Solvent kinetic isotope effects of human placental alkaline phosphatase in reverse micelles

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Human placental alkaline phosphatase was embedded in a reverse micellar system prepared by dissolving the surfactant sodium bis(2-ethylhexyl) sulphosuccinate (Aerosol-OT) in 2,2,4-trimethylpentane. This microemulsion system provides a convenient instrumental tool to study the possible kinetic properties of the membranous enzyme in an immobilized form. The pL (pH/p²H) dependence of hydrolysis of 4-nitrophenyl phosphate has been examined over a pL range of 8.5–12.5 in both aqueous and reverse micellar systems. Profiles of log *V* versus pL were H_a-bell shaped in the acidic region but reached a plateau in the basic region in which two p*K*_a values of 9.01–9.71 and 9.86–10.48, respectively, were observed in reverse micelles. However, only one p*K*_a value of 9.78–10.27 in aqueous solution was detected. Profiles of log *V*/*K* versus pL were bell-shaped in the acidic region. However, they were wave-shaped in the basic region in which a residue of p*K*_a 9.10–9.44 in aqueous solution and 8.07–8.78 in reverse micelles must be dehydronated for the reaction to reach an optimum. The *V*/*K* value shifted to a lower value upon dehydronation of a p*K*_a value of 9.80–10.62 in aqueous solution and 11.23–12.17 in reverse micelles. Solvent kinetic isotope

effects were measured at three pL values. At pL 9.5, the observed isotope effect was a product of equilibrium isotope effect and a kinetic isotope effect; at pL 10.4, the log *V*/*K* value was identical in water and deuterium. The deuterium kinetic isotope effect on *V*/*K* was 1.14 in an aqueous solution and 1.16 in reverse micelles. At pL 11.0 at which the log *V* values reached a plateau in either solvent system, the deuterium kinetic isotope effect on *V* was 2.08 in an aqueous solution and 0.62 in reverse micelles. Results from a proton inventory experiment suggested that a hydron transfer step is involved in the transition state of the catalytic reaction. The isotopic fractionation factor (*φ*) for deuterium for the transition state (*φ*^T) increased when the pH of the solution was raised. At pL 11.0, the *φ*^T was 1.07 in reverse micelles, which corresponds to the inverse-isotope effect of the reaction in this solvent system. Normal viscosity effects on *k*_{cat} and *k*_{cat}/*K*_m were observed in aqueous solution, corresponding to a diffusional controlled physical step as the rate-limiting step. We propose that the rate-limiting step of the hydrolytic reaction changes from phosphate releasing in aqueous solution to a covalent phosphorylation or dephosphorylation step in reverse micelles.

INTRODUCTION

Alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] from human placenta is a membrane-bound dimeric protein anchoring to the lipid bilayer through a phosphatidylinositol–glycan moiety [1,2]. The enzyme catalyses the hydrolysis of the phosphoester linkage of a wide range of substrates. Depending on their primary sequences, kinetic properties and antigenicity differences, the mammalian alkaline phosphatases are divided into four groups: the tissue non-specific (bone-liver-kidney), the intestinal, the placental (Regan), and the placental-like (Nagro) isoenzymes. These isoenzymes have attracted much interest because of their clinical association with many pathological conditions [3,4]. Less information is available for the mammalian enzymes with respect to structure and catalytic function when compared with the enzyme from *Escherichia coli*.

The three-dimensional structure of *E. coli* alkaline phosphatase was elucidated by X-ray crystallographic analysis more than a decade ago [5] and was achieved to 2.0 Å (10^{–10} m) resolution [6,7]. The amino-acid sequences of the mammalian enzymes were only 25–30 % similar to that of the *E. coli* enzyme [8,9]. However, computer-modelling results indicate that the mammalian

enzymes retain the core of the three-dimensional structure of the *E. coli* enzyme especially at the putative active-site region [10]. The reaction mechanism of all variants of alkaline phosphatase is the same irrespective of tissue source or species [6,7,10].

The reaction catalysed by the *E. coli* enzyme was suggested to follow a dissociative mechanism, called *S*_N1(P) [11–14]. The mechanism involved an initial dissociative loss of the alcoholic leaving group to generate a putative metaphosphate anion intermediate, which is then captured by a nucleophile, a zinc-activated serine residue, subsequently forming the phosphoryl–enzyme intermediate. Hydrolysis of the phosphoryl–enzyme by water or transfer of the phosphoryl group to an alcoholic acceptor completed the reaction cycle (Scheme 1). The rate-limiting step was the hydrolysis of phosphoryl–enzyme intermediate at acidic pH, but, at basic pH, changed to release of enzyme-bound inorganic phosphate. Han and Coleman [15] suggested that the mechanism depends on the p*K*_a value of the leaving group. When the p*K*_a value of the leaving group exceeds 15, phosphorylation becomes rate-limiting and the mechanism is primarily associative in character.

In *E. coli* cells, alkaline phosphatase is a soluble protein located in the periplasmic space between the cell membrane and cell wall, in contrast to the membrane-anchored mammalian

Abbreviations used: AMP, 2-amino-2-methyl-1-propanol; AOT, Aerosol-OT [sodium bis(2-ethylhexyl) sulphosuccinate]; NPP, 4-nitrophenyl phosphate; ω_o, the molar ratio of [H₂O]/[AOT]; pL, the equivalent of pH in mixed isotope solvents, corresponding to p²H in pure deuterium; ²H*V*, solvent kinetic isotope effect on *V*, i.e. *V*_{HOH}/*V*_{2HO2H}; ²H(*V*/*K*), solvent kinetic isotope effect on *V*/*K*, i.e. (*V*/*K*)_{HOH}/(*V*/*K*)_{2HO2H}; *φ*, isotopic fractionation factor.

