Modification of human placental alkaline phosphatase by periodate-oxidized 1,*N*⁶-ethenoadenosine monophosphate

Gu-Gang CHANG,*§ Ming-Shi SHIAO,† Kuan-Rong LEE‡ and Joe-Jen WU*

*Department of Biochemistry, National Defense Medical Center, P.O. Box 90048, Taipei, Taiwan,

†Department of Medical Research, Veterans General Hospital, Taipei, Taiwan, and ‡Institute of Life Science, National Tsing-Hua University, Hsinchu, Taiwan, Republic of China

Oxidation of 1,*N*⁶-ethenoadenosine monophosphate (ϵ AMP) with periodate cleaved the *cis*-diol of the ribose ring and resulted in the formation of a dialdehyde derivative (ϵ AMP-dial). At room temperature ϵ AMP-dial was unstable and underwent β -elimination to give 4',5'-anhydro-1,*N*⁶-ethenoadenosine dialdehyde acetal (A ϵ Ado-dial). These nucleotide analogues were found to inactivate human placental alkaline phosphatase in a time- and concentration-dependent manner. ϵ AMP-dial was shown to be an affinity label for the enzyme on the basis of the following criteria. (a) Kinetics of the enzyme activity loss over a wide range of ϵ AMP-dial concentration showed a saturating phenomenon. Removal of the phosphate group made the reagent (A ϵ Ado-dial) become a general chemical modifying reagent. (b) The artificial substrate *p*-nitrophenyl phosphate gave substantial protection of the enzyme against inactivation. (c) ϵ AMP-dial was a substrate and a partial mixed-type inhibitor for the enzyme. Results of the inhibition and protection studies indicated that the reagent and substrate could combine with the enzyme simultaneously. Besides the phosphate-binding domain, an induced hydrophobic region is proposed for the substrate-binding site for human placental alkaline phosphatase.

INTRODUCTION

Alkaline phosphatase [orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] catalyses the hydrolysis of phosphoester linkage of a broad range of substrates. Human alkaline phosphatase isoenzymes may be classified into four types according to their tissue-specificity: (a) the tissue-non-specific liver/bone/kidney type, (b) the intestinal type, (c) the term-placental type and (d) the placental-like type. Some of the tumour-associated enzymes are attributed to the placental-like alkaline phosphatase. They are structurally related to the term-placental alkaline phosphatase. For this reason the placental isoenzyme has attracted much interest (for reviews see refs. [1] and [2]).

Alkaline phosphatase is mainly present in plasma membranes, where extensive active transport takes place. Placental alkaline phosphatase is anchored to the plasma membrane of cells by a phosphatidylinositol-glycan anchor [3]. The enzyme may be involved in phosphate transport.

Oxidized nucleotides and nucleosides have been used extensively as affinity labels for various enzymes (for reviews see refs. [4] and [5]) and were found to have potent anti-tumour activity ([6–8] and references cited therein). Periodate-oxidized adenosine 5'-[β - γ -imido]triphosphate has been reported to be a potential affinity label for the rat intestinal alkaline phosphatase [9]. We found periodate-oxidized AMP to be a good affinity label for human placental alkaline phosphatase [10,11]. In the present study reactions between the enzyme and periodate-oxidized 1,*N*⁶-ethenoadenosine monophosphate (ϵ AMP), i.e. ϵ AMP-dial, or its dephospho derivative 4',5'-anhydro-1,*N*⁶-ethenoadenosine dialdehyde acetal (A ϵ Ado-dial) were characterized. ϵ AMP-dial may be a convenient fluorescent affinity label for this enzyme.

MATERIALS AND METHODS

Materials

Human placental alkaline phosphatase (type XVII), AMP, ϵ AMP, ϵ -adenosine, *p*-nitrophenyl phosphate and Bistris (Sigma Chemical Co., St. Louis, MO, U.S.A.) and NaBH₄, NaIO₄, sodium acetate, silica gel 60 F₂₅₄ t.l.c. plates, propan-2-ol, ³H₂O and dimethyl sulphoxide (E. Merck, Darmstadt, Germany) were purchased from the sources indicated. Distilled water, further purified with a Millipore Milli-Q system, was used throughout.

