Periodate-oxidized AMP as a substrate, an inhibitor and an affinity label of human placental alkaline phosphatase

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Human placental alkaline phosphatase (EC 3.1.3.1) was inactivated by periodate-oxidized AMP. The inactivation showed saturation kinetics and could be partially prevented by the substrate AMP or the product inhibitor inorganic phosphate. Oxidized AMP was itself a substrate for this enzyme, with an apparent K_m of 0.67 mM. The hydrolytic products of oxidized AMP were identified as oxidized adenosine hemiacetals. Oxidized AMP was also found to be a non-competitive inhibitor with respect to *p*-nitrophenyl phosphate, with identical K_{is} and K_{ii} values of 0.15 mM. Our results indicate that oxidized AMP could combine with the enzyme to form a binary complex, followed by reaction with the proximal lysyl amino group to yield a Schiff base. The latter was reduced with NaBH_4 and identified by t.l.c. The incorporation of only 1.5 molecules of oxidized [^{14}C]AMP per enzyme subunit resulted in a complete inactivation of the enzyme. The modified enzyme showed higher apparent K_m for the substrates and higher K_i for inorganic phosphate, but lower [^{32}P]phosphate incorporation, than the native enzyme. These results support the conclusion that a lysine residue is involved in the phosphate-binding site of human placental alkaline phosphatase.

Chemical modification with group-specific reagents is widely used in the study of structure–function relationships of enzymes and other functional proteins. The major limitation of many modifying reagents is the lack of active-site specificity. To solve this problem, the concept of ‘affinity label’ was developed, and many active-site-directed reagents were introduced (for reviews see Singer, 1967; Shaw, 1970). However, most affinity labels still lack group-specificity. The exact amino acid residue modified has to be determined individually. Oxidized nucleotides have been introduced (Rippa *et al.*, 1975; Ranieri-Raggi & Raggi, 1976) as enzyme-modifying reagents and proved to be site-specific reagents with group specificity. In the present paper we report the interactions between oxidized AMP and human placental alkaline phosphatase (EC 3.1.3.1). Both site- and group-specificities were demonstrated. A preliminary report has been presented (Chang *et al.*, 1981).

Experimental

Materials

Crude human placental alkaline phosphatase, *p*-nitrophenyl phosphate, α -naphthyl phosphate, AMP, adenosine, sodium periodate, Sephadex G-10,

G-25 and G-200 gels, Fiske–SubbaRow reducer, *N*- α -acetyl-lysine and D-glyceraldehyde (Sigma Chemical Co., St. Louis, MO, U.S.A.), sodium borohydride (Wako, Osaka, Japan), DEAE-cellulose (DE-52; Whatman, Maidstone, Kent, U.K.), silica gel (DF-5; Camag, Muttens, Switzerland) and [^3H]NaBH₄, [^{32}P]PP_i and [U- ^{14}C]AMP (The Radiochemical Centre, Amersham, Bucks., U.K.) were purchased from the designated sources. Distilled and deionized water was used throughout this work.

Alkaline phosphatase was purified from the crude product (Sigma type XVII) by DEAE-cellulose and Sephadex G-200 chromatography. Crude enzyme, 500 mg in 2 ml of the equilibrium buffer (10 mM-Tris/HCl, pH 7.7), was applied to a pre-equilibrated DEAE-cellulose column (2.6 cm $\times$ 61 cm). The column was washed with the same buffer and the enzyme was eluted with a linear gradient of 0–0.25 M-NaCl in the buffer. The fractions containing alkaline phosphatase activity (at around 0.14 M-NaCl) were pooled and concentrated by a Millipore (Bedford, MA, U.S.A.) immiscible membrane. The concentrated enzyme solution was then applied to a 1.5 cm $\times$ 62 cm column of Sephadex G-200 and was eluted with 10 mM-Tris/HCl buffer, pH 7.7, containing 0.1 M-NaCl. The enzyme solution was concentrated again. The purified enzyme

thus obtained has a specific activity of 295 units/mg of protein, which is close to the reported value (Sussman *et al.*, 1968). The homogeneity of the purified enzyme was as a routine checked by polyacrylamide-gel electrophoresis.

[^{32}P]P_i was prepared from [^{32}P]PP_i by hydrolysing the latter with 2M-KOH in a boiling-water bath for 2h. After being neutralized to pH 8.0, the solution was applied to a Dowex-1 column (0.9 cm $\times$ 10 cm) and washed with water. [^{32}P]P_i was eluted by 50 mM-HCl and neutralized to pH 7.5. Under these conditions PP_i was still bound to the column and could only be eluted by 100 mM-HCl.