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Scheme 1 Reaction mechanism of human placental alkaline phosphatase

Abbreviations: E, human placental alkaline phosphatase; NPP, 4-nitrophenyl phosphate; NP^- , 4-nitrophenolate anion; P_i , inorganic phosphate; ROH, a phosphoryl-group-accepting alcohol. The non-covalent association is represented by a dot and the covalent chemical bond is represented by a dash. Step 1, association of substrate with the enzyme forming a Michaelis complex; step 2, phosphorylation of enzyme serine residue; step 3, hydrolysis of phosphoryl-enzyme; step 4, transfer of phosphoryl group from phosphoryl-enzyme to an alcohol receptor; step 5, dissociation of enzyme-bound phosphate; Step 6, dissociation of enzyme-bound phosphorylated alcohol.

enzymes. The detailed kinetic mechanism may be different between bacterial and mammalian enzymes [16]. Almost all kinetic studies of the enzyme have been performed in aqueous solution and whether the data obtained represent the kinetic behaviour of the enzyme *in vivo* is an intriguing question. We have investigated the structure and function relationship of the membrane-anchored human placental alkaline phosphatase [17,18], especially its kinetic properties *in vivo* [19].

To study the kinetic properties of the membranous protein under conditions more closely approximating those that occur in tissues rather than in soluble preparations, we embedded the enzyme in a reverse-micellar system prepared by dissolving the surfactant Aerosol OT [sodium bis(2-ethylhexyl) sulphosuccinate, AOT] in 2,2,4-trimethylpentane, in which the tiny water droplets were dispersed in organic solvent. In this system, the enzyme in aqueous phase is surrounded by the surfactant molecules so that the polar heads are in contact with the aqueous phase and nonpolar tails point towards the organic phase, resulting in a transparent macro-homogeneous solution which can be examined by various optical instruments.

In our previous study, when the enzyme was embedded in AOT/2,2,4-trimethylpentane reverse micelles, the enzymic activity was retained but displayed different kinetic behaviour when compared with the enzyme in aqueous solution [19]. Our results imply that the enzyme may have different kinetic mechanisms in reverse micelles than when in aqueous solution. To clarify this point, we have examined the solvent kinetic isotope effect and proton-inventory effect of the enzyme in both aqueous and reverse micellar systems and the results indicated that the rate-limiting step of the reaction may be altered in different solvent systems.

MATERIALS AND METHODS

Materials

AOT, 2-amino-2-methyl-1-propanol (AMP) and deuterium (99.9 atom % ^2H) were purchased from Sigma-Aldrich. 2,2,4-Trimethylpentane was from Merck. Other chemicals used were as described previously [19]. The purity of AOT had been investigated previously and was used without further purification [19].

The partially purified human placental alkaline phosphatase (type XVII) from Sigma-Aldrich was further purified to apparent homogeneity using Q-Sepharose and concanavalin A-Sepharose (Pharmacia-Biotech) chromatography and the purity of the enzyme was routinely checked using SDS/PAGE [18].

Enzyme assay

Assay of the alkaline-phosphatase-catalysed hydrolysis of 4-nitrophenyl phosphate (NPP) in aqueous solution was performed as described previously [17]. The enzyme activity in reverse micelles was assayed by the injection method [19,20]. All concentrations specified in this study are the final concentration in terms of total volume and not the less definable volume enclosed in reverse-micellar vesicles. The $[\text{H}_2\text{O}]/[\text{AOT}]$ ratio (ω_o) employed was 22.6, which represented an inclusive volume of approx. 228 nm^3 of the reverse micelles with a core radius of 3.79 nm [21]. This volume is approximately equal to a dimeric enzyme molecule (227 nm^3) [19,22]. The enzyme molecules, therefore, could be regarded as being immobilized in the membranous micellar structure.

The reaction mixture contained 2.5 mM AMP/HCl buffer (pH 8.51–11.76), 20 μM or 2 mM NPP, 66.7 mM AOT and 1.01 μg of alkaline phosphatase in a total volume of 3 ml. The liberation of 4-nitrophenol was monitored continuously at an absorbance of 410 nm and at 30 °C. A molar absorption coefficient of $18.3 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for 4-nitrophenol [23] was used in calculations. The dimeric enzyme has an M_r of 115 000 and, at A_{280} , the enzyme solution has an absorption coefficient of $115 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [24].

pH studies

Tris/HCl and carbonate buffers produced different effects on enzyme activity [19]. To avoid this buffer effect we elected to use AMP/HCl buffer at all pH values. With this buffer, the $K_{m\text{NPP}}$ increased from 80 μM at pH 9.5 to 753 μM at pH 11.0 in aqueous solution, and from 66 μM at pH 9.5 to 285 μM at pH 11.0 in reverse micelles. The enzyme activities assayed at low substrate concentration (20 μM) divided by $[\text{S}]$ or at high substrate concentration (2 mM) corresponded to 80–97% V_{max}/K_m and 73–96% V_{max} in aqueous solution, and to 77–93% V_{max}/K_m and to 88–97% V_{max} in reverse micelles, respectively, at pH 9.5 and 11.0. The initial enzyme activities (v) measured at high substrate concentrations and the $v/[\text{S}]$ at low substrate concentrations thus approximated the V_{max} and V_{max}/K_m respectively.

