Ethylenediamine-*NN'*-tetra-acetate-Dependent Amino Acid-Stimulated Inactivation of Mouse Ovarian Alkaline Phosphatase Activity

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1. Preincubation of partially purified preparations of mouse ovarian alkaline phosphatase in the presence of both EDTA and glycine at alkaline pH resulted in a pronounced inactivation of alkaline phosphatase activity. Inactivation did not occur on preincubation with EDTA or glycine alone. 2. The rate of inactivation was first-order with respect to the concentration of enzyme, and was independent of EDTA concentration above a threshold value. 3. The process was pH-dependent with a pK at 9.85, and inactivation was not dependent on the stereochemistry of the amino acid. A free α -amino group and a free carboxyl group at a specific spatial separation were essential for inactivation. 4. Inactivation involved the formation of an enzyme-metal ion-amino acid complex, the amount formed being dependent on both the nature and concentration of the amino acid. This complex then decayed to a derivative that was then acted on by EDTA, yielding an inactive form of the enzyme.

Immature mouse ovaries possess an alkaline phosphatase activity (phosphatase I) that appears to be modified to a kinetically distinct form (phosphatase Ib) by gonadotrophin treatment *in vivo* (Bramley, 1974). In preliminary work on these activities amino acid buffers were used in the assay medium. As Zn^{2+} is a potent inhibitor of ovarian alkaline phosphatase activity, homogenates were preincubated briefly at 30°C with a small quantity of EDTA before measurements of alkaline phosphatase activity. However, instead of improving the reproducibility of the assay, preincubation led to considerable variability in measurements of specific activity. This was due to a rapid irreversible loss of activity on preincubation with EDTA in amino acid buffers, and this is the subject of the present paper.

Materials and Methods

All substrates and fine chemicals were obtained from the Sigma Chemical Co. Ltd., London S.W.6, U.K. All other reagents were of analytical quality. Human chorionic gonadotrophin (Pregnyl) was obtained from Organon Laboratories Ltd., Redhill, Surrey, U.K. A preparation of human pituitary follicle-stimulating hormone (CP_1) was prepared in this laboratory (Butt *et al.*, 1961).

Immature female mice of an outbred LACA strain were obtained from Olac Laboratories Ltd., King's Norton, Birmingham, U.K. They were 23–25 days old on arrival. Animals received three equal daily subcutaneous injections of 0.15M-NaCl–10mM-Tris–

HCl buffer, pH 7.4, containing 1% (w/v) of bovine serum albumin, or saline medium containing 10 i.u. of human chorionic gonadotrophin plus 2 i.u. of follicle-stimulating hormone. Mice were killed by chloroform anaesthesia on the morning of the fourth day, and the ovaries were removed, freed of fat and connective tissue, blotted, and collected in ice-cold 0.3M-sucrose–10mM-Tris–HCl buffer, pH 7.4. Pooled ovaries were homogenized by hand in an all-glass Potter homogenizer in sufficient sucrose–Tris medium to give approx. 5ml of medium/0.1g wet wt. of tissue. Homogenates were filtered through glass wool and stored at –20°C until required.

Before extraction of ovarian alkaline phosphatase I and Ib activities, homogenates were centrifuged at 5000 rev./min for 10 min in an MSE Minor centrifuge at room temperature. This removes erythrocytes, cell debris and nuclei, which interfere with the subsequent butanol extraction.

A 5ml portion of homogenate from control or gonadotrophin-treated mouse ovaries was diluted with 10ml of chilled extraction medium (0.3M-KCl–1mM-magnesium acetate–30mM-Tris-acetate buffer, pH 5.0), and 5ml of cold butan-1-ol was added. The mixture was homogenized by hand in a loosely fitting all-glass Potter homogenizer, and the homogenate was stirred magnetically for 60 min at 4°C. The mixture was centrifuged at 5000 rev./min for 10 min in an MSE Minor centrifuge and the lower aqueous layer was collected. The slightly turbid solution was adjusted to pH 8.5 with dilute NaOH, an equal volume of cold ethanol was added, and the solution was left for 60 min at 4°C. The mixture was centrifuged at 5000 rev./min for 10 min in an MSE

