Reverse micelles as a model system with which to study leaving group effects on alkaline phosphatase-catalysed hydrolysis

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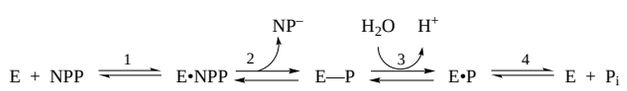
Ionisation of 4-nitrophenol in a reverse micellar system prepared by dissolving Aerosol OT (AOT), a surfactant with an anionic polar head, in isooctane, depends on the degree of hydration ([H_2O]/[AOT]) of the system. The model system provides a convenient instrumental tool with which to study the leaving group effect of alkaline phosphatase-catalysed hydrolysis of 4-nitrophenyl phosphate. The Brønsted constants, β_{lg}, for k_{cat} and k_{cat}/K_m were found to be −0.47 and −1.03, respectively. Assuming that phosphorylation of the enzyme is rate limiting, the strong leaving group effect on catalysis indicates that the apparent pK_a-values observed in reverse micelles are true measures of ionisation.

Introduction

Alkaline phosphatase [orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] catalyses the hydrolysis of a phosphoester linkage with broad substrate specificities.[1] The reaction involves an initial dissociative loss of the alcoholic leaving group to generate a presumable metaphosphate anion transition state, which is captured by a nucleophile, a zinc-activated serine residue, to produce the phosphorylated-enzyme intermediate. Hydrolysis of the phosphorylated-enzyme by H_2O or alcohols completes the reaction cycle (Scheme 1).[2] The *Escherichia coli* alkaline phosphatase was



Scheme 1 Proposed reaction mechanism for the hydrolysis by alkaline phosphatase. Abbreviations: E, human placental alkaline phosphatase; NPP, 4-nitrophenyl phosphate; NP^-, 4-nitrophenolate anion; P_i, inorganic phosphate. The non-covalent association is represented by a dot and the covalent chemical bond is represented by a dash.

suggested to follow a dissociative mechanism [$S_N1(P)$],[3] or an associative mechanism [$S_N2(P)$] depending on the pK_a value of the leaving group.[2] Under basic conditions, dissociation of the enzyme-bound phosphate (step 4 in Scheme 1) is the rate-limiting step. However, when the pK_a value of the leaving group is >15, phosphorylation becomes rate-limiting (step 2 in Scheme 1). The reaction catalysed by human placental alkaline phosphatase was assumed to follow the same pathway but the detailed mechanism is less clear.[4]

Hydrolysis of 4-nitrophenyl phosphate, the usual substrate employed in model studies, eliminates 4-nitrophenol. Ionic 4-nitrophenol at alkaline pH is readily monitored spectrophotometrically. Mechanistic aspects have been addressed by studying the susceptibility to the enzyme of compounds with various substituents in the benzene ring.[2,4] The substituents, due to field effects, endow the compounds with different reactivities in the leaving group. For 4-nitrophenyl phosphate, the substitution effect is reflected as a change in the pK_a values of the phenolic hydroxyl group. The reaction mechanism can thus be detailed by considering the Brønsted relationship for the substitution.

We found that in reverse micelles, which are formed upon dissolution of the anionic detergent AOT [sodium bis(2-ethylhexyl)sulfosuccinate] in isooctane (2,2,4-trimethylpentane), that the ionisation of the leaving group, 4-nitrophenol, depends on the inclusive volume of the reverse micelles. The reverse micellar system[5] thus provides a convenient system with which to examine the mechanism of catalysis by alkaline phosphatase.

Experimental

Materials

4-Nitrophenol, 4-nitrophenyl phosphate and AOT (Aerosol OT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of AOT was examined previously and was used without further purification.[6] Isooctane was obtained from Merck (Darmstadt, Germany). Other chemicals used were described previously.[6,7]

Human placental alkaline phosphatase from Sigma-Aldrich (Type XVII) was purified to apparent homogeneity according to our published procedure.[8] Lyophilised and commercially available powder of partially purified enzyme was freely soluble in water. After purification, an absorption coefficient of 115 × 10^3 dm^3 mol^{-1} cm^{-1} at 280 nm was utilised for protein estimation.[9] An M_r of 115 000 was used for the calculation of the enzyme concentration.[9]