The buffering capacity and pH value of the reverse micellar system were measured before initiation and after completion of the enzyme assay and were found to be unchanged and were the same as in aqueous solution. These results support the assumption that at $\omega_o > 10$ –15, the pH value of the aqueous phase in reverse micelles should be the same as the bulk aqueous solution [20,25].

The data were analysed by the non-linear regression program SigmaPlot (Jandel Scientific) to estimated the pK_a values of the possible amino acid residues involved in substrate binding or catalysis. The pH–log V profiles were fitted to eqn. (1) or eqn. (2) for one or two hydronation sites respectively:

$$\log V = \log \frac{V_{\text{max}}}{1 + \frac{[\text{H}^+]}{K_{\text{es1}}}} \quad (1)$$

$$\log V = \log \frac{V_{\text{max}}}{1 + \frac{[\text{H}^+]}{K_{\text{es1}}} + \frac{[\text{H}^+]^2}{K_{\text{es1}} \cdot K_{\text{es2}}}} \quad (2)$$

where V denotes the parameter measured at various pH values, V_{max} represents the pH-independent value of V , and K_{es1} and K_{es2} are the dissociation constants for the enzyme groups.

The pH–log V/K profiles were fitted to eqn. (3) according to a mechanism involving two V_{max} , in which all three enzyme

species (E^{n+1} , E^n and E^{n-1}) can bind with the substrate and both E^nS and $E^{n-1}S$ could catalyse the reaction with $V_{\max}$ and $V_{\max}'$ respectively, where $V_{\max}'$ equals $\gamma V_{\max}$ and $\gamma < 1$ [19].

$$\log \frac{V}{K} = \log \frac{V_{\max} \left(1 + \frac{\gamma K_{es2}'}{[H^+]} \right)}{K_m \left(1 + \frac{[H^+]}{K_{e1}} + \frac{K_{e2}}{[H^+]} \right)} \quad (3)$$

Solvent kinetic isotope effect

The same experiments in pH studies were performed in deuterium medium. The p^2H values were determined by addition of a factor of 0.4 to glass-electrode measurements [26]. Solvent kinetic isotope effects of human placental alkaline-phosphatase-catalysed reactions in aqueous and reverse micellar systems were examined at three pL values (9.5, 10.4 and 11.0) by determining the initial velocity with various substrate concentrations. The ratio of the enzyme rate in water and deuterium (v_H/v_{2H}) were fitted as a function of the fractional substrate saturation (f) of the enzyme according to eqn. (4) and eqn. (5) [27]:

$$v_H/v_{2H} = f \cdot {}^2H V + (1-f) \cdot {}^2H (V/K) \quad (4)$$

where

$$f = \frac{[NPP]}{K_{mNPP(HOH)} + [NPP]} \quad (5)$$

and ${}^2H V$ is the solvent kinetic isotope effect on V (i.e. V_{HOH}/V_{2HO^2H}) and ${}^2H (V/K)$ is the solvent kinetic isotope effect on V/K , i.e. $(V/K)_{HOH}/(V/K)_{2HO^2H}$.

The $K_{mNPP(HOH)}$ was calculated by fitting the initial velocity (v) determined at various concentrations of NPP to the Michaelis-Menten equation [eqn. (6)]:

$$v = \frac{V_{\max} \cdot [NPP]}{K_{mNPP} + [NPP]} \quad (6)$$

Proton-inventory effect

The solvent-isotope effect was measured in H_2O - 2H_2O buffers. The glass electrode reading in H_2O - 2H_2O mixtures is related to pL by eqn. (7) [26]:

$$pL = pH (\text{meter reading}) + 0.221n + 0.173n^2 \quad (7)$$

where n is the atom fraction of deuterium $\{[{}^2H]/([{}^2H] + [H])\}$. The initial velocities of the enzyme reaction were determined in H_2O - 2H_2O buffers of different atom fractions of deuterium. The general dependence of v_{2H} on n is given by eqn. (8) [26,28,29]:

$$v_{2H} = v_H \frac{\prod_i (1 - n + n\phi_i^T)}{\prod_j (1 - n + n\phi_j^R)} \quad (8)$$

where ϕ_i^T and ϕ_j^R denote the isotopic fractionation factors (ϕ) for deuterium in the i th site of the transition state and the j th site of the reactant state respectively, p represents the number of hydrogens transferred.

If we assume that most protons do not change states on activation and the exchangeable protons of proteins should have $\phi^R \cong 1$, with the exception of those with sulphhydryl groups where $\phi^R \cong 0.5$, eqn. (8) can be simplified to eqn. (9) or eqn. (10) for the transition state of a single or multiple contributing site

respectively. In the latter case, each transferred proton is assumed to have an identical ϕ_i^T :

$$v_{2H} = v_H (1 - n + n\phi^T) \quad (9)$$

$$v_{2H} = v_H (1 - n + n\phi_i^T)^p \quad (10)$$

A plot of v_{2H} versus n according to eqn. (9) will give a straight line with the intercept at v_H and slope = $v_H \cdot (\phi^T - 1)$. By comparison, a plot of v_{2H} versus n , according to eqn. (10), will be non-linear at $p > 1$. It is, thus, possible to distinguish between one or multiple hydrogenic sites with carefully performed investigations.