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Minor centrifuge and the active precipitate was resuspended in a small volume of 1 mM-magnesium acetate–30 mM-Tris–HCl buffer, pH 8.5, and re-centrifuged at 5000 rev./min for 10 min in an MSE Minor centrifuge. The inactive sediment was discarded. The enzyme solution was dialysed overnight against 1 mM-magnesium acetate–30 mM-Tris–HCl buffer, pH 8.5, and the inactive precipitate was removed by centrifugation. This procedure resulted in a 15–30-fold purification of mouse ovarian alkaline phosphatase I and Ib activities.

Alkaline phosphatase activity was measured as described previously (Bramley, 1974), with *p*-nitrophenyl phosphate as substrate.

Results

Alkaline phosphatases I and Ib were found to behave in a very similar fashion with respect to inactivation by EDTA and amino acids. Therefore most experiments were performed with the more active phosphatase Ib preparation. However, results obtained for phosphatase Ib were confirmed with phosphatase I preparations.

Both ovarian alkaline phosphatase activities required the presence of Mg^{2+} and were inactive in the presence of excess of EDTA. Addition of excess of Mg^{2+} after preincubation at 30°C with EDTA in the presence of a non-amino acid buffer (piperazine–HCl, pH 10.4) resulted in an almost complete and rapid reactivation. However, similar preincubation in the presence of an amino acid-containing buffer (glycine–NaOH, pH 10.4) resulted in a pronounced and time-dependent decrease in the amount of activity regenerated by Mg^{2+} . Semi-logarithmic plots of alkaline phosphatase activity against the duration of preincubation with EDTA and glycine were linear, indicating that inactivation was a first-order process (Fig. 1). This decrease was not observed in the absence of EDTA, nor when EDTA had been removed by adding a slight excess of Zn^{2+} or Mg^{2+} , suggesting that EDTA acted by the chelation of a metal ion. Ethanedioxybis(ethylamine)-*NNN'*-tetra-acetate ('ethylene glycol bis(amino-methyl ether) tetra-acetate'), a chelating agent specific for Ca^{2+} at low concentrations, was not as effective as EDTA (Fig. 1). Preincubation with various substrates failed to protect against inactivation, and addition of oxidized or reduced glutathione failed to regenerate activity (T. A. Bramley, unpublished work). The rate of inactivation was unaffected by the presence of 10 mM-*N*-ethylmaleimide or 10 mM-iodoacetate.

Effects of chelating agents

Preincubation with low concentrations of EDTA (1 μ M) had no effect (Fig. 2), but as the concentration

of EDTA was increased there was a sudden fall in alkaline phosphatase activities observed after preincubation. Further increases in EDTA concentrations up to quite high values had no effect. The extent of inactivation could, however, be manipulated by altering the duration of preincubation. This suggests that reaction with EDTA is not a rate-limiting step.

Fig. 1. Inactivation of ovarian alkaline phosphatase activity on preincubation with EDTA and glycine

Alkaline phosphatase Ib was isolated from gonadotrophin-treated mouse ovaries and preincubated at 30°C for the period indicated in the presence of 150 mM-piperazine–HCl buffer, pH 10.4, containing: 50 mM-glycine–NaOH buffer, pH 10.4 (□), 0.5 mM-EDTA–NaOH buffer, pH 10.4 (Δ), 50 mM-glycine–NaOH buffer plus 0.5 mM-ethanedioxybis(ethylamine)tetra-acetate (○), or 50 mM-glycine–NaOH buffer plus 0.5 mM-EDTA (●). Magnesium acetate was then added (final concn. 3 mM) and the enzyme reaction was started by the addition of *p*-nitrophenyl phosphate (final concn. 3 mM). Alkaline phosphatase activity was measured after further incubation at 30°C, and log(alkaline phosphatase activity) was plotted against duration of preincubation.