Preparation of AOT-reverse micelles in systems of various degrees of hydration

AOT reverse micellar stock solution (0.24 mol dm^{-3}) was prepared by mixing AOT (10.67 g, 24 mmol) in isooctane (100 cm^3) until a clear solution was obtained. For preparing the working solution, AOT concentration was maintained constant at 100 mmol dm^{-3}. The [H_2O]/[surfactant] ratio was adjusted by varying the amount of water so as to change the dimensions of the reverse micelles. Aqueous solution was introduced into the system by an injection technique.[10] The water droplet was solubilised by mechanical agitation (vortex). The core radius (R_c) of the inner cavity of the AOT-reverse micelles was estimated by the empirical eqn. (1),[11] where ω_o denotes the molar ratio of [H_2O]/[AOT].

$$R_c/\text{nm} = 0.15\omega_o + 0.4 \quad (1)$$

Ionisation of 4-nitrophenol in AOT-reverse micelles

The absorption of 4-nitrophenol (100 μmol dm^{-3}) in aqueous $NaHCO_3$–Na_2CO_3 buffer (50 mmol dm^{-3}, pH 9.8) was determined spectrophotometrically at 402 nm. The absorption of this solution in AOT/isooctane reverse micelles of various degrees of hydration ([H_2O]/[AOT] = 4.44–40.0) was also exam-

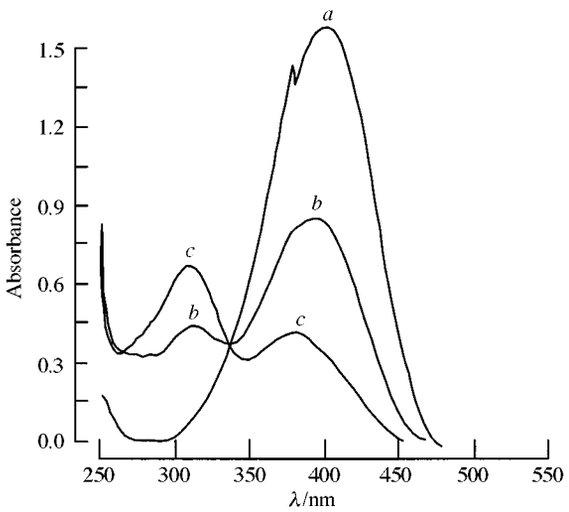


Fig. 1 Absorption spectra of 4-nitrophenol in aqueous and reverse micellar systems. (*a*) 4-Nitrophenol (100 μmol dm^{-3}) in aqueous $NaHCO_3$–Na_2CO_3 buffer solution (6.7 mmol dm^{-3}, pH 9.8). (*b*) 4-Nitrophenol (100 μmol dm^{-3}) in AOT-reverse micelles (100 mmol dm^{-3}) containing $NaHCO_3$–Na_2CO_3 buffer (6.7 mmol dm^{-3}, pH 9.8) at ($[H_2O]/[AOT]$) = 19. (*c*) Same as (*b*) at ($[H_2O]/[AOT]$) = 10. Each spectrum was scanned 2–4 times.

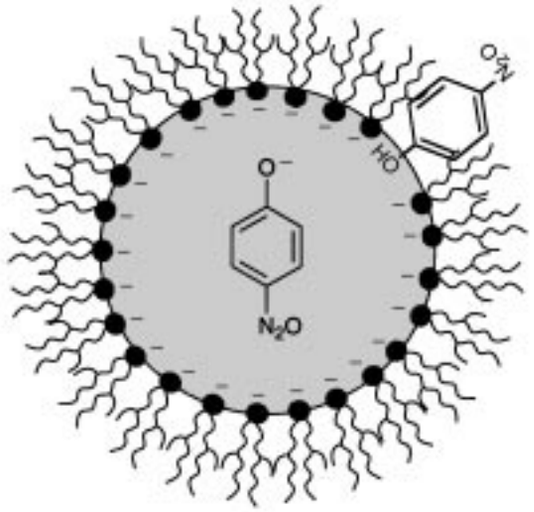


Fig. 2 Schematic model showing 4-nitrophenol entrapped in AOT-reverse micelles. The two populations of 4-nitrophenol in the reverse micelles are shown according to Menger and Saito.[15] The polar head of AOT molecule is drawn into the water pool (grey area) according to Martinek *et al.*[5]

ined. We used an absorption coefficient of 18.3×10^3 dm^3 mol^{-1} cm^{-1} for the ionised 4-nitrophenol in aqueous solution for the calculation.[12]

