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New Insights into the Mechanism of Purple Acid Phosphatase through ^{1}H NMR Spectroscopy of the Recombinant Human Enzyme

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Purple acid phosphatases (PAPs) are ubiquitous iron-containing enzymes characterized by their acidic pH optima and their intense purple color due to a TyrO-to-Fe^{III} charge-transfer transition.[1] The best-studied mammalian PAPs are those from bovine spleen (BSPAP), porcine uterine fluids (uteroferrin, Uf), and rat bone, which have been proposed to be involved in iron transport,[2] the immune response,[3] and bone resorption.[4] Consequently, PAP is a potential therapeutic target for the structure-based design of inhibitors for the treatment of osteoporosis and other disorders.

The amino acid residues coordinating the metal ions are conserved in all PAPs.[1] Crystal structures of inactive pH or redox forms of the PAPs from kidney bean,[5] recombinant rat bone,[6] and Uf[7] have shown that active PAPs contain an Fe^{III} ion coordinated to Tyr Oη, a His Nϵ, and an Asp Oδ2, in addition to a divalent metal ion (Fe, Zn, or Mn) coordinated by a His Nϵ, a His Nδ, and an Asn Oδ. A hydroxide ion and an Asp Oδ2 bridge the two metal ions. To elucidate the enzymatic mechanism, we have measured ^{1}H NMR spectra of recombinant single polypeptide human PAP (recHPAP)[8] in its enzymatically active $Fe^{III}-Fe^{II}$ form at, above, and below its pH optimum.[9] The spectra of inhibited forms of the enzyme, containing fluoride and phosphate, have also been acquired. The latter anions are generally considered as analogues of the substrates hydroxide and phosphate esters, respectively. Our results demonstrate that the group responsible for p$K_{a,1}$ functions as a "gatekeeper", whose protonation state controls anion binding to the mixed-valent dinuclear site.

The downfield region of the ^{1}H NMR spectrum of recHPAP in H_2O buffer at pH 5.5 (Figure 1A) shows the presence of six broad hyperfine downfield-shifted signals (I−VI), as well as two broad upfield-shifted signals at −27.8 and −69.0 ppm (not shown). These signals are assigned to protons of ligands to the dinuclear paramagnetic iron center, as found for other PAPs.[10,11] This conclusion is further supported by their very short T_1 longitudinal relaxation times. The hyperfine-shifted NMR signals for recHPAP, their chemical shifts, and the related relaxation times, as well as the difference spectra obtained upon selective saturation, are virtually identical to those previously reported for Uf.[10b,c] These NMR data, together with the close resemblance of the EPR spectra of these two enzymes,[8] indicate that the molecular and electronic structure of the active site of recHPAP is essentially identical to that of the recently reported structure of Uf.[7]

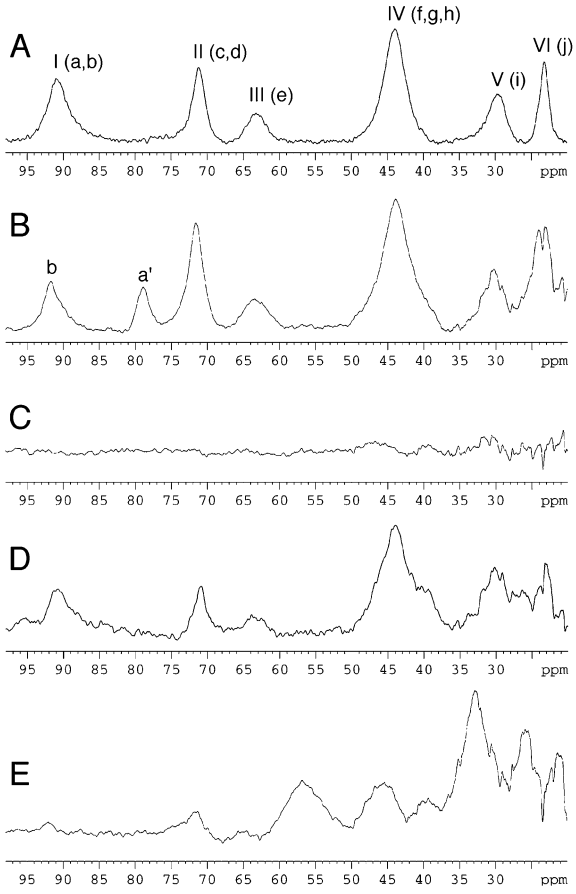

Figure 1. ^{1}H NMR (500 MHz) spectra at 292 K of native recHPAP at pH 5.5 (A) and pH 4.1 (B). The spectra obtained in the presence of fluoride at pH 4.1 (C) and in the presence of phosphate at pH 5.5 (D) and 4.1 (E) are shown. Other sample conditions are given in the text.

