Mechanism of the Reaction of Phosphate with Purple Acid Phosphatase†

Manuel A. S. Aquino, Joo-Sang Lim and A. Geoffrey Sykes*
Department of Chemistry, The University, Newcastle upon Tyne, NE1 7RU, UK

The rate-determining step in the binding of phosphate to the pink active form ($Fe^{II}Fe^{III}$) of purple acid phosphatase monitored at 620 nm is independent of phosphate, indicating rapid binding to Fe^{II} as a first stage, followed by rate-determining bridging to the chromophoric iron(III) centre ($pK_a \leqslant 4.02$).

The active form ($Fe^{II}Fe^{III}$) of purple acid phosphatase (PAP) catalyses the hydrolysis of phosphate esters. The most extensively studied PAPs are those obtained from the porcine uterus (also referred to as uteroferrin) and from bovine spleen.[1–5] Both enzymes are glycoproteins, $M_r \approx 35\,000$, with a monomeric peptide structure and containing a novel binuclear iron centre the precise structure of which is not yet known. There is a 90% sequence homology between the two uteroferrin and beef spleen forms,[6,7] and Mössbauer,[8,9] EPR,[4,10] and resonance Raman studies[11,12] have indicated similarities between the active sites of the two proteins.

The reduced pink $Fe^{II}Fe^{III}$ form of the enzyme has a peak at 515 nm (ε = 4000 M^{-1} cm^{-1} per dimer) whereas the inactive purple form has two antiferromagnetically coupled iron(III) centres, and an absorption maximum at 550 nm (ε 4000 M^{-1} cm^{-1}).[13] The visible absorption arises in large part from tyrosine–Fe^{III} ligand-to-metal transfer (l.m.c.t.) at the non-redox active iron(III) site.[4,14] Histidine co-ordination to both irons has been proposed from extended X-ray absorption fine structure spectroscopy (EXAFS),[5] NMR,[16,17] and pulsed-EPR studies.[18] For the purple form, magnetic susceptibilities of $-J \leqslant 40$ and $\leqslant 150$ cm^{-1} have been reported indicating antiferromagnetic coupling.[4,12,19] On reduction to the pink form $-J$ values of 5 to 11 cm^{-1} are obtained.[12,16,20] Despite the range of values obtained the most likely explanation is that a μ-oxo ligand becomes protonated upon reduction to the $Fe^{II}Fe^{III}$ form.

Other binuclear non-haem iron enzymes include hemerythrin,[21,22] and ribonucleotide reductase,[23] crystal structures of which are known, and methane monooxygenase.[24,25] Purple acid phosphatases from plant and microbiological sources have also been identified. That from the kidney bean is of particular interest because it contains Fe^{III} and Zn^{II} at the active site.[19] Inhibitors for PAP activity include phosphate and its analogues, arsenate, vanadate and molybdate.[26]

The mechanism of interaction of oxyanions with the pink active form of PAP ($Fe^{II}Fe^{III}$) has not so far been ascertained, and there is clearly no understanding of the precise function of the Fe^{II} and Fe^{III} combination. We report here on a stopped-flow kinetic investigation (25 °C) of the interaction of phosphate with uteroferrin PAP. The protein was isolated from pig allantoic fluid and purified by a procedure described in the literature.[13] A large excess of phosphate (pK_a values of 2.12, 7.21 and 12.7)[27] was employed in the kinetic measurements. Acetic acid–sodium acetate was used as a buffer to give a pH range of 3.2–6.5, and the ionic strength adjusted to 0.100 M (NaCl). Spectrophotometric changes corresponded to a shift from 510 nm for the $Fe^{II}Fe^{III}$ form to 535–560 nm (pH dependent) for the phosphato product. A slower change to *ca.* 550 nm occurs subsequently consistent with oxidation to the inactive $Fe^{III}Fe^{III}$ form. First-order rate constants k_{obs} were obtained by monitoring absorbance changes corresponding to a uniphasic process at 620 nm.