Enzyme assay

Enzymic activity was measured spectrophotometrically in a Gilford 250, Beckman 24 or Varian 635 spectrophotometer. The reaction mixture consisted of 50 mM-Na₂CO₃/NaHCO₃ buffer, pH 9.8, containing 10 mM-MgCl₂ and 10 mM-*p*-nitrophenyl phosphate in a total volume of 1 ml. The release of *p*-nitrophenol was monitored continuously at 410 nm. A unit of enzyme activity is defined as the amount that catalyses the hydrolysis of 1 μ mol of substrate/min under the assay conditions.

Protein determination

Protein concentration was determined spectrophotometrically at 280 nm, assuming an absorption coefficient of 1.0 for a 1 mg/ml solution (Sussman *et al.*, 1968). For the stoichiometry study, the protein was determined by the use of Coomassie Blue (Bradford, 1976). *M_r* 115 000 (Sakiyama *et al.*, 1979) was used for calculation of the enzyme concentration.

Preparation of oxidized AMP

Oxidation of AMP by NaIO₄ was performed essentially as described by Easterbrook-Smith *et al.* (1976). However, we found that further purification of the oxidized AMP by Sephadex G-10 chromatography was not necessary, because all preparations were homogeneous as shown by t.l.c. in two solvent systems (Chang *et al.*, 1981). Furthermore, in the control experiment, the reagent mixture (containing NaIO₄ and excess of ethylene glycol but without AMP) caused no inactivation of enzyme activity, indicating that all NaIO₄ has been consumed by ethylene glycol and that the latter has no effect on enzyme activity.

Oxidized adenosine and radioactive oxidized AMP were prepared from adenosine and [U- ^{14}C]-AMP (513 Ci/mmol) respectively by the same procedure.

Modification of alkaline phosphatase with oxidized AMP

The enzyme was incubated with different con-

centrations of freshly prepared oxidized AMP in 20 mM-sodium borate buffer, pH 7.5, at 37°C. Immediately after the addition of the reagent to the enzyme solution, a sample was withdrawn and assayed for the zero-time phosphatase activity. At intervals samples of the reaction mixture were removed and immediately assayed for enzymic activity.

Protection experiments were similarly performed with further addition of substrate or inhibitor before the oxidized AMP.

Identification of the hydrolytic product from oxidized AMP

The hydrolysis of oxidized AMP by alkaline phosphatase was performed as described for the regular enzyme assay except that the substrate was replaced by oxidized AMP and the phosphate released was determined by the Fiske & SubbaRow (1925) method. The reaction mixture was also subjected to t.l.c. on silica gel with ethyl acetate/acetone (3:2, v/v) (solvent system 1) or ethyl acetate/ethanol (3:2, v/v) (solvent system 2), and the nucleotide or nucleoside was located and identified with a u.v. lamp.

Identification of the amino acid residue that reacted with oxidized AMP

N- ϵ -Formylmethyl-lysine

was prepared from *N*- α -acetyl-lysine and D-glyceraldehyde as described by Dallochio *et al.* (1976). Alkaline phosphatase was allowed to react with non-radioactive oxidized AMP. After the enzyme activity had decreased to a constant value, a few crystals of [^3H]NaBH₄ (9.5 Ci/mmol) were added to reduce the protein. The solution was then dialysed exhaustively against water and hydrolysed with 6M-HCl in an evacuated and sealed ampoule at 110°C for 24 h. The hydrolysate was then subjected to t.l.c. with propan-1-ol/25% (v/v) conc. aq. NH₃ (67:33, v/v). After the solvent front reached the top, the plate was dried and marked every 1 cm. Each section was removed carefully with a micro-spatula, suspended in toluene-based scintillation solution and counted for radioactivity in a Packard 3320 liquid-scintillation spectrometer.

Stoichiometry of the inactivation reaction

Alkaline phosphatase was incubated with oxidized [^{14}C]AMP (1.9–11.6 mM; 100 c.p.m./nmol) in 20 mM-sodium borate buffer, pH 7.5, at 24°C. Samples were withdrawn and assayed for enzyme activity at zero time and after 15 h incubation. The remaining portion was then reduced with NaBH₄ and dialysed against 50 mM-Tris/HCl buffer, pH 7.5, until no radioactivity was detectable in the diffusate.