Viscosity effect

Kinetic parameters were determined at 30 °C in water containing various amounts of glycerol (0–35 %, w/v) or sucrose (0–36 %, w/v) as viscogens. Assays typically contained 50 mM $Na_2CO_3/NaHCO_3$ buffer (pH 9.8) or 2.5 mM AMP/HCl buffer (pH 9.8), 10 μ M or 10 mM NPP and 1.01 μ g or 2.02 μ g of enzyme. The initial enzyme activities measured at high substrate concentrations and $v/[S]$ at low substrate concentrations were used to estimate the k_{cat} and k_{cat}/K_m respectively.

RESULTS

pH studies

Our previous work has demonstrated that Tris/HCl and carbonate buffers caused quite different effects on k_{cat} or k_{cat}/K_m of the enzyme [19]. To explore further the pH effect on the reaction rate of the enzyme, a phosphoryl-group-accepting buffer (AMP) was used for the entire pH range of our experiments (pH 8.51–11.76, a range over which the enzyme is stable during the experimental period). All values are means $\pm$ S.E.M. Figure 1 shows that only the hydronation of an ES group with an apparent pK_a value of 9.78 ± 0.6 was detected in aqueous solution (Figure 1a). In reverse micelles, however, the data can be optimally fitted to a model that involves two hydronated sites with apparent pK_a values of 9.01 ± 0.15 and 9.86 ± 0.11 respectively (Figure 1b). Both groups have to be dehydronated to give maximum reaction rate. Figures 1(c) and 1(d) summarize the effects of pH on V/K . The curves show a normal bell-shape at the acidic region. However, the curves level off at greater pH values, thereby conforming to a mechanism having two $V_{\max}$ values ($V_{\max}$ and $V_{\max}'$) because E^nS and $E^{n-1}S$ are both enzymically active where $V_{\max}'$ is equal to $\gamma V_{\max}$ (Scheme 2). The γ value was found to be 0.007 in aqueous solution and 0.004 in reverse micelles. The γ value could be > 1 with a different buffer system [19]. The pK_a values from computer fitting of the above results are summarized in Table 1.

Solvent kinetic isotope effect

When the experiments described above were performed in deuterium medium, similar results were obtained with apparent pK_a values shifted to the basic region. In aqueous solution, the pK_a value of the amino acid residue involved in catalysis was shifted by 0.49 ± 0.02 unit to give a value of 10.27 ± 0.4 . Two apparent pK_a values were also detected in reverse micelles with ΔpK_{es1} and ΔpK_{es2} equal to 0.7 ± 0.01 and 0.62 ± 0.01 respectively. The pH versus $\log V/K$ profiles in deuterium medium were similar to those in water. The ΔpK_{e1} and ΔpK_{e2} were shifted by 0.34 ± 0.06 and 0.82 ± 0.03 unit in aqueous solution and 0.71 ± 0.06 and 0.94 ± 0.05 unit in reverse micelles respectively (Table 1).

Figure 1 Effects of pL on the reaction catalysed by human placental alkaline phosphatase in aqueous and reverse micellar systems

The effects of pL on V (a and b) or V/K (c and d) were determined with NPP as substrate at various pL values in water (○) or deuterium (●). (a) and (c), aqueous system; (b) and (d), reverse micellar system ($\omega_0 = 22.6$). The symbols are experimental values and the curves are computer-generated fittings according to eqn. (1) in (a), eqn. (2) in (b) and eqn. (3) in (c) and (d). The broken lines in (b) are computer fittings according to eqn. (1). The precision of the determinations was shown in the duplicate assays in one of the lines (○) in (d). The amount of enzyme used in each assay was $1.01 \mu\text{g}$.

Scheme 2 Proposed kinetic mechanism of human placental alkaline-phosphatase-catalysed reaction

Assuming that binding of NPP with the enzyme is rapid in comparison with subsequent covalent bond formation or breakdown and that $\alpha = \beta = 1$ for rapid equilibrium of substrate and H^+ , the binding processes shown in the grey rectangles are not considered in eqn. (3). Eqn. (3) does not distinguish between the two covalent steps, phosphorylation and dephosphorylation. The abbreviations are as shown in Scheme 1.

Based on the values of V_{lim} and $(V/K)_{\text{lim}}$ (Table 1), the pL-independent isotope effect values on V and V/K were calculated to be ${}^2\text{H}V_{\text{lim}} = 2.02 \pm 0.13$ and ${}^2\text{H}(V/K)_{\text{lim}} = 7.1 \pm 3.4$ in aqueous solution and ${}^2\text{H}V_{\text{lim}} = 0.84 \pm 0.06$ and ${}^2\text{H}(V/K)_{\text{lim}} = 1.07 \pm 1.51$ in reverse micelles. These values were obtained at high and low substrate concentrations. To examine the detailed isotope effect at various pH values, we performed the substrate saturation experiment at three pL values (9.5, 10.4 and 11.0), which corresponded to regions having a large shift in dissociation constant, minimum effect on V/K and constant V respectively. A typical result at pL 9.5 is presented in Figure 2.