Purification and assay of human placental alkaline phosphatase

Partially purified human placental alkaline phosphatase from Sigma Chemical Co. was further purified to homogeneity by DE-52 DEAE-cellulose and concanavalin A-Sepharose chromatographies [12]. Protein concentration was determined spectrophotometrically at 279 nm, by using an absorption coefficient of 1.0 for a 0.1% (w/v) protein solution [13].

Alkaline phosphatase activity was assayed in a Gilford 2600 spectrophotometer by continuously monitoring the hydrolysis of *p*-nitrophenyl phosphate at 410 nm. 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. If a nucleotide was used as substrate, the hydrolysis of substrate was measured by monitoring the phosphate release, which was measured by the absorbance change at 350 nm after mixing the reaction mixture with zinc acetate and ammonium molybdate [14,15].

Preparation of periodate-oxidized nucleotides and its reduced compounds

ϵ AMP-dial was prepared essentially as described for AMP-dial [10] with AMP replaced by ϵ AMP. Since iodide was not

Abbreviations used: ϵ AMP, 1,*N*⁶-ethenoadenosine monophosphate; ϵ Ado, 1,*N*⁶-ethenoadenosine; ϵ AMP-dial, ϵ AMP-dialdehyde (periodate-oxidized ϵ AMP); ϵ Ado-dial, ϵ Ado-dialdehyde; A ϵ Ado-dial, 4',5'-anhydro-1,*N*⁶-ethenoadenosine-2',3'-dialdehyde acetal; ϵ AMP-diol, ϵ AMP-2',3'-acyclic dialcohol; A ϵ Ado-diol, 4',5'-anhydro-1,*N*⁶-ethenoadenosine-2',3'-acyclic dialcohol.

§ To whom correspondence should be addressed.

Fig. 1. Elution profiles of ϵ AMP-dial and AeAdo-dial on a Mono-Q f.p.l.c. column

The nucleotides were separated on a Mono-Q column (5 mm $\times$ 50 mm). Solvent A was 20 mM-Bistris buffer, pH 6.4. Solvent B was 0.7 M-sodium acetate in solvent A. The nucleotides were eluted with linear gradient of 0–100% (v/v) solvent B in solvent A at a flow rate of 1 ml/min. (a) Freshly prepared ϵ AMP-dial. (b) AeAdo-dial prepared by incubation of fresh ϵ AMP-dial at 30 °C for 6 h. The peak marked with asterisk (*) was an unknown impurity in ethylene glycol used in the preparation of the reagent.

completely separated from the nucleotides by the Sephadex G-10 column, the Sephadex G-10 step was omitted and a Pharmacia Mono-Q f.p.l.c. column was used in the final purification step (the elution conditions are given in Fig. 1 legend). The completeness of the oxidation was confirmed by t.l.c. and the f.p.l.c. system. All ϵ AMP was converted into ϵ AMP-dial after periodate treatment. ϵ AMP-dial was used immediately after preparation.

AeAdo-dial was prepared by incubating an aqueous solution of ϵ AMP-dial at 30 °C for 6 h. ϵ AMP-dial was completely converted into AeAdo-dial under these conditions. ϵ AMP-diol and AeAdo-diol were prepared by reducing the corresponding nucleotide dialdehyde with NaBH_4 [16]. Purification of AeAdo-dial, ϵ AMP-diol and AeAdo-diol was also performed by use of the Mono Q f.p.l.c. column.

Spectroscopic analysis of the modified nucleotides

U.v.-visible-absorption spectra were recorded on a Cary 2290 spectrophotometer from 200 to 700 nm. Emission and excitation

fluorescence spectra of the nucleotides were scanned with a Farrand System 3 spectrofluorimeter. ^1H - and ^{13}C -n.m.r. spectra were obtained with Bruker AM 400 and ACP 300 n.m.r. spectrometers at ambient temperature as described previously [17]. N.m.r. spectra of samples in $^2\text{H}_2\text{O}$ (99.8% deuterated) were reported as p.p.m. downfield from tetramethylsilane ($\delta = 0$) with dimethyl sulphoxide ($\delta = 39.7$) as reference. The water suppression technique was used.