Fig. 2. Effects of EDTA concentration on inactivation of ovarian alkaline phosphatase activity

Alkaline phosphatase Ib isolated from gonadotrophin-treated mouse ovaries was preincubated at 30°C for either 10 min (○) or 15 min (●) in the presence of 150 mM-piperazine-HCl buffer, pH 10.4, 50 mM-glycine-NaOH buffer, pH 10.4, and EDTA at the concentration indicated. After preincubation, sufficient magnesium acetate was added to bring the concentration to 3 mM. The enzyme reaction was started by the addition of *p*-nitrophenyl phosphate (final concn. 3 mM), and alkaline phosphatase activity was measured after incubation at 30°C. Points represent the means, and the vertical bars the ranges, of a single experiment in quadruplicate.

Fig. 3. Effects of pH on rate of inactivation of ovarian alkaline phosphatase activity

Alkaline phosphatase Ib was isolated from gonadotrophin-treated mouse ovaries and preincubated at 30°C in medium containing 150 mM-piperazine-HCl buffer, 50 mM-glycine-NaOH buffer and 0.5 mM-EDTA for at least six periods ranging from 0 to 30 min at each pH value (20°C). Appropriate controls were included. After the addition of Mg^{2+} and *p*-nitrophenyl phosphate, alkaline phosphatase activities were measured, and log (alkaline phosphatase activity) was plotted against duration of preincubation at each pH value. Inactivation rates were calculated from:

$$\text{Rate} = \frac{\ln 2}{t_{50}}$$

where t_{50} is time when 50% of the activity remains. All rates were corrected for controls. Finally, log (inactivation rate) was plotted against pH.

Effects of pH on inactivation

The rate of inactivation of alkaline phosphatase on preincubation at 30°C with glycine and EDTA was pH-dependent. Plots of the logarithm of the inactivation rates measured at several pH values showed a pK at pH 9.85 (Fig. 3). Although the pK measured may have been due to the ionization of a charge within the enzyme molecule, it seems most likely that it was due to the ionization of the α -amino group of glycine (pK 9.78 at 20°C), indicating that the unprotonated form of the amino acid was required to inactivate the enzyme.

Essential structures for inactivation

A range of substances was tested for the ability to inactivate ovarian alkaline phosphatase activity on preincubation with EDTA at pH 10.4 and at 30°C. Such studies should indicate those structures that are essential for inactivation.

(a) Various α -oxo carboxylic acids were ineffective, indicating that the α -amino group of the amino acid was essential for inactivation, as expected from the pK of the process (Table 1).

(b) Various primary and secondary amines were ineffective, as were amino acid esters, indicating the involvement of the free carboxyl group of the amino acid.

(c) The free α -amino group and the free carboxyl group appeared to be required at a particular spatial separation, since several dipeptides (in which the free amino and carboxyl groups are widely separated), and β -alanine (in which the free groups are separated by an additional carbon atom), were very much less effective.

(d) The D, L and DL forms of the amino acids tested were equally effective, indicating that the process did not discriminate between their different stereoisomers.

Table 1. *Effects of various compounds on inactivation of ovarian alkaline phosphatase*

A preparation of mouse ovarian alkaline phosphatase Ib was preincubated at 30°C for at least six different periods of time in media containing 150 mM-piperazine-HCl buffer, pH 10.4, 0.5 mM-EDTA, and the appropriate substances at the concentrations indicated (after adjustment to pH 10.4). Mg^{2+} and *p*-nitrophenyl phosphate were then added, and alkaline phosphatase activity was measured after a further period of incubation at 30°C. Rates of inactivation were calculated as described in the legend to Fig. 3.