A control sample was treated with [^{14}C]AMP simultaneously. The radioactivity incorporated in the sample minus the non-specific absorption from [^{14}C]AMP was determined and expressed as molecules of oxidized [^{14}C]AMP incorporated per enzyme subunit.

[^{32}P]Phosphate-incorporation studies

The native and modified alkaline phosphatases were labelled with [^{32}P]P_i (98×10^6 c.p.m./mmol) at pH 5.0 at 0°C for 10 min as described by Milstein (1964). After exhaustive dialysis against water, the covalently bound [^{32}P]P_i was determined.

Results

Oxidized AMP as a substrate for human placental alkaline phosphatase

Oxidized AMP, an analogue of the substrate AMP with the aldehyde groups at the 2'- and 3'-positions of the ribose moiety (cf. Scheme 1), was hydrolysed by alkaline phosphatase. The release of P_i was determined by the Fiske & SubbaRow (1925) method. The reaction was linear with time and enzyme concentration. The reaction was activated by Mg²⁺ and inhibited by high concentrations of EDTA. The apparent K_m for oxidized AMP was 0.67 mM, which was much lower than that for AMP (3 mM). The other hydrolytic product was identified as oxidized adenosine hemiacetals by t.l.c. in two solvent systems. Oxidized AMP shows only one spot in both solvent systems. The R_F values were 0.31 and 0.64 respectively in solvent systems 1 and 2.

The hydrolysed mixture and the oxidized adenosine, on the other hand, gave two spots with identical R_F values (0.15 and 0.26 in solvent 1; 0.5 and 0.58 in solvent 2) in both chromatograms. The two spots probably represent the two isomers of oxidized adenosine hemiacetal. As shown in Scheme 1, stable six-membered intramolecular hemiacetals resulting from the reaction between the 5'-OH and 2'-CHO groups were assumed. During the hemiacetal formation, a new chiral centre (2') was created, and thus we have α - and β -isomers corresponding to the two spots.

Oxidized AMP as an inhibitor for human placental alkaline phosphatase

The hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase was inhibited by oxidized AMP. The inhibition showed a classical non-competitive pattern (Fig. 1) with identical inhibition constants for slope and intercept (K_{is} and K_{ii}) of 0.15 mM (Fig. 1 inset). The study of inhibitory action of AMP hydrolysis by oxidized AMP or the oxidized AMP hydrolysis by *p*-nitrophenyl phosphate were precluded by the fact that both are substrates, and the P_i released from the inhibitor will interfere with the analysis of the P_i from the substrate.

Inactivation of the alkaline phosphatase by oxidized AMP

In the absence of Mg²⁺ and substrate, oxidized AMP inactivated the alkaline phosphatase rapidly. The dependence of the loss of enzymic activity on oxidized AMP concentration showed saturation

Scheme 1. *Proposed mechanism for the formation of oxidized adenosine hemiacetals*
Abbreviations: oAMP, oxidized AMP; oAdenosine, oxidized adenosine.

Fig. 1. Inhibition of *p*-nitrophenyl phosphate hydrolysis by oxidized AMP

The assays were performed as described in the Experimental section, except that concentrations of *p*-nitrophenyl phosphate and oxidized AMP (■, 0 mM; □, 0.27 mM; ●, 0.54 mM; ○, 0.81 mM) were varied as indicated. The slope (●) and intercept (○) re-plots are linear, as shown in the inset. The amount of enzyme used in each assay was 4.5 μ g.

kinetics. A plot of inactivation half-time ($t_{0.5}$) against the reciprocal of oxidized AMP concentration was linear (Fig. 2). The substantial ordinate intercept indicates the formation of an enzyme–oxidized AMP complex before the enzyme inactivation. The limiting $t_{0.5}$ value and the dissociation constant of the presumed enzyme–oxidized AMP binary complex were calculated to be 55 min and 6.8 mM respectively.

Protection by substrate and inhibitor against the enzyme inactivation

Fig. 3 shows the effects of AMP and P_i on the inactivation of the alkaline phosphatase by oxidized AMP. Protection was afforded by substrate AMP or P_i . The incomplete protection was probably due to the higher affinity of the enzyme with oxidized AMP, which has lower K_m and K_i values (Table 1). Other substrates gave similar results. Higher concentrations of P_i or substrates did not give further protection. These results suggest the binding of the inactivator at or near the active site.

