearing on the proposed catalytic mechanism of δ -aminolaevulinate synthase. Scholnick *et al.* (1972) have postulated that δ -aminolaevulinate synthase has a thiol-dependent active site (making δ -aminolaevulinate synthase the only pyridoxal enzyme of this type),

on the basis of studies with thiol-blocking agents. Although this was later supported by Whiting & Granick (1976), neither study was made with homogeneous native δ -aminolaevulinate synthase. The observation in the present work that Pb^{2+} activates rather than inhibits native δ -aminolaevulinate synthase suggests that further work on the active site is required.

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