At pL 9.5, the isotope effect on V was similar in aqueous solution (3.16 ± 0.09) or in reverse micelles (3.32 ± 0.17). The isotope effect on V/K , on the other hand, was increased from 1.09 ± 0.11 in aqueous solution to 3.43 ± 0.22 in reverse micelles

(Table 2). At pL 10.4, the $\log V/K$ value is identical in water or deuterium. The deuterium isotope effect on V/K was almost identical in aqueous solution (1.14 ± 0.04) to that in reverse micelles (1.16 ± 0.07). These results probably indicate small isotope effect on the phosphorylation step in both solvent systems. However, by examining the data shown in Figure 1, these isotope effects clearly result from the product of equilibrium isotope effect for an acid ionization and a kinetic isotope effect, i.e., $k_{\text{obs}} = K \cdot k$.

If both systems are compared, the $\log V$ values plateau only at pL 11. By analogy with the acylation and deacylation in the reaction mechanism of protease [26], the kinetic parameter k_{cat} has an effective reactant state as the phosphoryl enzyme. The effective transition state is that for the dephosphorylation process. The parameter k_{cat}/K_m has an effective reactant state as the free substrate and free enzyme. The effective transition state is that for the phosphorylation process. The isotope effect on V/K and V at pL 11.0 thus reflects the effect on the phosphorylation and dephosphorylation steps respectively. Quite different results were obtained in aqueous or reverse micellar solution. Normal isotope effects of 1.21 ± 0.03 and 2.08 ± 0.08 in an aqueous solution but an inverse isotope effect of 0.46 ± 0.04 and 0.62 ± 0.06 in reverse micelles were observed for ${}^2\text{H}(V/K)$ and ${}^2\text{H}V$ respectively (Table 2).

In an aqueous solution, the K_{mNPP} in water was 80 ± 20 , 370 ± 20 and $753 \pm 60 \mu\text{M}$ at pL 9.5, 10.4 and 11.0 respectively. These values changed to 19 ± 7 , 170 ± 10 and $449 \pm 40 \mu\text{M}$ in deuterium. In reverse micelles, the K_{mNPP} differences between water and deuterium were always smaller than those in aqueous solution. At pL 9.5, 10.4 and 11.0, the K_{mNPP} values in water were 66 ± 8 , 116 ± 22 and $285 \pm 60 \mu\text{M}$ respectively. These values changed to 72 ± 7 , 105 ± 17 and $169 \pm 20 \mu\text{M}$ in deuterium.

The isotope effect on K_m was high in an aqueous solution but disappeared in reverse micelles. The dependency of ${}^2\text{H}K_m$ on pL was opposite in an aqueous solution and in reverse micelles. At

Table 1 Summary of the pK_a values from the pL dependence of kinetic parameters

All values are shown as mean $\pm$ S.E.M. ($n = 15$ for H_2O and $n = 17$ for 2H_2O).

Solvent system	Medium	pL range	Kinetic parameters	Eqn. fitted	pK_a Values and kinetic constants
Aqueous	H_2O	8.51–11.76	V/K	3	$pK_{e1} = 9.10 \pm 0.52$, $pK_{e2} = 9.80 \pm 0.40$, $(V/K)_{lim} = 0.2 \pm 0.17 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$
	2H_2O	8.88–12.42	V/K	3	$pK_{e1} = 9.44 \pm 0.8$, $pK_{e2} = 10.62 \pm 0.21$, $\Delta pK_{e1} = 0.34 \pm 0.06$, $\Delta pK_{e2} = 0.82 \pm 0.03$, $(V/K)_{lim} = 0.028 \pm 0.003 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$, $^2H(V/K)_{lim} = 7.1 \pm 3.4$
	H_2O	8.51–11.76	V	1	$pK_{es1} = 9.78 \pm 0.6$, $V_{lim} = 6.46 \pm 0.54 \text{ s}^{-1}$
	2H_2O	8.88–12.42	V	2	$pK_{es1} = 10.27 \pm 0.4$, $\Delta pK_{es1} = 0.49 \pm 0.02$, $V_{lim} = 3.2 \pm 0.15 \text{ s}^{-1}$, $^2H V_{lim} = 2.02 \pm 0.13$
Reverse micelles	H_2O	8.51–11.76	V/K	3	$pK_{e1} = 8.07 \pm 0.73$, $pK_{e2} = 11.23 \pm 0.71$, $(V/K)_{lim} = 1.72 \pm 2.48 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$
	2H_2O	8.88–12.42	V/K	3	$pK_{e1} = 8.78 \pm 0.62$, $pK_{e2} = 12.17 \pm 0.61$, $\Delta pK_{e1} = 0.71 \pm 0.06$, $\Delta pK_{e2} = 0.94 \pm 0.05$, $(V/K)_{lim} = 1.61 \pm 2.22 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$, $^2H(V/K)_{lim} = 1.07 \pm 1.51$
	H_2O	8.51–11.76	V	1	$pK_{es1} = 9.01 \pm 0.15$, $pK_{es2} = 9.86 \pm 0.11$, $V_{lim} = 3.87 \pm 0.38 \text{ s}^{-1}$
	2H_2O	8.88–12.42	V	2	$pK_{es1} = 9.71 \pm 0.22$, $pK_{es2} = 10.48 \pm 0.24$, $V_{lim} = 4.61 \pm 0.21 \text{ s}^{-1}$, $\Delta pK_{es1} = 0.7 \pm 0.01$, $\Delta pK_{es2} = 0.62 \pm 0.01$, $^2H V_{lim} = 0.84 \pm 0.06$