Compound	Concentration (mM)	Inactivation rate (%/min)
Amino acids		
Glycine	62.5	2.27
	31.2	1.79
L-Asparagine	62.5	1.85
	31.2	1.28
L-Serine	62.5	2.50
	31.2	2.27
D-Serine	62.5	2.50
	31.2	2.27
L-Histidine	10.0	4.00
D-Histidine	10.0	3.95
DL-Histidine	10.0	4.00
Dipeptides		
Gly-Leu	31.2	0.50
Gly-Gly	22.0	0.40
Gly-Asp	27.5	0.0
Esters		
Ethylglycine	50	0.0
Methylglycine	50	0.0
Oxo carboxylic acids		
Oxoglutarate	40	0.0
Pyruvate	40	0.0
Bases		
Ethanolamine	150	<0.3
	40	<0.1
Piperazine	300	0.02
Histamine	50	0.1

It is known that amino acids and bivalent metal ions form chelates that satisfy these criteria. Both the free amino and carboxyl groups interact with the metal ion, and both groups are required for chelation. Since only two-point attachment is involved, however, the process is independent of the stereochemistry of the amino acid. Further, the separation of these groups is critical, since a stable chelate will not be formed if the separation is too great.

As the side chain of the amino acid is not directly involved in co-ordination and actually projects away from the co-ordinating sites, its nature would be expected to have only a minimal influence on chelation, and thereby on inactivation of alkaline phosphatase.

Fig. 4. *Effects of nature and concentration of amino acid on inactivation of ovarian alkaline phosphatase activity*

Alkaline phosphatase Ib was isolated from gonadotrophin-treated mouse ovaries and preincubated (30°C, pH 10.4) in media containing 150 mM-piperazine-HCl, 0.5 mM-EDTA, and a range of concentrations of different amino acids adjusted to pH 10.4 with NaOH or HCl. They were preincubated for at least six preincubation periods (from 0 to 30 min) at each concentration of each amino acid, and the appropriate controls were included. After addition of Mg^{2+} and substrate, alkaline phosphatase activities were measured and log (activity) was plotted against duration of preincubation for each concentration of each amino acid. Inactivation rates were calculated as described in the legend to Fig. 3. Finally double-reciprocal plots of inactivation rate against concentration were constructed for each amino acid. ○, L-Histidine; △, L-phenylalanine, L-leucine; ●, glycine; ■, D- and L-serine; □, L-asparagine, L-glutamine.

phatase. However, in the present study the nature of the amino acid side chain was found to have a considerable influence.

Effects of concentration and the nature of the amino acid side chain on inactivation

Both the nature and concentration of the amino acid present during preincubation with EDTA affected the rate of alkaline phosphatase inactivation. Fig. 4 shows double-reciprocal plots of inactivation rate versus concentration for a range of different amino acids. Different amino acids gave curves with the same intercept but markedly different slopes. This implies that the inactivation of alkaline phosphatase takes place in two separate and fundamentally different stages, one being independent of amino acid, the other dependent on both the nature

Fig. 5. Arrhenius plots for inactivation of ovarian alkaline phosphatase activity

Double-reciprocal plots were constructed as described in the legend to Fig. 4 from inactivation rates measured at each of a range of temperatures. At least five amino acid concentrations were used to construct each double-reciprocal plot at each temperature. From these plots, the maximum inactivation rate, V (reciprocal of intercept on $1/v$ axis), and the apparent association constant, K_{AA} (reciprocal of intercept on $1/[amino\ acid]$ axis), were calculated. $\log V$ (a) and pK_{AA} ($-\log K_{AA}$) (b) were plotted against $1/T$ (°K). Activation energies were calculated from the computed regressions of each line. $\square$, Glycine; $\bullet$, asparagine; $\square$, alkaline phosphatase I; $\circ$, $\bullet$, alkaline phosphatase Ib.

and concentration of amino acid present. By analogy with enzyme-substrate reactions, the amino acid-dependent stage reflects the interactions of amino acid with the enzyme. The intermediate formed breaks down to give the reaction product, which in this case is an inactive enzyme, the rate of breakdown being dependent only on the concentration of the intermediate.