Inactivation of alkaline phosphatase by ϵ AMP-dial or AeAdo-dial

Inactivation of the enzyme by ϵ AMP-dial or AeAdo-dial was studied by continuously monitoring the substrate hydrolysis in the presence of a modifying reagent (for review see ref. [18]). ϵ AMP-dial was unstable at room temperature. At 30 °C a β -elimination product appeared within 30 min with $t_{1/2}$ 4 h. At 4 °C the $t_{1/2}$ was 12 days. Inactivation studies were performed at 8 °C by circulating coolant to the cuvette holder. At this temperature ϵ AMP-dial was stable in the reaction mixture for at least 4 h. The reaction progress was monitored with a Gilford programmed printer, which recorded reaction rate as well as absorbance at programmed time intervals.

The substrate consumed in all experiments was less than 2%, and thus no product inhibition was considered. Inactivation of the enzyme by oxidized nucleotides was analysed by plotting $-\ln(v_t/v_c)$ versus time in accordance with Pierce *et al.* [19], where v_t and v_c represented the reaction rates at time t with or without oxidized nucleotide added respectively. The slope of the resulting straight line represented the observed inactivation rate constant (k_{obs}).

Protection experiments

The reaction progress curve was analysed as described above, unless inactivation was performed at several reagent and substrate concentrations. The results were analysed by the double-reciprocal plot of $1/k_{\text{obs}}$ versus $1/[\epsilon\text{AMP-dial}]$ at several p -nitrophenyl phosphate concentrations.

Inhibition studies

Inhibition of the enzyme by oxidized nucleotide was studied by measuring the initial reaction rate at several inhibitor concentrations. The p -nitrophenyl phosphate concentrations ranged from 0.2 to 1.67 mM. The double-reciprocal plot was used in examining the inhibition pattern.

Data analysis

Data from kinetic studies were analysed with a non-linear-regression program (DNRP-53) [20]. Fitting of the experimental data to various kinetic models was based on the relative fit error and errors in the constants.

Covalent labelling studies

The traditional chemical-modification technique was used. Modification was performed by addition of ϵ AMP-dial to an enzyme solution in borate buffer at pH 7.0 and 25 °C. The progress of enzyme inactivation was monitored by assaying the enzyme activity on small samples withdrawn at designated time intervals. At the same time other samples were withdrawn for determination of labelling. The product of labelling was stabilized by NaBH_4 reduction. The free ϵ AMP-dial was separated from the protein molecules by using a Millipore membrane filter. The protein retained on the membrane was eluted with the minimum amount of borate buffer. The protein concentration and fluorescence of the labelled protein solution were determined. Freshly prepared ϵ AMP-dial solution was used as a calibration standard, assuming no quench of the nucleotide fluorescence after covalent bonding.

Table 1. Separation of various nucleotides and nucleosides by t.l.c. and f.p.l.c.

The solvent system used for t.l.c. was propan-2-ol/water (1:1, v/v).
For f.p.l.c. elution conditions see Fig. 1 legend.

Nucleotide or nucleoside	T.l.c. R_F value	F.p.l.c. retention time (min)
ϵ AMP	0	11.0
ϵ Adenosine	0.33	1.2
ϵ AMP-dial	0	11.8
ϵ Ado-dial	0.64	3.0
A ϵ Ado-dial	0.7	1.8