Figure 2 Solvent kinetic isotope effects of human placental alkaline-phosphatase-catalysed hydrolysis of NPP in aqueous and reverse micellar systems

Hydrolysis of NPP was assayed in water or deuterium media at pL 9.5 at various substrate concentrations. Solvent kinetic isotope effects were expressed as function of fractional substrate saturation. (○), Aqueous system; (●), reverse micellar system ($\omega_0 = 22.6$); DOD, $^2HO^2H$. The lines are computer fittings to eqn. (4). The amount of enzyme used in each assay was 1.01 μg .

pL 11.0, no difference was observed for $^2H K_m$ in these two solvent systems (Table 2).

Proton inventory effect

The reaction rate was determined in H_2O – 2H_2O buffers with various atom fractions of deuterium at the above mentioned three pL values. When v_{2H} was plotted versus the mole fraction of deuterium (n), linear proton inventory plots were obtained at all pL values. Figure 3 shows the results of a representative experiments performed at pL 11. It is not possible to distinguish between one-hydrogenic-site or two-hydrogenic-site models from

Table 2 Solvent kinetic isotope effect and proton inventory effect of human placental alkaline-phosphatase-catalysed hydrolysis of NPP

All values are means $\pm$ S.E.M. ($n = 20$ in isotope effect determinations and $n = 9$ in proton inventory experiments at each pL value). Values in parentheses are the results fitting a two-hydrogenic-site model.

pL	Solvent kinetic isotope effect			Fractional factor for deuterium (ϕ^T)
	$^2\text{H}(V/K)$	$^2\text{H}V$	$^2\text{H}K_m$	
Aqueous system				
9.5	1.09 ± 0.11	3.16 ± 0.09	4.07 ± 1.25	0.26 ± 0.04 (0.53 ± 0.03)
10.4	1.14 ± 0.04	2.52 ± 0.06	2.18 ± 0.12	0.37 ± 0.03 (0.62 ± 0.02)
11.0	1.21 ± 0.03	2.08 ± 0.08	1.68 ± 0.14	0.52 ± 0.04 (0.73 ± 0.03)
Reverse-micellar system				
9.5	3.43 ± 0.22	3.32 ± 0.17	0.92 ± 0.10	0.15 ± 0.02 (0.44 ± 0.02)
10.4	1.16 ± 0.07	1.52 ± 0.07	1.10 ± 0.19	0.50 ± 0.05 (0.70 ± 0.03)
11.0	0.46 ± 0.04	0.62 ± 0.06	1.68 ± 0.28	1.07 ± 0.16 (1.03 ± 0.08)

the data. From Figure 3 and similar results obtained at pL 9.5 and 10.4, the deuterium fractionation factors in the transition state (ϕ^T) for the simple one- or two-hydrogenic-site model were estimated. The ϕ^T increases at larger pL values (Table 2). Taking values of the one-hydrogenic site, the deuterium solvent kinetic isotope effects at pL 9.5, 10.4 and 11.0 were estimated to be 3.85, 2.70 and 1.92 in aqueous solution and 6.7, 2.0 and 0.93 in reverse micelles respectively. These values closely correspond to the $^2H V$ values determined separately in substrate saturation experiments (Figure 2 and Table 2).

The data were also fitted to eqn. (10) to examine the possibility that multiple hydrogenic sites were involved. Large standard errors were obtained for the fitted data. The data were also not compatible with another linear (Gross–Butler) equation, $v_{2H} = v_H / (1 - n + n\phi_j^R)$, for the reactant state.

Viscosity effect

In a study involving the isotope effects on malic enzyme, Karsten et al. [30] compared the solvent isotope and viscosity effects and

Figure 3 Proton-inventory effects of human placental alkaline-phosphatase-catalysed hydrolysis of NPP in aqueous and reverse micellar systems

Hydrolysis of NPP was assayed in H_2O – $^2\text{H}_2\text{O}$ buffer at pL 11 and various deuterium (D) fractions. (○), Aqueous solution; (●), reverse micelles ($\omega_0 = 22.6$). Lines are computer fittings to a one-hydrogenic site model [eqn. (9), solid lines] or a two-hydrogenic site model [eqn. (10) at $p = 2$, dashed lines]. The amount of enzyme used in each assay was 1.01 μg .

concluded that the inverse solvent isotope effects observed on malic enzyme are the result of the viscosity difference between water and deuterium. We therefore performed the viscosity experiment as a control. Figure 4 shows that the second-order rate constant, k_{cat}/K_m , decreased exponentially and the first-order rate constant, k_{cat} , decreased linearly with increasing solvent viscosity irrespective of whether glycerol or sucrose was used as the viscogen. Above normal viscosity effects correlate with a diffusional controlled physical step as the rate-limiting step in aqueous solution, similar to that found for the *E. coli* enzyme [31].