The pH of reverse micellar solution was determined by directly immersing a glass electrode into the transparent reverse micellar solution. The pH of the reverse micellar solution was the same as that of the aqueous solution, which indicates that the pH of the water pool is close to that of the initial buffer system, in accordance with the generally accepted conception of pH of a reverse micellar system at $[H_2O]/[surfactant] > 10$–15.[5]

Entrapping of human placental alkaline phosphatase in AOT-reverse micelles and assay of enzyme activity

The final total volume, not the less definable volume entrapped in reverse micelles,[10] was used for measurement of concentrations. The substrate, 4-nitrophenyl phosphate (17 μmol dm^{-3}–1.0 mmol dm^{-3}) in the presence of Mg^{2+} (0.3 mmol dm^{-3}) in Tris-Cl buffer (50 mmol dm^{-3}, pH 9.5), was added to AOT/isooctane solution (100 mmol dm^{-3}) and solubilised (vortex mixer). The resulting transparent solution was incubated for 5 min at 30 °C. Aliquots (10 mm^3, 0.39 μg protein) of the enzyme solution were added to the above AOT/isooctane/substrate solution (total volume 3 cm^3) by an injection technique.[10] The resulting clear microemulsion was then transferred to a cuvette and absorbance at 402 nm was continuously monitored at 30 °C. The slope of the resulting linear tracing was taken as the initial rate (v). The observed reaction rate was fitted to the Michaelis–Menten equation for estimation of kinetic parameters, eqn. (2), where

$$v = V_{max}[S]/(K_m + [S]) \quad (2)$$

V_{max} is the maximum reaction rate, K_m is the Michaelis constant, [S] represents the substrate concentration and k_{cat} was obtained by dividing V_{max} by $[E_t]$, the total concentration of the enzyme.

Results and discussion

Ionisation of 4-nitrophenol in AOT/isooctane reverse micelles

4-Nitrophenol has a pK_a value of 7.14 in aqueous solution.[5,13] The concentration of 4-nitrophenol in aqueous $NaHCO_3$–Na_2CO_3 buffer (pH 9.8) determined spectrophotometrically correlates well to the weighed amount indicating that the commercial sample of 4-nitrophenol is of sufficient purity. The UV–VIS spectrum of 4-nitrophenol in aqueous buffer solution (pH 9.8) shows a maximum peak at 402 nm (Fig. 1). Injecting the same solution into AOT/isooctane reverse micelles caused the extinction coefficient of the solution at 402 nm to decrease but a new absorption peak at 310 nm to appear (Fig. 1). One possible explanation for this hypochromicity is the screening effect from the molecules closer to the light source. However, it is more likely that ionisation of 4-nitrophenol is hindered in the reverse micellar system, which have negatively charged polar head groups. We have observed the pK_a value of the glutathione sulfhydryl group to decrease from 9.169 in aqueous solution to 8.095 in AOT reverse micelles and this change in pK_a value did not occur in positively charged cetyltrimethylammonium bromide (CTAB) reverse micelles.[14]

Impaired ionisation of 4-nitrophenol in AOT/isooctane reverse micelles is reported by Menger and Saito,[15] who suggested that 4-nitrophenol distributes in reverse micelles in two populations. That located in the water pool has a pK_a value of 7.6–7.9, close to that in the bulk aqueous solution, while that located in the interface has a pK_a value of 11.5. Ionisation at the interface is impaired due to embedding of the molecule into the interfacial phase of the system, thus causing contacts of the phenolic hydroxide group to the anionic sulfonates of AOT (Fig. 2). We observed that the pK_a value of the phenolic –OH increased by 2–3 pH units in organic solvent systems. Change in apparent pK_a values clearly demonstrates that 4-nitrophenol exists in two populations and the observed pK_a values represent the resultant of these populations. The two absorption peaks of 4-nitrophenol in reverse micelles thus represent those of the ionised form 4-nitrophenolate (absorbs at 402 nm) and those of the non-ionised form 4-nitrophenol (absorbs at 310 nm).