RESULTS

Characterization of ϵ AMP-dial, A ϵ Ado-dial, ϵ AMP-diol and A ϵ Ado-diol

The purity of ϵ AMP used in the periodate oxidation was checked by its characteristic ^1H -n.m.r. spectrum in $^2\text{H}_2\text{O}$ ($^1\text{H}^2\text{HO}$ proton signal was set at 5.00 p.p.m.). Two sets of doublet (δ 7.71 p.p.m., d, $J = 5$ Hz, and δ 8.11 p.p.m., d, $J = 5$ Hz) were assigned to H-11 and H-10 respectively. Signals corresponding to H-8 (δ 8.83 p.p.m., 1H) and H-2 (δ 9.24 p.p.m., 1H) were also observed [21]. Oxidation of ϵ AMP with periodate resulted in the formation of ϵ AMP-dial, with R_F value similar to that of the original compound on the t.l.c. plate and similar retention time in f.p.l.c. (Table 1). ϵ AMP-dial was unstable in aqueous solution. When kept at 30 °C for 6 h it was almost completely converted into a compound (A ϵ Ado-dial) that was not absorbed on the anion-exchanger Mono-Q column (Fig. 1). The periodate-treated preparation, which contained ϵ AMP-dial, gave complicated ^1H -n.m.r. spectra. However, the spectra clearly indicated the presence of ϵ AMP-dial and showed that both aldehyde groups were hydrated. Through careful examination of the splitting patterns and signal intensities given by ϵ AMP-dial and comparison with those of periodate-oxidized ATP preparation previously reported by Lowe & Beechey [16], we concluded that both the acyclic form and three cyclic forms of the hydrated ϵ AMP-dial were present in $^2\text{H}_2\text{O}$. The 2',3'-diaxial cyclic isomer of the four possible cyclic forms of ϵ AMP-dial was not detectable by ^1H -n.m.r. spectroscopy in $^2\text{H}_2\text{O}$ solution, presumably because of it being stereochemically less stable. The elimination product of ϵ AMP-dial, namely A ϵ Ado-dial, still gave an intact 1, N^6 -ethenoadenosine ring skeleton, as shown by its ^1H -n.m.r. signals at δ 7.75 (1H, s, H-11), 8.16 (1H, s, H-10), 8.62 (1H, bs, H-8) and 9.31 (1H, s, H-2). Its H-1' proton appeared at δ 6.60 p.p.m. (1H, d, $J = 4$ Hz) and both the hydrated aldehyde and two olefinic protons (H-5') were resonated at δ 5.5–5.9 p.p.m. ϵ AMP-dial was further characterized by reduction with NaBH_4 to give the corresponding 2',3'-acyclic dialcohol (ϵ AMP-diol), which was more stable for spectroscopic measurement. ϵ AMP-diol gave almost identical olefinic proton signals (δ 7.78, H-11; 8.18, H-10; 8.73, H-8; 9.20 p.p.m., H-2) and also showed a characteristic H-1' signal and splitting pattern (δ 6.41 p.p.m., 1H, t, $J = 5.4$ Hz). The remaining H-2', H-3', H-4' and H-5' protons resonated at δ 3.85–4.40 p.p.m. The proton-noise-decoupled ^{13}C -n.m.r. spectrum and DEPT (distortionless enhancement by polarization transfer) experiment (135° pulse) were also recorded for ϵ AMP-diol. The C-2' and C-3' carbon atoms resonated at δ 62.6 and 59.9 p.p.m. respectively, with both signals appearing as CH_2 type in the DEPT experiment. Seven olefinic carbon atoms resonated at δ 113–141 p.p.m. with varied signal intensities were due to the presence of three quaternary carbon atoms (C-4, C-5 and C-6).

Fig. 2. Hydrolysis of ϵ AMP-dial by human placental alkaline phosphatase

(a) Hydrolysis of ϵ AMP-dial (●) was measured by monitoring the released phosphate in carbonate buffer, pH 9.8. From top to bottom, the ϵ AMP-dial concentrations were 1.7, 0.85, 0.51 and 0.34 mM. A ϵ Ado-dial (■) was tested at 1.7 mM under the same conditions. (b) Double-reciprocal plot of $1/[\epsilon\text{AMP-dial}]$ versus $1/v$ from (a).

Through extensive chromatographic purification we found that this ϵ AMP-diol preparation was completely devoid of A ϵ Ado-diol. The latter compound showed olefinic proton signals in the ^1H -n.m.r. spectrum at δ 5.0–6.3 p.p.m. A ϵ Ado-diol could also be recovered through the above-mentioned purification procedure.