DISCUSSION

Chemical modification and site-directed mutagenesis results have identified the essential arginine, tyrosine, lysine and histidine residues in the active centre of human placental alkaline phosphatase [17,32–35]. The enzyme has been observed to undergo phosphorylation–dephosphorylation during the catalytic cycle (cf. [36]). Multiple amino-acid sequence alignments with the *E. coli* enzyme reveal that the putative active-site region of placental alkaline phosphatase is comprised of a conserved Asp⁹¹–Ser⁹²–Ala⁹³ sequence, the metal triplet (Zn1–Zn2–Mg) and Arg¹⁶⁶ and other groups nearby [8,9]. Mammalian alkaline phosphatases are 20- to 30-fold more active than the corresponding bacterial enzymes. Histidine-153 could be responsible for the higher activity of mammalian enzymes [37]. We have constructed several mutant placental alkaline phosphatases and the specific activity of His¹⁵³ → Asp and His¹⁵³ → Ala is only 7.4% and 0.23%, respectively, of the wild-type value [35].

In reverse micelles, the magnesium ion induces a conformational change in the enzyme and a curved plot is obtained when the initial rate is measured, thereby complicating the initial rate analysis [38]. For this reason we did not include magnesium ions in the assay mixture and the modulatory role of Mg^{2+} is not considered in the following discussion. If the two-metal-centre

Figure 4 Viscosity effects of human placental alkaline-phosphatase-catalysed reaction

Hydrolysis of NPP by alkaline phosphatase in 50 mM $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer, pH 9.8, at 30 °C in the presence of various amounts of (a) glycerol or (b) sucrose. The effects of viscogens on the second-order rate constant k_{cat}/K_m (●), or the first-order rate constant k_{cat} (○) were plotted against the percentage of viscogen. The lines for k_{cat} are linear regression lines. The lines for k_{cat}/K_m are exponential fitting lines.

reaction mechanism for the *E. coli* enzyme [6] is adopted, the mechanism involves an activation of serine-92 by the zinc ion at the M2 site, which results in a better nucleophile, serine- O^- , with which to attack the phosphorus atom of the substrate molecule, resulting in formation of a phosphoryl–enzyme intermediate. The zinc ion at the M1 site assists the alcoholic product to leave the active centre. Our experimental data on the pH and isotope effects studies may help in elucidating the chemical mechanism of the human placental alkaline-phosphatase-catalysed reaction.

The most significant result is the finding of an inverse isotope effect at pL 11.0 in reverse micelles. An inverse isotope effect could be augmented by: (a) the acid dissociation of a sulphhydryl group, (b) solvent isotope effects due to the medium, (c) a viscosity effect resulting from viscous deuterium when compared with water or (d) dissociation of a metal-bound water molecule. Of these possibilities, dissociation of sulphhydryl group could be ruled out because the ΔpK_a values shown in Table 1 are markedly larger than that reported for a sulphhydryl group (~ 0.2 – 0.3) [30]. The medium effect is characterized by the large number of hydrons involved, with each contributing a small isotope effect. The results shown in Figure 3 also discount this possibility. The change in solvent viscosity due to deuterium results in an inverse viscosity effect. However, we observed a normal viscosity effect (Figure 4). Correction for the viscosity effects, if they really exist, will give an even larger inverse isotope effect. None of the above provides a satisfactory explanation for the observed inverse

Scheme 3 Proposed chemical mechanism of human placental alkaline-phosphatase-catalysed hydrolysis of the phosphomonoester in reverse micelles

The two-metal site catalytic mechanism for *E. coli* alkaline phosphatase [6] was adopted. The rate-limiting step is changed from dissociation of phosphate ion in aqueous solution to phosphorylation step in reverse micelles because of the hindered dissociation of the leaving group 4-nitrophenol (see the text for details). Only part of the reverse micellar vesicle is shown. The active-site region of the enzyme is highly schematic showing only some of the essential parts. See Scheme 1 for abbreviations.

isotope effects of placental alkaline phosphatase in reverse micelles. The only explanation left is the dissociation of the metal-chelated water molecule. The fractionation factors for most metal–water complexes range from 0.7 to 1.0 [30], which is very close to the value obtained in the present study. The inverse isotope effect observed in this investigation could be attributed to displacement of a Zn-bound water molecule upon binding of the substrate.

In general, pK_a values deduced from the pH–log V/K plot reveal the dissociating groups of the free enzyme or substrate (see [39]). The dissociation of two hydrons of the phosphate group ($pK_{a1} = 6.8$, $pK_{a2} \cong 12$) may not fall in the pH range 8.51–11.76. In alkaline phosphatase, the active site is a small pocket barely large enough to accommodate a phosphate group, thereby leaving the remainder of the substrate in the solvent region, which accounts for the broad substrate specificities of the enzyme [6]. Two enzyme groups with pK_a values of 9.10 and 9.80 were detected in the pH–log V/K plot (Table 1). The former decreased to 8.07 and the latter increased to 11.23 in reverse micelles. These groups may participate in the phosphate binding. The most likely candidates in these groups are tyrosine-169, arginine-166 and the

hydrogen-bonded water-459 and water-457 [7]. The latter water molecule, which co-ordinates Mg or hydrogen bonding to lysine-328 in the *E. coli* enzyme, has been proposed to play a role in hydron transfer [6,10]. However, in placental alkaline phosphatase a histidine residue is substituted at position 328.

The difference between pK_{e1} and pK_{e2} was 0.7 in aqueous solution suggesting a negatively co-operative hydronation process, which indicates that the ionization of a group changes value depending on the ionic state of other groups. Each titrating group is the sum of the fractions of the molecular pK_a values [40]. In reverse micelles, the $pK_{e2} - pK_{e1}$ value increased to 3.16 indicating that the titration constants become effectively equal to the molecular pK_a values [41,42].