We proceeded to modulate occupancy in the two populations by adjusting the degrees of hydration of the system (represented by the molar ratio of $[H_2O]/[AOT]$). The ionisation of 4-nitrophenol in reverse micelles at various hydration degrees of the system was calculated according to the Henderson–Hasselbalch equation (3).

$$pH = pK_a + \log([4\text{-nitrophenolate}]/[4\text{-nitrophenol}]) \quad (3)$$

Fig. 3 shows a plot of the apparent pK_a values of 4-nitrophenol *versus* the degrees of hydration of the system. At smaller $[H_2O]/[AOT]$ ratios, the inclusive volume of the reverse micelles decreased and more 4-nitrophenol molecules are par-

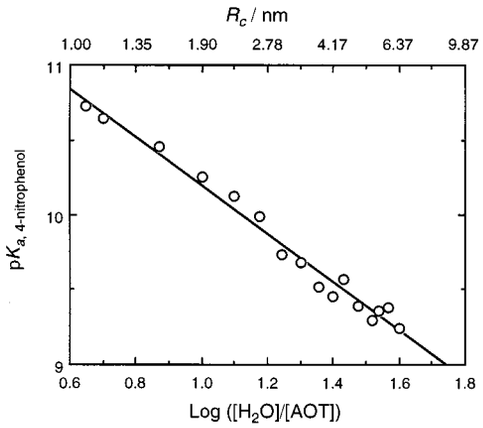


Fig. 3 Dissociation of 4-nitrophenol in AOT-reverse micelles. The core radius (R_c) of the inner cavity of the reverse micelles is shown on the right scale. The line was fitted to the equation: $pK_a = 11.81 - 1.61 \cdot \log([H_2O]/[AOT])$ ($r = 0.98$).

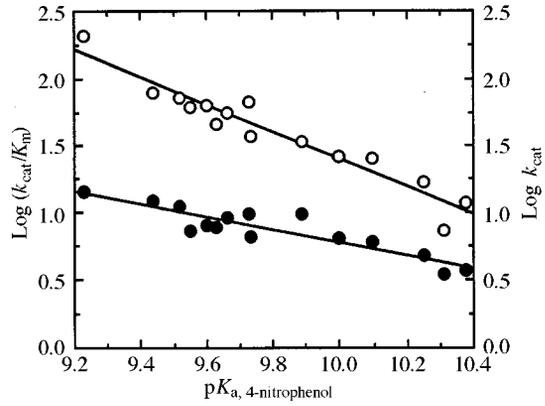


Fig. 4 Leaving group dependence of the alkaline phosphatase-catalysed hydrolysis of 4-nitrophenyl phosphate. The [H_2O]/[AOT] ratio was adjusted by varying the water content for fixed AOT concentration. The lines were fitted to the equation: $\log (k_{cat}/K_m) = 11.72 - 1.03pK_a$ ($r = 0.92$) (○) and $\log k_{cat} = 5.52 - 0.47pK_a$ ($r = 0.83$) (●), respectively.

titioned at the interface, thus the apparent pK_a value was high. The apparent pK_a value of 4-nitrophenol increased exponentially as the hydration degree of the system decreased. There is a good correlation between the apparent pK_a value of 4-nitrophenol and the [H_2O]/[AOT] ratio of the system. Thus, modulation of the inclusive volume of the small reverse micelles might offer an advantage over that of the tedious, traditional approach of synthesizing numerous derivatives.

The above observation prompted us to examine the utility of reverse micelles as a tool with which to study the substitution effect of the alkaline phosphatase-catalysed reaction. The enzymic hydrolysis of 4-nitrophenyl phosphate was therefore performed in reverse micelles with various degrees of hydration.

Alkaline phosphatase-catalysed hydrolysis of 4-nitrophenyl phosphate in reverse micelles

We found that the rate of enzymic hydrolysis of 4-nitrophenyl phosphate decreased in AOT/isooctane reverse micelles.[6] This rate inhibition can be explained by assuming an increase of pK_a value of 4-nitrophenol. Fig. 4 shows the correlation of apparent pK_a values with the first-order (k_{cat}) and second-order (k_{cat}/K_m) rate constants of the enzymic reaction. The correlation obtained is consistent with a Brønsted catalytic relationship between the pK_a value and the rate enhancement, which confirms the possible usefulness of reverse micellar systems as a model system with which to study the leaving group effect in alkaline phosphatase-catalysed reaction. However, it should be pointed out that the explanation advanced above for the decrease in k_{cat} and k_{cat}/K_m may not be unique. It is very likely that the reactant 4-nitrophenyl phosphate also partitions to some extent between the aqueous and micelle surface phases. As the [H_2O]/[AOT] ratio decreases the amount of reactant in the aqueous phase decreases, causing at least a part of the observed decrease in the kinetic parameters. Furthermore, although we have observed an identical fluorescence spectrum of the enzyme in reverse micelles and aqueous systems more global electrostatic effects involving the enzyme and/or binding interactions cannot be completely ruled out. On the other hand, in view of the dynamics of reverse micelles, the possibility that the enzyme molecule might take too much room and exhaust the substrate in that particular reverse micelle, while not having access to substrate in other aggregates, may be ruled out. The reverse micelles are constantly coalescing between micelles and exchange of contents takes place through a transient dimer, which is the prerequisite for reaction between enzyme and substrate.[5] With these caveats in mind, we interpret our results in the following manner.