The above ^1H - and ^{13}C -n.m.r. data suggested that β -elimination of ϵ AMP-dial resulted in the formation of A ϵ Ado-dial. Direct evidence for the dephosphorylation of ϵ AMP-dial is shown in Fig. 2. Incubation of ϵ AMP-dial with alkaline phosphatase resulted in a time-dependent release of phosphate. Thus ϵ AMP-dial was shown to be a substrate of human placental alkaline phosphatase with K_m 2.87 mM and V_{max} 0.1 $\Delta A_{350}/\text{min}$. Under identical conditions no phosphate was released from A ϵ Ado-dial (Fig. 2a).

On the basis of our n.m.r. data and several other characterized oxidized nucleotides [22,23], we conclude that ϵ AMP-dial also underwent β -elimination instead of hydrolysis as the major breakdown pathway. The chemical changes of various nucleotides are summarized in Scheme 1.

Inhibition of human placental alkaline phosphatase by ϵ AMP-dial and A ϵ Ado-dial

ϵ AMP-dial was found to inhibit the hydrolysis of p -nitrophenyl phosphate catalysed by alkaline phosphatase. Double-reciprocal plots of $1/v$ versus $1/[p\text{-nitrophenyl phosphate}]$ at several ϵ AMP-dial concentrations gave a linear intercepting pattern with all lines converged at a point in the second quadrant (Fig. 3a). However, the slope and vertical intercept replots were non-linear and concave-down curves. The slope replot is shown in Fig. 3(b).

Scheme 1. Proposed reaction products derived from ϵ -AMP and NaIO_4

Key: (I), ϵ -AMP; (II), ϵ -AMP-dial; (III), monohydrate form of (II); (IV), dihydrate form of (II); (V), A ϵ Ado-dial; (VI), ϵ -AMP-diol; (VII), A ϵ Ado-diol.

This result indicated that inhibition of the enzyme by ϵ AMP-dial was consistent with a partial mixed-type inhibition, i.e. substrate and inhibitor can combine with the enzyme simultaneously to form a productive ESI ternary complex with a lower reaction rate (k') as shown in Scheme 2. The rate equation derived from this mechanism is given in eqn. (1) [24]:

$$v = \{V_{\max} \cdot (1 + k'[I]/kK'_i)/(1 + [I]/K'_i)\} / \{1 + K_s/[S] \cdot (1 + [I]/K_i)/(1 + [I]/K'_i)\} \quad (1)$$

where K_s , K_i and K'_i are the dissociation constants for the ES and EI complexes and ESI (to ES and I) respectively. The K_s , K_i and K'_i values calculated by the non-linear-regression analysis fitting to eqn. (1) were 4.3 ± 0.08 , 0.34 ± 0.09 and 0.24 ± 0.09 mM respectively. The ratio of the inhibited and non-inhibited reaction rates (k'/k) was 0.3 ± 0.1 . The K'_s value was calculated to be 3.04 mM from the definition of equilibrium constants that $K_i/K'_i = K_s/K'_s$ [24].

Fig. 3. Inhibition of human placental alkaline phosphatase by ϵ AMP-dial

Hydrolysis of *p*-nitrophenyl phosphate catalysed by alkaline phosphatase was assayed in carbonate buffer, pH 9.8, in the absence and presence of various concentrations of ϵ AMP-dial. (a) Double-reciprocal plot. From top to bottom, the ϵ AMP-dial concentrations were 0.68, 0.476, 0.34, 0.136, 0.068 and 0 mM. (b) Slope replot versus $[\epsilon\text{AMP-dial}]$ from (a).

Scheme 2.

In a similar experiment $\text{A}\epsilon\text{Ado-dial}$ was also found to be a partial mixed-type inhibitor for the enzyme. The K_s , K'_s , K_i , K'_i and k'/k values were found to be 2.1 ± 0.6 mM, 2.94 mM, 0.05 ± 0.007 mM, 0.07 ± 0.02 mM and 0.25 ± 0.09 respectively. Similar K_s values were obtained in different experiments within experimental errors.