Identification of the modified amino acid residue

The specificity of the reaction was demonstrated in our case. The essential amino acid residue modified by oxidized AMP was identified as a lysine by t.l.c. of an acid hydrolysate of the oxidized-AMP-modified and $[^3H]NaBH_4$ -reduced enzyme. A chromatogram developed in the propan-1-ol/25% NH_3 solvent system showed that the modified enzyme gave a single radioactive peak with R_F value

Fig. 2. Effect of oxidized AMP concentration on the rate of inactivation

Alkaline phosphatase (26 μ g) was incubated with different concentrations of oxidized AMP at 37°C and pH 7.5. For experimental details see the text. The inactivation half-time ($t_{0.5}$) was plotted against reciprocal of oxidized AMP concentration.

(0.75) identical with that of the *N*- ϵ -formylmethyl-lysine standard. This result indicates that the aldehyde groups of oxidized AMP can form a reducible Schiff base with a lysine residue at or near the active site of this enzyme.

Stoichiometry of the inactivation

The stoichiometry was studied by incubating the alkaline phosphatase, a dimeric protein composed of subunits of equal molecular weight (Gottlieb & Sussman, 1968), with oxidized [^{14}C]AMP and then reducing the product with NaBH_4 . The incorporation of oxidized [^{14}C]AMP increased linearly as the enzyme activity gradually decreased (results not shown). Extrapolation to complete inactivation

showed incorporation of 1.5 molecules of oxidized AMP/enzyme subunit. However, it should be pointed out that there was substantial non-specific incorporation observed in the control experiment. Under the same conditions, the incorporation from [^{14}C]AMP was about 10–20% of that from oxidized [^{14}C]AMP. Both the enzyme activity and the incorporation of oxidized [^{14}C]AMP were partially protected by *p*-nitrophenyl phosphate and Mg^{2+} . For example, in one experiment, an unprotected enzyme incorporated 0.85 molecule of oxidized [^{14}C]AMP/subunit after 67% inactivation, whereas under the same conditions the protected enzyme incorporated 0.57 molecule/subunit with 48% inactivation.

Kinetic properties of the modified enzyme

We have previously shown that oxidized-AMP-modified enzyme has an increased electrophoretic mobility and a higher K_m value for substrates (Chang *et al.*, 1981). The $V_{\max}$ was decreased, however (Table 1). P_i is a competitive product inhibitor for this enzyme. Its inhibition constant (K_i) was found to be increased from 0.35 to 2.1 mM after 82% inactivation. These results suggested that the binding affinities between the enzyme and the substrate or inhibitor were decreased after modification. The kinetic parameters of the native and modified enzyme are summarized in Table 1.

It was found that alkaline phosphatase could be specifically labelled at a serine residue with [^{32}P]P_i (Engström, 1961a,b). We have checked this property of the enzyme after modification. The native enzyme incorporated 0.73 molecule of [^{32}P]P_i/enzyme molecule, whereas the incorporation of [^{32}P]P_i into the modified enzyme was only about 19% of that in the native enzyme.

Fig. 3. Protection of the alkaline phosphatase against inactivation by oxidized AMP

The reaction mixture contained $0.17\ \mu\text{M}$ -alkaline phosphatase in 27 mM-borate buffer, pH 7.5, and 3 mM-oxidized AMP, with the following additions: ○, none; ●, 5.3 mM-AMP; □, 21.2 mM-AMP; ■, 26.7 mM-potassium phosphate buffer, pH 7.5.

Table 1. Kinetic parameters of the native and modified human placental alkaline phosphatase
For experimental details see the text.

Substrate or inhibitor	Native enzyme			Modified enzyme		
	K_m (mM)	K_i (mM)	Relative $V_{\max}$ (%)	K_m (mM)	K_i (mM)	Relative $V_{\max}$ (%)
<i>p</i> -Nitrophenyl phosphate	1.0		100	1.6 2.5		4.3 2.2
α -Naphthyl phosphate	1.3		45	1.7 2.4 3.3 5.6		14.2 6.5 2.7 1.1
AMP	3.0		52			
Oxidized AMP	0.67	0.15	52			
L-Phenylalanine		1.5			1.8 ^(a)	
P_i		0.35			1.5 ^(b) 2.1 ^(c)	

* Residual activity was: (a) 21%; (b) 29%; (c) 18%.