The linear Gibbs-energy relationship of k_{cat} and k_{cat}/K_m of the enzymic reaction to the apparent pK_a values of 4-nitrophenol shown in Fig. 4 suggests that a uniform mechanism is utilised in reverse micelles. The first-order rate constant, k_{cat}, is a measure of how active the enzyme is in bringing about the reaction of a substrate in the E·S binary complex.[16] For an alkaline phosphatase-catalysed reaction, k_{cat} measures the dephosphorylation step. The second-order rate constant, k_{cat}/K_m, is a measure of the catalytic effectiveness of the free enzyme, including the specific binding of the substrate to the enzyme.[16] For an alkaline phosphatase-catalysed reaction, k_{cat}/K_m measures the phosphorylation step.

The Brønsted constants, β_{lg}, which measure the dependence of rate constants on the pK_a of the leaving group, were obtained from the slopes of $\log k_{cat}$ and $\log k_{cat}/K_m$ *versus* pK_a plots. From the Brønsted-type plots shown in Fig. 4, β_{lg} of -0.47 and -1.03 were obtained for k_{cat} and k_{cat}/K_m, respectively. The large leaving group effect on catalysis for both k_{cat} and k_{cat}/K_m suggests that a chemical step is the rate-limiting step. Assuming that phosphorylation of the enzyme is rate limiting, the strong leaving group effect on catalysis indicates that the apparent pK_a values observed in reverse micelles are true measures of ionisation.

We have observed normal viscosity effects on both k_{cat} and k_{cat}/K_m for the human placental alkaline phosphatase-catalysed reaction in aqueous solution (T. M. Huang, H. C. Hung, T. C. Chang and G. G. Chang, unpublished results), and these correlate with a diffusional-controlled physical step as the rate-limiting step, similar to those for *Escherichia coli* alkaline phosphatase.[17] We have suggested that the rate-limiting step of the human placental alkaline phosphatase-catalysed reaction may be different in reverse micellar and aqueous systems.[6] More recently, we observed a normal solvent kinetic isotope effect on k_{cat} and k_{cat}/K_m in aqueous solution, but an inverse isotope effect on k_{cat} and k_{cat}/K_m in AOT-reverse micelles for the enzyme (T. M. Huang, H. C. Hung, T. C. Chang and G. G. Chang, unpublished results). All these observations support our argument that a change of rate-limiting step occurs in aqueous or reverse micellar systems.

Our present results suggest that dissociation of non-covalently bound phosphate is the rate-limiting step in aqueous solution, whereas phosphorylation may be the rate-limiting step in reverse micelles owing to the rise in pK_a value of the leaving group. This finding could have important biological significance. Human placental alkaline phosphatase is a membrane-bound protein, anchoring to the lipid bilayer *via* a phosphoinositol–glycan moiety.[18] Reverse micelles represent a simple system that mimics the membranous structure. We propose that the properties of the enzyme in reverse micelles represent those in physiological conditions. The kinetic properties of human alkaline phosphatase in reverse micelles may have important implications *in vivo*.

In summary, we describe in this paper a reverse micellar system prepared by dissolving a surfactant with an anionic polar head group in isooctane, which hinders ionisation of the phenolic OH of the leaving group 4-nitrophenol and provides a convenient method of studying the leaving group effect for alkaline phosphatase-catalysed hydrolysis of 4-nitrophenyl phosphate.

Acknowledgements

This work was supported by the National Science Council, Republic of China (Grant NSC86-2314-B016-011).

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Paper 7/01918A
Received 19*th March* 1997
Accepted 29*th August* 1997