Inactivation of human placental alkaline phosphatase by various oxidized nucleotides

The progress of *p*-nitrophenyl phosphate hydrolysis catalysed by alkaline phosphatase was continuously monitored in the presence of various nucleotides. In the absence of modifying agent, the absorbance change at 410 nm was linear with time. Addition of ϵ AMP-dial into the reaction mixture caused the plot of absorbance change versus time to become curved. Under identical conditions, $\epsilon\text{Ado-dial}$ was less active, whereas $\text{A}\epsilon\text{Ado-dial}$ was more active than ϵ AMP-dial. NaBH_4 -reduced dialdehydes (ϵ AMP-diol and $\text{A}\epsilon\text{Ado-diol}$) gave linear plots of absorbance change versus time.

The inactivation of enzyme activity by various dialdehydes was not reversible. The downward curvature of the progress curve was obtained whether the substrate was added before or after the reagent (for review see ref. [25]).

Concentration-dependence of the enzyme inactivation by ϵ AMP-dial and $\text{A}\epsilon\text{Ado-dial}$

The inactivation of alkaline phosphatase by ϵ AMP-dial and $\text{A}\epsilon\text{Ado-dial}$ was concentration-dependent. When the observed

Fig. 4. Saturation kinetics of the inactivation of human placental alkaline phosphatase by ϵ AMP-dial

The progress of substrate hydrolysis was monitored in the presence of various concentrations of ϵ AMP-dial. The observed inactivation rate constant (k_{obs}) was calculated as described in the Materials and methods section. (a) Plot of k_{obs} versus $[\epsilon\text{AMP-dial}]$. (b) Double-reciprocal plot of (a).

Scheme 3.

inactivation rate constants were plotted against ϵ AMP-dial concentrations, a hyperbolic saturation curve was obtained (Fig. 4a). This result was consistent with the mechanism shown in Scheme 3, indicating the formation of an enzyme–reagent binary complex before inactivation. The observed inactivation rate constant ($k_{\text{obs.}}$) according to this scheme is:

$$k_{\text{obs.}} = k_{\max.} [I] / ([I] + K_I) \quad (2)$$

where $k_{\max.}$ is the maximum inactivation rate constant at saturating reagent concentration, K_I is the reagent concentration when $k_{\text{obs.}}$ equals half $k_{\max.}$, and $[I]$ is the ϵ AMP-dial concentration. From the intercepts of the plot of $1/k_{\text{obs.}}$ versus $1/[\epsilon\text{AMP-dial}]$ (Fig. 4b) the $k_{\max.}$ and K_I were calculated to be 0.67 min^{-1} and 1.33 mM respectively.

On the other hand, a linear relationship between the enzyme inactivation rate constants and concentrations of AeAdo-dial up to 2 mM was obtained (results not shown). The double-logarithmic plot of the data was also linear with a slope of 1.2. Removal of a phosphate group thus made the reagent become a general chemical modifying reagent.

Scheme 4.

Substrate protection of the enzymic activity from the inactivation by oxidized nucleotides

The inactivation of alkaline phosphatase by oxidized nucleotides was retarded in the presence of substrate. The inactivation rates by various concentrations of ϵ AMP-dial were measured at different substrate concentrations. The results are shown in Fig. 5. The different lines intercepted at a point in the second quadrant, similarly to a mixed-type inhibition pattern. The data can be treated in the manner shown in Scheme 4, where EI is the enzyme–reagent binary complex, $E' - I$ represents the covalently modified enzyme molecule and ESI is the enzyme–substrate–modifier ternary complex, which will not give covalent modified enzyme owing to protection by the substrate.

The steady-state treatment of this mechanism is very complicated. Brocklehurst [26] indicated that an equilibrium as-

Fig. 5. Protection by substrate of human placental alkaline phosphatase from the inactivation by ϵ AMP-dial

The progress of substrate hydrolysis was monitored in the presence of various concentrations of p -nitrophenyl phosphate and ϵ AMP-dial. (a) Double-reciprocal plot of $1/k_{\text{obs.}}$ versus $1/[\epsilon\text{AMP-dial}]$. From top to bottom, the p -nitrophenyl phosphate concentrations were 3.4, 2.38, 1.36, 1.02 and 0.68 mM. (b) Slope (●) and vertical intercept (■) replots of (a).