NMR spectra of recHPAP were measured at pH < p$K_{a,1}$ = 4.6, at the optimal pH = 5.5 for catalytic activity,[8] and at pH > p$K_{a,2}$ = 6.7. No differences were observed in the hyperfine-shifted signals at pH 5.5 and pH 7.1, indicating that p$K_{a,2}$ does not involve a metal ligand. We suggest that p$K_{a,2}$ is instead due to ionization of one of the two conserved His residues near the dinuclear site. In contrast, decreasing the pH to <5.5 results in modest shifts for all signals with the exception of signal *a*, which is shifted by 12 ppm upfield (signal *a*′, Figure 1B) at pH 4.1. The position of signal *a*′ remains unaltered at pH < 4.8, while its intensity, which is unchanged in D_2O, increases with decreasing pH over the pH range 4.1−4.8,

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with a concomitant decrease in the intensity of signal *a*. Thus, the two species (deprotonated at pH > p$K_{a,1}$ and protonated at pH < p$K_{a,1}$) represented by signals *a* in Figure 1A and *a′* in Figure 1B, respectively, are in slow exchange on the NMR time scale. The observed changes in the NMR signals indicate that p$K_{a,1}$ is due to a metal-bound moiety that is deprotonated in the active enzyme. A plausible candidate for such a group is a metal-bound water, which upon deprotonation has been proposed to act as the nucleophile in phosphate ester hydrolysis by PAPs. Two alternatives have been proposed for the catalytic nucleophile: a hydroxide terminally bound to Fe^{III},[12] and the bridging hydroxide.[13]

To probe the nature of the nucleophile, we used ^{1}H NMR to monitor the interaction of recHPAP with fluoride, an uncompetitive inhibitor that forms an inactive ternary E·S·F complex,[14] and with phosphate, a product of the reaction and a competitive inhibitor. ^{1}H NMR spectra of recHPAP in the presence of fluoride were recorded at pH 7.1, 5.5, and 4.1. The spectra at pH 5.5 and 7.1 are identical to those in the absence of fluoride, indicating that fluoride does not bind at pH ≥ 5.5. At pH 4.1, however, addition of fluoride results in the disappearance of all hyperfine-shifted signals (Figure 1C), indicating that fluoride significantly perturbs the magnetic interaction between the iron ions. The most plausible explanation is that fluoride replaces a bridging ligand. The fact that this occurs only when the group responsible for p$K_{a,1}$ is protonated supports the proposal that p$K_{a,1}$ corresponds to deprotonation of a coordinated water molecule.[12,13]

The NMR spectra of recHPAP in the presence of saturating concentrations of phosphate over the pH range 7.1−5.5 (Figure 1D) are essentially identical to those of the native enzyme, indicating that phosphate does not bind to the dinuclear center under these conditions. On the other hand, significant spectral changes are observed at pH 4.1 (Figure 1E), showing that phosphate does bind to the dinuclear site of recHPAP at pH < p$K_{a,1}$. In contrast with fluoride binding at this pH, the observation of broad hyperfine-shifted signals in the spectrum of the phosphate complex indicate that, as with Uf,[15−16] binding of phosphate only slightly perturbs the magnetic interaction between the metal ions. This conclusion implies that phosphate does not replace a bridging ligand. Most importantly, the NMR spectra demonstrate that phosphate does *not* interact directly with the dinuclear metal center at the optimal pH for enzymatic activity. This result suggests that the substrate interacts only with protein residues prior to nucleophilic attack by a coordinated hydroxide.

In conclusion, the present work demonstrates that binding of inhibitory anions to the dinuclear mixed-valent site of recHPAP is controlled by protonation of a ligand to the dinuclear center. The correlation between the pK_a values observed in kinetics studies and the pH-dependent spectroscopic changes strongly suggests that this ligand is the nucleophilic hydroxide that attacks the phosphate ester substrate.

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Supporting Information Available: Equations for ring-flipping process (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) NMR experiments were performed on Bruker Avance spectrometers operating at 500 and 600 MHz. All spectra were calibrated by assigning a chemical shift of 4.88 ppm to the residual water signal at 292 K. Data acquisition and processing were performed with a Bruker software package (XWINNMR). 1D NOE spectra on hyperfine-shifted signals were recorded in difference mode after selective irradiation of hyperfine-shifted and fast-relaxing resonances.[16] Two reference frequencies were set to the immediate left and right of the irradiated signal, according to the scheme on−off (left)−on−off (right). Both repetition (acquisition time + relaxation delay) and irradiation times were 45 ms. NMR experiments with native and fluoride-bound enzyme were performed under aerobic conditions, while those with phosphate were carried out under anaerobic conditions (10−15 cycles of argon flushing before phosphate addition). For NOE and T_1 relaxation experiments at pH 5.5, the sample contained 750 μM recHPAP in a 50 mM Na-acetate, 50 mM MES, 50 mM HEPES, and 0.5 M KCl buffer. For pH-dependent studies, the sample was split into three portions (500 μL, 360 μM), and the pH of each aliquot was adjusted by three cycles of 1:10 buffer exchange using a Microcon (10 kDa cutoff). D_2O buffers had the same buffer composition. The pH of the sample was controlled by measuring the pH of the flow-through. Visible spectra were recorded throughout the experiments on a Cary 50 in order to ensure the $Fe^{III}-Fe^{II}$ state of the enzyme. For ligand binding studies 10 mM fluoride or 50 mM phosphate from a stock solution at the correct pH was used.

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(11) The assignment of the hyperfine shifted signals previously proposed for Uf[10b,c] was largely confirmed for recHPAP through selective saturation experiments. However, the strength (35−70% of the saturated signals) of the observed connectivities (in Figure 1) involving resonances a (91.0 ppm) and k (14.2 ppm), and c (71.3 ppm) and e (63.1 ppm) prompted us to interpret them as due to exchange phenomena rather than to NOE. We therefore assign these signals to the Fe(III)-bound Tyr Hϵ and Hδ, rather than to the β-CH_2 protons of tyrosine as proposed for Uf.[10b,c] All other observed dipolar connectivities support the proposed assignment. The coordinated tyrosinate must give rise to such magnetization transfer via a ring-flipping process (estimated upper limit for rate = 130 s^{-1}; see Supporting Information). Because signals b and f disappear in D_2O, they must be due to exchangeable NH protons of coordinated His residues, as proposed previously.[10b,c]

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