Discussion

Results obtained in the present study indicate that oxidized AMP is a unique reagent for human placental alkaline phosphatase. It is a substrate and an inhibitor, as well as an affinity label. Oxidized AMP can form a binary complex with the enzyme. Then the bound reagent may be hydrolysed to give P_i and oxidized adenosine. The latter further cyclizes to give oxidized adenosine hemiacetals. Alternatively the reactive aldehyde groups of the bound reagent may react with the amino group of the lysyl side chain to give a Schiff base and result in enzyme inactivation. The reaction products of both routes have been identified. Clues for these two routes came from the inactivation experiments. It was found that the kinetics of the inactivation were not consistent with any simple scheme. The inactivation proceeded rapidly in the beginning, until about 50% activity was lost. The inactivation rate then slowed down, but further inactivation could be observed when fresh oxidized AMP was added into the reaction mixture. We also observed that P_i was released during the inactivation process, indicating the hydrolysis of oxidized AMP. Mg^{2+} may be one of the factors that influence the bound reagent to function as a substrate, or as an inactivator. In the presence of Mg^{2+} , the hydrolysis of oxidized AMP may be accelerated.

When oxidized AMP was added to the assay mixture containing the enzyme, Mg^{2+} and the substrate *p*-nitrophenyl phosphate, the hydrolysis of the substrate was inhibited. However, the non-competitive inhibition pattern of oxidized AMP versus *p*-nitrophenyl phosphate was unexpected. Although unlikely, one explanation for this result is that oxidized AMP occupies the phosphorylation site, which is distinct from the substrate site. However, the wide specificity of alkaline phosphatase casts doubt on the existence of a 'fussy' substrate site. Even if this were true, one would expect to find some competition between oxidized AMP and *p*-nitrophenyl phosphate for the active site, bearing in mind the relative K_m values. Inhibition patterns would then appear 'mixed' competitive and non-competitive. A more attractive explanation for the non-competitive behaviour is that oxidized AMP caused a rapid irreversible inactivation of the enzyme, so lowering the V_{max} . The incomplete protection by substrate suggests that this possibility should be taken into serious consideration. The fact that incorporation of oxidized [^{14}C]-AMP occurs in the presence of Mg^{2+} and *p*-nitrophenyl phosphate supports the above suggestion.

Oxidized nucleotides have been shown to be rather successful affinity labels for those enzymes with nucleotides as their substrates (Easterbrook-Smith *et al.*, 1976; Fayat *et al.*, 1978; Bellini *et al.*,

1979; Chang & Huang, 1979). In three cases, the amino acid residue that reacted was identified to be the lysine residue (Easterbrook-Smith *et al.*, 1976; Dallochio *et al.*, 1976; Bellini *et al.*, 1979). We have also demonstrated in the present study that lysine was the only amino acid residue that reacted with oxidized AMP. Several lines of evidence also suggest that oxidized AMP is an affinity label of this enzyme: (a) inactivation by oxidized AMP exhibits saturation kinetic behaviour; (b) it is a substrate for this enzyme and a non-competitive inhibitor with respect to *p*-nitrophenyl phosphate; (c) the inactivation is partially protected against by substrate or P_i ; (d) incorporation of only 1.5 molecules of the reagent/enzyme subunit resulted in a complete inactivation of the enzyme. The large incorporation of [^{14}C]AMP would tend to make the incorporation result unreliable. The nature of this incorporation of AMP is unclear at this moment. However, the small number of incorporated oxidized [^{14}C]AMP molecules/subunit together with the group-specificity led us to conclude that oxidized AMP is an excellent modifying reagent for lysine residues. It has the advantage of both group- and site-specificities. The availability of this reagent provides a good tool for exploration of the geometry of the active centre of this enzyme.

Lysine residues have been suggested to be involved in the substrate-binding of human placental alkaline phosphatase (for reviews see Fishman & Ghosh, 1967; Fishman, 1974). Our kinetic and phosphate incorporation results also indicate the same conclusion. The binding affinity between the modified enzyme and substrate is probably decreased, as reflected by the higher K_m value. The fact that the dissociation constant of the enzyme- P_i complex (K_{is}) was increased 6-fold for the modified enzyme with 18% residual activity strongly suggests the interaction between the positively charged ϵ -amino group of the lysine residue and the negatively charged phosphate group of the substrate. Direct evidence was provided by the phosphorylation experiments. The incorporation of [^{32}P]- P_i into the enzyme molecule was much less after modification. The positively charged amino group of the lysine residue has been replaced by a negatively charged phosphate group in the bound oxidized AMP molecule. Charge repulsion was assumed to be the cause of the lowered affinity between substrate and the modified enzyme. However, since oxidized AMP is a bulky molecule, steric effects could not be completely ruled out.

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