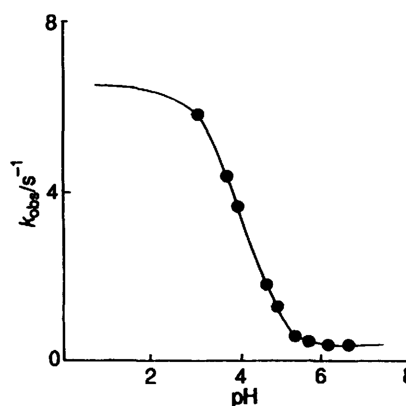
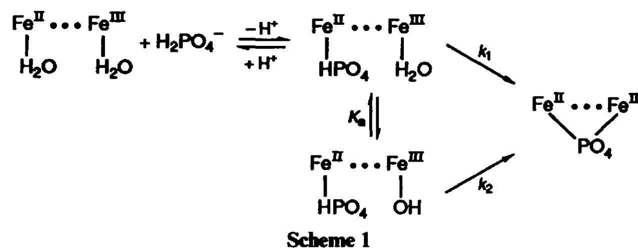


Fig. 1 Variation of k_{obs} (25 °C) with pH for the reaction of phosphate with purple acid phosphatase in the active state ($Fe^{II}Fe^{III}$), I = 0.100 M (NaCl)



Scheme 1

A surprising feature is that the reaction is in fact independent of the concentration of phosphate ($H_2PO_4^-$) in the range 4–45 mM. The reaction is also independent of acetate buffer (25–55 mM). Rate constants are however dependent on pH, Fig. 1. The mechanism proposed (Scheme 1) involves initial rapid binding of phosphate to the Fe^{II}, followed by a rate-determining pH-dependent intramolecular substitution of H_2O on Fe^{III} resulting in bridge closure. The reaction is slower at higher pH due to acid dissociation of the water (K_a). The OH^- of the conjugate-base form is displaced more slowly by the phosphate, and this may of course take place by a different mechanism involving

† *Non-SI unit employed:* M = mol dm^{-3}.

substitution of the OH^- into the phosphate moiety. The mechanism proposed gives a rate dependence (1), and from an

$$k_{obs} = \frac{k_1[H^+] + k_2K_a}{[H^+] + K_a} \quad (1)$$

unweighted non-linear fit $pK_a = 4.02 \pm 0.04$, $k_1 = 6.6 \pm 0.2$ s^{-1}, and $k_2 = 0.34 \pm 0.06$ s^{-1}. Owing to some protein instability at low pH we assume $pK_a \leqslant 4.02$. The pK_a can be assigned to acid dissociation of a water attached to the Fe^{III}. The first acid dissociation for $[Fe(H_2O)_6]^{3+}$ has a pK_a of 3.0, whereas Fe^{II} remains in the aqua form until pH $\geqslant$ 7.[28]

It is known that high-spin $[Fe(H_2O)_6]^{2+}$ has a water-exchange rate constant of *ca.* 10^6 s^{-1}, which is 10^2–10^3 times faster than the corresponding value for $[Fe(H_2O)_6]^{3+}$.[29,30] Whether the Fe^{II} in the enzyme is five- or six-co-ordinate with H_2O in the sixth position remains to be established. Either way rapid initial binding of phosphate is to be expected. This is followed by slower substitution into the iron(III) co-ordination sphere, in the phosphate-independent step. The iron(III) chromophore of the PAP gives rise to the intense colour of both oxidation states with no UV/VIS absorbance changes observable for reaction at the iron(II) site. Because of the proximity of the phosphate to Fe^{III} a somewhat faster bridge-closure substitution process at the iron(III) relative to the iron(II) site might have been expected. Bridge closure of PO_4^{3-} has previously been studied for a binuclear cobalt(III) complex.[31]

The observation that rate constants are independent of acetate suggests that there is no significant co-ordination of acetate prior to phosphate as observed in the reaction of phenyl phosphate with the model complex $[Fe_2(\mu\text{-}O)(\mu\text{-}O_2CCH_3)_2L^2]^{2+}$, L = 1,4,7-trimethyl-1,4,7-triazacyclononane.[32] The reaction of phenyl phosphate (pK_a values of 0.30 and 5.63[33]) with the enzyme PAP was also shown to be independent of phenyl phosphate concentration at pH 4.6, and gives a similar rate constant consistent with Scheme 1.

These studies are being extended to include consideration of the mechanism of hydrolysis of different phosphate esters. Of interest is the ability of zinc(II) to replace iron(II) in plant PAP and initiate the same chemistry. The lability and non-redox activity of zinc supports such a functional role, and other metal ions could be implicated in a similar way.

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