Spectroscopic Characterization of a Ternary Phosphatase−Substrate−Fluoride Complex. Mechanistic Implications for Dinuclear Hydrolases

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Ser/Thr protein phosphatases (PP1 and PP2B)[1] and the purple acid phosphatases (PAPs)[2] comprise a subset of hydrolases with closely related bimetallic active sites[3] (Scheme 1). The protein phosphatases are involved in cell signaling and transduction,[1] while the PAPs perform an as yet undetermined role.[2] For PP2B and the PAPs, the active-site metal ions are Fe(III) and either Fe(II) or Zn(II). Crystal structures[1a,d,2b] and EXAFS analysis[4] show that the substrate analogue phosphate and other tetraoxo anions bind as a bidentate bridge to the bimetallic center. If this binding mode applies to the phosphate ester substrate (Scheme 1A), then the only nucleophile available for hydrolysis is the hydroxo bridge, which is appropriately oriented to achieve the inversion of configuration observed at the P center.[5] A proposed alternative mechanism is the attack of a terminal Fe(III)−OH on a monodentate substrate coordinated to the M(II) ion (Scheme 1B).[6] It is difficult to distinguish between these two options due to the difficulty of characterizing the short-lived activated enzyme−substrate complex. The use of an anion, such as fluoride, that can replace the nucleophilic hydroxide may be a useful approach to trap the activated complex and probe the hydrolase mechanism. In this paper, we report the spectroscopic characterization of such a ternary enzyme−substrate−fluoride complex for uteroferrin (Uf, the PAP from porcine uterus) that sheds light on the mechanism of phosphate ester hydrolysis at a bimetallic active site.

Steady-state kinetic measurements show that fluoride ion is an uncompetitive inhibitor of Fe(III)Zn(II)uteroferrin (FeZnUf) at pH 5, the pH optimum for enzyme activity, with a K_i value of 0.2 mM. The uncompetitive behavior is indicated by parallel lines in the Lineweaver−Burk plot as well as nonlinear least-squares fits of the FeZnUf data to the Michaelis−Menten equation (Figure S1).[7] The uncompetitive behavior of F^- shows that it can replace the nucleophilic hydroxide and suggests that a ternary FeZnUf·substrate·F complex can be formed.

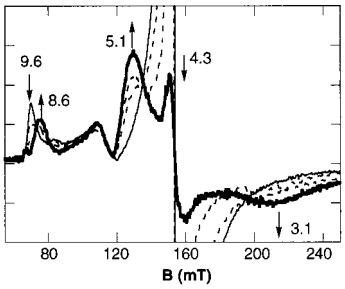
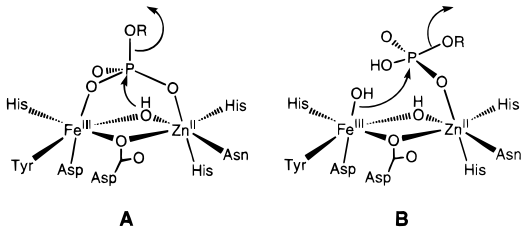


Figure 1. Titration of FeZnUf·PO_4 with fluoride ion as monitored by EPR at 4.2 K. The light solid line corresponds to FeZnUf·PO_4, and the dark solid line corresponds to the ternary FeZnUf·PO_4·F complex. Dashed-line spectra reflect intermediate stages of the titration. [FeZnUf] = 0.3 mM; [PO_4] = 40 mM; [F] = 0, 0.24, 0.50, 2.0, and 20 mM.

Scheme 1. Alternative Mechanistic Schemes for Phosphate Ester Hydrolysis at the Dinuclear Site of Purple Acid Phosphatases