Fig. 6. Protection by substrate of human placental alkaline phosphatase from the inactivation by AeAdo-dial

The progress of substrate hydrolysis was monitored in the presence of various concentrations of p -nitrophenyl phosphate and AeAdo-dial. (a) Semi-logarithmic plot to obtain the $k_{\text{obs.}}$. From top to bottom, the p -nitrophenyl phosphate concentrations were 0.34, 0.68, 1.02, 1.36, 2.38 and 3.4 mM. The concentration of AeAdo-dial was 1.16 mM. (b) Plot of $1/k_{\text{obs.}}$ versus $[p\text{-nitrophenyl phosphate}]$ from (a).

sumption may be applied to most irreversible inhibitors in modification reactions. The rate equation in accordance with Scheme 4 was thus derived by assuming an equilibrium condition. Eqn. (3) shows the rate equation for the inactivation (a similar stepwise derivation for the equation was described by Price & Stevens [27]):

$$k_{\text{obs}} = k_{\text{max}} \cdot ([I]/K_i) / (1 + [I]/K_i + [S]/K_s + [S][I]/K_i K_s') \quad (3)$$

The double-reciprocal plot of $1/k_{\text{obs}}$ versus $1/[I]$ will give straight lines with slope and vertical intercept equal to $\{K_i(1 + [S]/K_s)\}/k_{\text{max}}$ and $(1 + [S]/K_s')/k_{\text{max}}$, respectively (Fig. 5b). The K_s , K_s' , K_i and K_i' values were found to be 1.63 ± 0.79 , 3.96 ± 1.35 , 0.24 ± 0.08 and 0.58 mM respectively.

It should be noted that the major difference between the inhibition and protection experiments was that in inhibition studies the linear initial rate of substrate hydrolysis was recorded, whereas in the protection experiment described by Scheme 4 we were measuring the enzyme inactivation rate by monitoring the concave-down part of the progress curve in the next $\frac{1}{2}$ –1 h. These different approaches gave comparable K_s and K_s' values.

Protection of the enzyme from inactivation by A ϵ Ado-dial was also afforded by *p*-nitrophenyl phosphate (Fig. 6). However, the result did not conform to the mechanism shown in Scheme 4. Instead, a plot of $1/k_{\text{obs}}$ versus [substrate] in accordance with Mildvan & Leigh [28] gave a straight line. This result indicated that A ϵ Ado-dial competed with the substrate for the enzyme without formation of an enzyme–reagent complex. The K_s value calculated from this plot was 2.2 mM, which was in good agreement with the value obtained from the inhibition experiment.

Covalent labelling of human placental alkaline phosphatase with ϵ AMP-dial

The results described above demonstrated that ϵ AMP-dial was an affinity label for the enzyme whereas A ϵ Ado-dial was not. We then analysed the covalent labelling of the enzyme during inactivation after stabilizing the labelling with NaBH₄. The fluorescence of ϵ AMP-dial was used in quantification of the covalent labelling. Oxidation by periodate did not affect the fluorescence spectra of ϵ AMP. Both ϵ AMP-dial and A ϵ Ado-dial gave maximum absorption when excited at 337 nm and the maximum fluorescence emitted at 407 nm. The correlation between labelling and enzyme inactivation was determined. Despite various kinetic evidence to support the view that ϵ AMP-dial was an affinity label for the enzyme, ϵ AMP-dial reacted with some non-essential groups after prolonged incubation. However, the essential group related to enzyme activity seemed to be supra-active with the reagent. Extrapolating the initial part of the inactivation gave a stoichiometry of 2.0 molecules of ϵ AMP-dial labelled per enzyme dimer. Since the fluorescence quantum yield of the nucleotide might change when bound to the enzyme, it was difficult to interpret the precise stoichiometry at the present stage. The maximum enzyme activity lost was about 50%. Prolonged incubation did not inactivate the enzyme further but produced non-specific labelling, because the data points coincided with those of the A ϵ Ado-dial labelling. As many as nine molecules of labelled A ϵ Ado-dial per enzyme molecule were obtained after prolonged incubation of the enzyme with the reagent.