Effects of temperature

Arrhenius plots of the overall rate of inactivation of alkaline phosphatase (with glycine and EDTA) were linear over a wide temperature range (0–40°C), and showed that the activation energy for the overall process was high [153 kJ/mol (36.5 kcal/mol); T. A. Bramley (unpublished work)]. Double-reciprocal plots of inactivation rate against amino acid concentration were constructed from values calculated at several temperatures for two amino acids with different association constants and for alkaline phosphatase I and Ib preparations. From these plots values of the maximum rate and amino acid association constants (K_{AA}) were calculated at each temperature, Arrhenius plots were constructed, and activation energies for both the formation and

breakdown of the intermediate complex were calculated (Fig. 5). Both processes were found to have similar high activation energies [approx. 153 kJ/mol (36.5 kcal/mol)]. The activation energies were similar with either glycine or asparagine, and with both phosphatase I and Ib preparations.

Dialysis experiments

Dialysis of alkaline phosphatase Ib preparations at 4°C against iso-osmotic sucrose containing EDTA and amino acids gave results similar to those of pre-incubation experiments at 30°C (although, as predicted from the high activation energy, the inactivation rate was very much lower at 4°C than at 30°C). Both EDTA and glycine were necessary: very little inactivation occurred on dialysis against either alone.

The first step in the inactivation process may be either the interaction of the enzyme with amino acid, followed by interaction with EDTA, or vice versa. Dialysis against iso-osmotic sucrose containing EDTA, followed by extensive dialysis against iso-osmotic sucrose alone to remove free EDTA, yielded a preparation with very low alkaline phosphatase activity, which could, however, be restored to control

Fig. 6. *Dialysis experiments in inactivation of ovarian alkaline phosphatase activity*

Samples (2ml) of a diluted alkaline phosphatase Ib preparation extracted from gonadotrophin-treated mouse ovaries were dialysed for 24 h (4°C; pH 10.4) against 50 ml of (a) 0.3 M-sucrose–15 mM-piperazine–HCl buffer, (b) 0.3 M-sucrose–piperazine–HCl buffer containing 0.5 mM-EDTA, or (c) 50 mM-glycine–NaOH buffer. The preparations were then re-dialysed for 4 h at 4°C against three 50 ml lots of 0.3 M-sucrose–piperazine buffer only. Portions of each sample were then preincubated (30°C; pH 10.4) for the period indicated, in media containing 150 mM-piperazine–HCl buffer alone (□) or piperazine–HCl buffer containing 50 mM-glycine–NaOH buffer (○), 0.5 mM-EDTA (▲) or 50 mM-glycine plus 0.5 mM-EDTA (●). After the addition of Mg^{2+} and substrate and incubation at 30°C, alkaline phosphatase activity was measured and plotted on a log scale against duration of preincubation. Similar results to these were obtained in three separate, less complete, series of experiments.

values by adding Mg^{2+} . Incubation of this preparation at 30°C with glycine alone did not result in inactivation, although incubation with glycine plus EDTA did so (Fig. 6b). This indicates that exposure to EDTA before incubation with glycine will not result in inactivation.

Dialysis against iso-osmotic sucrose containing glycine, followed by further dialysis against iso-osmotic sucrose alone to remove free amino acid, should yield an enzyme–amino acid complex. If EDTA can interact with this complex, inactivation should occur on preincubation of this preparation at 30°C with EDTA alone. Some inactivation was indeed observed (Fig. 6c), although the rate was increased if glycine was once more added during preincubation. Although the enzyme–glycine complex would tend to dissociate during the dialysis necessary to remove free amino acid, sufficient survived to demonstrate inactivation with EDTA alone, indicating that the complex must be quite stable.

Discussion

Irreversible inactivation of mouse ovarian alkaline phosphatase activity occurred on exposure of the enzyme to EDTA and amino acids. The charac-

teristics of inactivation were very similar for alkaline phosphatase I and Ib preparations, despite the marked differences in both specific activity and K_m .

The reaction sequence and mechanism of inactivation can be defined unequivocally only by further experiments with highly purified alkaline phosphatase preparations. However, some limited conclusions can be drawn from these experiments that indicate a probable reaction sequence.