The rich and well-characterized spectroscopic properties of uteroferrin[5,8,9] provide a unique opportunity to characterize this ternary enzyme−substrate−fluoride complex. FeZnUf·PO_4 exhibits an EPR spectrum with signals at $g = 9.6$ and 4.3 associated with the high-spin Fe(III) center ($S = {}^5/_2$, $E/D = 0.33$). Titration of FeZnUf·PO_4 with F^- results in the disappearance of the $g = 9.6$ and 4.3 signals concomitant with the appearance of signals at $g = 8.6$ and 5.1, corresponding to an $S = {}^5/_2$ center with an E/D of 0.18 (Figure 1). The spectral changes are associated with a K_d value for F^- of 0.4 mM, which is in reasonable agreement with the kinetically determined K_i of 0.2 mM. This dramatic change in the EPR properties suggests that F^- binds to the high-spin Fe(III) ion. The $E/D = 0.18$ species can thus be associated with a ternary FeZnUf·PO_4·F complex. Identical EPR spectra (Figure S2) are obtained for other ternary complexes wherein a slow substrate, such as phenyl phosphate or AMP, or arsenate is used in lieu of phosphate, implying that the substrate binds to the bimetallic site in the same manner as the product phosphate.[10]

EXAFS analysis of the Fe and Zn K-edge spectra of the ternary complex (Figure 2, Table S1) shows that the phosphate remains bridged to the dinuclear center. A phosphorus scatterer is observed at ca. 3.2 Å in both the Fe and Zn spheres. Furthermore, the Fe−Zn distance of ca. 3.4 Å is comparable to the distances observed for FeZnUf·PO_4 (3.3 Å) and FeZnUf·MoO_4 (3.4 Å),[4] suggesting that the ternary fluoride complex is similarly tribridged. Since the metal ions are six-coordinate in FeZnUf·PO_4, fluoride most likely replaces hydroxide as the third bridging ligand to form the ternary FeZnUf·PO_4·F complex (Figure 2, inset). However, the

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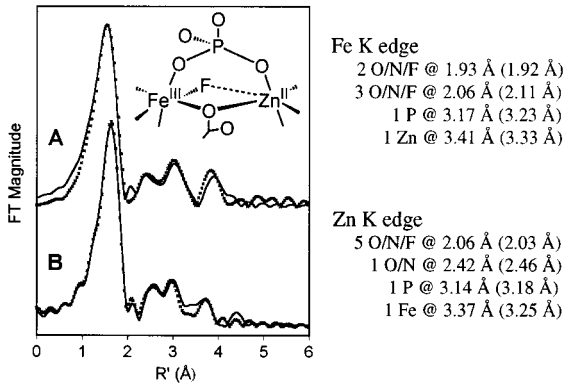

Figure 2. EXAFS data and the corresponding best fits for FeZnUf·PO_4·F. (A) Fe K-edge and (B) Zn K-edge. The inset shows the proposed structure of the ternary complex. The numbers in parentheses represent the best fit data for FeZnUf·PO_4.

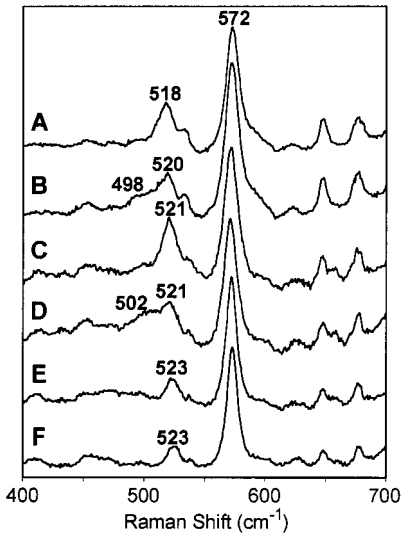

Figure 3. Resonance Raman spectra of (A) FeZnUf ($I_{521/572}$ = 0.50), (B) FeZnUf $H_2{}^{18}O$ ($I_{521/572}$ = 0.28), (C) FeZnUf·AsO_4 in $H_2{}^{16}O$ ($I_{521/572}$ = 0.56), (D) FeZnUf·AsO_4 in $H_2{}^{18}O$ ($I_{521/572}$ = 0.33), (E) FeZnUf·AsO_4·F in $H_2{}^{16}O$ ($I_{521/572}$ = 0.27), and (F) FeZnUf·AsO_4·F in $H_2{}^{18}O$ ($I_{521/572}$ = 0.30) obtained at 20 °C with 514.5-nm excitation at 20-mW power at the sample.

EXAFS analysis of the ternary fluoride complex also shows that, relative to FeZnUf·PO_4, the Fe coordination sphere contracts as indicated by the 0.05-Å decrease of the 2.1-Å subshell, while the Zn coordination sphere expands by 0.03 Å (Figure 2). Thus, the formation of the ternary complex appears to induce a shift of ligand from the Zn sphere to the Fe sphere, presumably caused by the substitution of fluoride for the hydroxide bridge.