The catalytic group detected in the pH–log V plot has a pK_a value of 9.78. The identity of this group is not obvious. Neither Zn1 or Zn2 has an ancillary amino acid nucleophile to aid hydronation and dehydronation steps. However, water-465, which is near the Mg co-ordination sphere, could be involved in the hydronation of serine-92 [6]. The pK_a value of $HOH-Mg^{2+}$ is known to ionize with a pK_a of 10.6–11.4, whereas the pK_a of $HOH-Zn$ is 9.6 [39,43]. The $HOH-Zn$ interaction is strong

enough to reduce the pK_a from 15.7 to 7 or less [6]. Our data suggest that the pK_a 9.78 may contribute to ionization of the water molecule in HOH-Zn.

To obtain a complete separation of solvent isotope effects on pK_a and k_{cat} , pL-independent isotope effects on V at pL 11.0 were compared. The linear v_{2H} versus n plot corresponds to generation of the isotope effect by a single transition-state site and is expected for a simple one-site general acid-base catalysis. The kinetic isotope effect can be considered as the ratio of a ground-state and transition-state fractionation factor $k_H/k_{2H} = \phi^R/\phi^T$, on the assumption that a single hydron bond has undergone force-constant changes in the transition state. V_{max}/K_m and V_{max} reflect the conversion of the ES complex to phosphorylated enzyme and the hydrolysis of this intermediate to free enzyme and product respectively. The inverse isotope effect observed on V/K and V signifies a tightening at the hydrogenic site upon formation of the transition state, which then suggests phosphorylation or dephosphorylation as the rate-limiting step.

Another indication of a change in the rate-limiting step in these two solvent systems originated from K_{mNPP} values determined at different pL values. There was a 9.4-fold increase in K_{mNPP} from pH 9.5 to 11.0 in aqueous solution, which is related to the phosphate dissociation as the rate-limiting step at high pH values. In reverse micelles, the increase in K_{mNPP} at high pH is only 4.3-fold, which is not so large when compared with that in aqueous solution. However, since the non-covalent $E \cdot P_i$ complex involves ionic interactions between the negatively-charged P_i and the positively charged arginine-166 and Zn^{2+} groups, the strength of such interactions might be different in water and deuterium and will make the observed isotope effects more complex. With these caveats in mind, we have interpreted our experimental data as follows.

For *E. coli* alkaline phosphatase, when the pK_a value of the leaving group is greater than 15, phosphorylation of the enzyme molecule becomes rate-limiting and k_{cat} shows a dependence on the pK_a value of the leaving group [15]. More recently we found that the reaction rate of the placental enzyme in reverse micelles depends on the pK_a value of the leaving group [44]. This observation also suggests that the rate-limiting step in reverse micelles is phosphorylation of the active-site serine-92 hydroxy group rather than product dissociation. The pK_a of 4-nitrophenol is 7.15 in aqueous solution, 7.5–7.9 in the water pool of reverse micelles, but 11.5 in the interfacial region [45,46]. At $\omega_o = 22.6$, the inclusive volume of the reverse micellar vesicle was presumably barely sufficient to accommodate a dimeric enzyme molecule and thus only a monolayer of bound water at the interface is available for 4-nitrophenol, which, with a pK_a value of 11.5, explains the different rate-limiting step in the reverse micelles.

Based on above discussion, the following chemical mechanism catalysed by human placental alkaline phosphatase is proposed (Scheme 3). The concentration of AMP buffer used in this investigation was low, thereby preventing transphosphorylation from competing with the hydrolysis reaction and, thus, only hydrolysis is of concern. In the basic pH region, a dianion of a phosphomonoester is the normal substrate, which binds with the enzyme forming an $E \cdot ROP$ binary complex where one of the ester oxygen co-ordinates with Zn1 and the second oxygen co-ordinates with Zn2, forming a phosphate bridge between Zn1 and Zn2. The other two oxygen atoms form hydrogen bonds with the guanidinium group of arginine-166. Serine-92, activated by Zn2, serves as a nucleophile attacking the phosphorus atom with formation of phosphoryl-enzyme ($E-P$). A Zn1 co-ordinated water dissociates a hydron to become $Zn-OH$, which functions as the nucleophile for hydrolysis of the phosphoryl-enzyme. The non-covalently enzyme-bound phosphate ($E \cdot P$)

forms as the phosphate moves away from serine-92. One of the oxygen atoms of the phosphate again co-ordinates Zn1 to re-establish a phosphate bridge.

In alkali pH, dissociation of non-covalently associated phosphate is the rate-limiting step. In reverse micelles the enzyme is surrounded by membrane-bound water and negatively-charged polar heads of the surfactant, which hinders the ionization of 4-nitrophenol and makes 4-nitrophenolate a poor leaving group. Covalent phosphorylation or dephosphorylation becomes the rate-limiting step.

In summary, we propose that the rate-limiting step of human placental alkaline-phosphatase-catalysed hydrolysis changes from dissociation of inorganic phosphate in aqueous solution to the phosphorylation or dephosphorylation step in reverse micelles. Since the placental enzyme is a membrane-anchored protein and reverse micelles mimic the membranous structure, the kinetic properties of the enzyme in the reverse-micellar system might represent *in vivo* conditions. Our experimental results, thus, might have significant biological implications.

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