DISCUSSION

We have characterized the chemical changes of ϵ AMP-dial in aqueous solution. At lower temperature (8 °C) ϵ AMP-dial was stable for several hours. Under this condition ϵ AMP-dial was found to be an affinity label for human placental alkaline phosphatase with the following criteria. (a) ϵ AMP-dial was a

substrate for the enzyme. (b) ϵ AMP-dial was a partial mixed-type inhibitor for the enzyme. (c) Saturation kinetics was obtained when inactivation was performed at several concentrations of ϵ AMP-dial. (d) Substrate could protect the enzyme from inactivation by ϵ AMP-dial.

The above conclusion was further supported by the parallel experiments with A ϵ Ado-dial, a derivative without the phosphate group. (a) A ϵ Ado-dial was not a substrate for the enzyme. (b) No saturation kinetics was observed. (c) Non-specific labelling of the enzyme was observed for A ϵ Ado-dial. However, A ϵ Ado-dial and ϵ AMP-dial gave the same partial mixed-type inhibition pattern, showing that either reagent may combine with the enzyme in the presence of substrate.

The above discussion shows that A ϵ Ado-dial, although it does not contain a phosphate group, competes for the substrate-binding site. Therefore, at the active centre of human placental alkaline phosphatase, besides the phosphate-binding site, there must be a 'hydrophobic region' that fits for an aromatic group. There may be some overlap between the hydrophobic region and phosphorylation site, because L-phenylalanine, the specific uncompetitive inhibitor of the placental isoenzyme, might bind to the hydrophobic site and prevent the dephosphorylation step of the catalytic cycle, as suggested by Fernley and his colleagues [29]. From transient-kinetic and inhibition studies, Butterworth and colleagues [30,31] suggested that the hydrophobic binding region of alkaline phosphatase may be induced by the substrate binding. This would explain that A ϵ Ado-dial, lacking a phosphate group, is a partial mixed-type inhibitor of the enzyme. The tertiary structure of human placental alkaline phosphatase around its active site is not known. The refined structure of *Escherichia coli* alkaline phosphatase at 0.28 nm resolution showed that the active-site pocket can barely accommodate an inorganic phosphate group. Thus the alcoholic or phenolic part of the substrate would have to be exposed on the surface of the enzyme [32]. The amino acid sequence of human placental alkaline phosphatase has been deduced through cDNA nucleotide sequence analysis [33]. The amino acid sequence of placental enzyme shows 30% identity with the sequence of the *E. coli* enzyme [34], especially in the region of the putative active site and metal-binding sites [35]. The postulated hydrophobic region of placental enzyme thus has to wait until the detailed tertiary structure of the enzyme–substrate complex becomes available.

The reason for the higher inactivation rate with A ϵ Ado-dial than with ϵ AMP-dial may be explained by the higher concentration of free aldehyde group of A ϵ Ado-dial in aqueous solution. The active $\alpha\beta$ -unsaturated allelic aldehyde group of A ϵ Ado-dial is seldom hydrated [36]. Dephosphorylation by β -elimination introduces a non-specific factor for the oxidized nucleotide, as shown in the fluorescence labelling experiment. A differential labelling technique might be preferred in using ϵ AMP-dial as an affinity label for specific labelling of the active centre, i.e. modification proceeding via two sequential steps with AMP-dial and ϵ AMP-dial in the presence and in the absence of protecting ligand respectively (see ref. [37]).

Oxidized nucleotide has been demonstrated to be an affinity label specific for the lysine residue. A Schiff base or a morpholino derivative between the aldehyde group and the ϵ -amino group of the lysine residue has been postulated. Experiments with model compounds suggested the latter to be the major product [38]. The irreversibility of the inactivation in our case suggested a morpholino derivative to be the reaction product. The covalent labelling experiment indicated that prolonged incubation of the enzyme with ϵ AMP-dial caused non-specific labelling, presumably due to the effect of A ϵ Ado-dial produced by β -elimination of ϵ AMP-dial. With either ϵ AMP-dial or A ϵ Ado-dial, the maxi-

mum inactivation obtained was 50 %, even at high concentrations of the reagent used. This result suggested that the modified lysine residue was not essential for catalysis, but that its modification might interfere with the substrate binding, as indicated by the increased K_s value in the presence of reagent.

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