Resonance Raman studies support the notion that fluoride displaces the hydroxo bridge. Excitation into the ca. 520-nm band of FeZnUf complexes affords vibrations typical of iron−tyrosinate chromophores.[9] In the 500−600-cm^{-1} region, there are features at 521 and 572 cm^{-1}, the latter being assigned to the ν(Fe−O−Tyr) mode. Superimposed on the former is another vibration that is sensitive to $H_2{}^{18}O$. This mode downshifts 20 cm^{-1} for FeZnUf and FeZnUf·AsO_4 (Figure 3B and D),[11] and the ratio of the intensities of the 521- and 572-cm^{-1} peaks (I_{521}/I_{572}) is concomitantly halved. Since the hydroxo bridge is the only solvent-derived ligand that remains when tetraoxo anions bind to the dinuclear center,[2b,4] the solvent-sensitive mode can be unequivocally assigned to the Fe(III)−μ-OH mode. This vibration is likely resonance enhanced by coupling to the Fe−tyrosinate chromophore trans to it. The addition of F^- causes the disappearance of this Fe(III)−μ-OH mode in FeZnUf·AsO_4 (Figure 3E), as indicated by the small I_{521}/I_{572} ratio and the lack of an ^{18}O effect on its Raman spectrum (Figure 3F), suggesting that the hydroxo bridge is displaced by fluoride in the ternary complex.[12]

Surprisingly, the resonance Raman spectrum of FeZnUf·(PO_4) does not show an Fe(III)−μ-OH vibration (Figure S3, I_{521}/I_{572} = 0.33 and no ^{18}O effect), suggesting that this bond weakens when phosphate binds. Furthermore, CD/MCD studies on Fe(III)Fe(II)Uf show that the Fe(II)−μ-OH bond is strengthened in the phosphate complex.[13] These observations suggest that, in the phosphate complex, the hydroxide bridge is more strongly bound to the M(II) ion. This binding mode is supported by the decrease, from 20 to 6 cm^{-1} ($H = JS_1 \cdot S_2$), of the antiferromagnetic coupling between the metal centers in Fe(III)Fe(II)Uf upon phosphate binding.[14]

The spectroscopic data obtained suggest that the structure of the FeZnUf·(substrate)·F complex consists of a bridging substrate and a bridging fluoride with no ligated hydroxide (Figure 2, inset). If the ternary fluoride complex is, indeed, a good model of the activated enzyme−substrate complex, then these studies would favor a mechanism wherein the nucleophile is the hydroxo bridge. However, the lower nucleophilicity of the bridging hydroxide argues against mechanism A in Scheme 1. We thus propose that substrate binding triggers the shift of the hydroxide bridge toward the M(II) ion, converting it into a quasi-terminal and more nucleophilic M(II)−OH moiety, which is ideally set up for an in-line back-side attack on the phosphorus center. The idea of the bridging hydroxide as the attacking nucleophile has also been suggested for other hydrolases with (μ-hydroxo)(μ,η^1-carboxylato)dimetal centers and supported by crystallographic results on dizinc leucine aminopeptidase and dimanganese arginase complexed with transition-state analogues.[15] This proposed mechanistic strategy thus provides a rationale for the ubiquity of such (μ-hydroxo)dimetal centers in the active sites of peptidases, amidases, and phosphatases.[3]

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Note Added in Proof. An EPR spectrum similar to that in Figure 1 has just been reported for the ternary enzyme−phosphate−fluoride complex of the bovine spleen PAP.[16]

Supporting Information Available: Figure S1, giving steady-state kinetic data demonstrating that fluoride is an uncompetitive inhibitor; Figure S2, showing the EPR spectra of ternary fluoride complexes with slow substrates; Figure S3, showing the resonance Raman spectra of FeZnUf·PO_4 in H_2O and $H_2{}^{18}O$; and Tables S1 and S2, showing the EXAFS fits for FeZnUf·PO_4·F (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Similar solvent-dependent downshifts were also observed for Fe(III)Fe(II)Uf and its anion complexes. Sanders-Loehr, J.; David, S. S.; Que, L., Jr., unpublished observations.

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