The first reaction appears to be the formation of an enzyme–amino acid complex. The amounts of the complex formed depend on both the concentration and the nature of the side chain of amino acid used. Complex-formation has a high activation energy, and specificity studies indicate that both the α -amino and the carboxyl groups of the amino acid are co-ordinated to a metal ion that is tightly bound to the enzyme. The second stage of the reaction sequence involves the decay of the enzyme–amino acid complex in the presence of EDTA to give an inactive form of enzyme that can no longer be reactivated by Mg^{2+} . This may take place in one of two ways: (i) the intermediate is acted on rapidly by EDTA to give a metal ion-free form that then decays in a time-dependent manner to an inactive enzyme; or (ii) the intermediate decays in a time-dependent manner to a form that is rapidly attacked by EDTA, yielding an inactive enzyme.

The preferred reaction sequence is (ii). This results in the production of a modified form of the metal ion-enzyme that is then susceptible to attack by EDTA, giving an inactive metal ion-free enzyme. Sequence (i), however, implies the existence of an active metal ion-free enzyme, which is less readily contemplated. However, whether this reaction occurs via (i) or (ii), the decay stage is independent of the nature of the amino acid used, and has a very similar activation energy to that for the formation of the intermediate.

Hofstee (1955) concluded from studies of the dialysis of calf duodenal alkaline phosphatase against EDTA that the essential metal ion was tightly bound, and not normally removed by EDTA. The chelator did not remove the metal ion, but complexed it at the enzyme surface, giving an inactive EDTA-metal ion-enzyme complex that was rapidly reactivated by the addition of bivalent metal ions, which displaced EDTA from the enzyme. One can envisage that the metal ion is situated in a crevice at the enzyme surface ($E_1 \cdot M$) in such a way that there is sufficient room for the co-ordination of an amino acid, but insufficient room to allow EDTA to wrap around the metal ion to fully co-ordinate with, and remove, it. The co-ordination of the enzyme-bound metal ion with amino acids to give $E_1 \cdot M \cdot AA$ has a high activation energy, implying the imposition of a constraint on the conformation of the active centre. This may cause a rearrangement or disruption of existing metal ion-enzyme bonds. Dissociation of the modified amino acid-metal ion-enzyme complex ($E_2 \cdot M \cdot AA$) then occurs. This step has a similar activation energy to that of complex-formation, and is unaffected by the nature of the amino acid side chain. This gives a modified form of metal ion-enzyme ($E_2 \cdot M$) in which the metal ion may be more accessible and/or less tightly bound than in the initial structure. EDTA then acts rapidly to remove the metal ion, so completing the inactivation of the enzyme. Other schemes may be proposed, but the simple sequence outlined adequately accounts for the experimental findings. Further studies will be required to establish the exact sequence and mechanism of the reaction (particularly the nature of the

critical conformational change occurring between $E_1 \cdot M \cdot A$ and $E_2 \cdot M \cdot A$) and to establish the reversibility and the appropriate thermodynamic parameters of each intermediate reaction.

No similar effects of EDTA and amino acids have been reported, except for a brief observation by Mathies (1958), who noted that dialysis of a purified pig kidney alkaline phosphatase preparation against alanine greatly facilitated the removal of the essential metal ion (and inactivation of the enzyme) by subsequent dialysis against EDTA.

Certain features of the inactivation reaction cannot yet be satisfactorily explained. The nature of the amino acid side chain exerted a considerable influence on the formation of the enzyme-amino acid complex, although it was remote from the groups involved in co-ordination. If spatial considerations are important in the formation of the enzyme complex, then the steric bulk and charge of the side chain may then exert a marked influence. However, values of K_{AA} for different amino acids did not appear to correlate well with either of these factors.

The nature of the tightly bound metal ion of ovarian alkaline phosphatase is an enigma. Treatment with chelating agents alone will not remove the ion, whereas treatment with chelating agents plus amino acids remove the ion, but in the process the enzyme appears to be irreversibly inactivated. Thus the usual stratagem of dialysis of the enzyme preparation against chelating agents, followed by reactivation with various metal ions, cannot be pursued. However, treatment with chelating agent and amino acid will specifically remove the ion from the enzyme. It may then be possible to isolate the ion and chemically characterize it. Alternatively, the use of narrow-spectrum chelating agents may indicate the nature of the essential metal ion.

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