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Characterization of the Phosphoserine of Pepsinogen Using ^{31}P Nuclear Magnetic Resonance: Corroboration of X-ray Crystallographic Results†

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ABSTRACT: The endogenous phosphoserine residue in porcine pepsinogen has been titrated with use of phosphorus-31 nuclear magnetic resonance (^{31}P NMR). It has an observed pK_{a_2} of 6.7 and a narrow line width ($\simeq$10 Hz). The phosphate can be readily removed by an acid phosphatase from potato; however, it is resistant to hydrolysis by several alkaline phosphatases. The X-ray crystal structure of porcine pepsinogen at 1.8-Å resolution [James, M. N. G., & Sielecki, A. (1986) *Nature (London) 319*, 33–38] shows a rather weak and diffuse region of electron density in the vicinity of the phosphorylated serine residue. This suggests considerable dynamic mobility or conformational disorder of the phosphate. In order to define more fully this behavior, the NMR data have been used to corroborate these crystallographic results. All these physical data are consistent with a highly mobile phosphoserine residue on the surface of the zymogen and freely exposed to solvent. In addition, certain properties of this phosphoserine moiety on pepsinogen are similar to those of one of the phosphorylated residues of ovalbumin. The possible significance of this is discussed.

Both pepsin and pepsinogen have long been known to contain one atom of phosphorus per molecule (Northrop, 1939), this being present as phosphoserine (Flavin, 1954). Enzymatic removal of this phosphate group has no effect on the activity of either pepsin or pepsinogen (Perlmann, 1952), and its function is still unknown. Early studies on the enzymic dephosphorylation of pepsinogen (Perlmann, 1955, 1958) suggested that the phosphate was present in a diester linkage (i.e., linking together two regions of the protein, as, for example, does a disulfide bridge). Later studies on the kinetics of dephosphorylation using a potato phosphatase (Clement et al., 1970) led these workers to favor a phosphate monoester moiety. This was further confirmed by ^{31}P NMR (Edmondson & James, 1979). However, the only evidence provided for this were the chemical shifts of the phosphoryl group of pepsinogen at two pH extremes. These values were quoted to be identical with those of free *O*-phosphoserine.

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The crystal structure of pepsinogen is known at 1.8-Å resolution (James & Sielecki, 1986). During the course of the X-ray structure refinement, a diffuse, electron-poor peak associated with an extension of the O^{γ} of serine-68 (sequential residue numbering of porcine pepsin) was observed in the electron density maps. Conclusive identification of this feature as a phosphate moiety was not possible from the X-ray data alone, as it behaved poorly during the least-squares refinement (Sielecki and James, unpublished data). In order to provide further evidence of the phosphoserine residue in this preparation of pepsinogen and to characterize the NMR spectrum more completely, we undertook to study this phosphoprotein in more detail using ^{31}P NMR.[1]

Materials and Methods

Chemicals. Pepsinogen from porcine intestinal mucosa, all acid and alkaline phosphatases, and *O*-phospho-L-serine (free acid form) were purchased from Sigma. D_2O (99.8%) and Chelex 100 (100–200 mesh) were from Bio-Rad. Ultrapure urea (Schwarz) was recrystallized before use. D_2O and all buffers were treated with Chelex before use.

Treatment of Enzyme. Pepsinogen was dissolved in either 10 mM MOPS, 20 mM Tris-HCl, and 1 mM EDTA, pH 8, or 10 mM MES and 20 mM Tris-HCl, pH 8, and dialyzed vs. the appropriate buffer containing added Chelex resin. Some samples were also applied to a column of Sephadex G100 prior to Chelex treatment. These procedures were performed at 4 °C. Protein solutions were concentrated with a Millipore CX-10000 immersible filter with agitation as described previously (Williams et al., 1985). The protein concentration was determined from a molar extinction coefficient of 51.2×10^3 at 278 nm (Arnon & Perlmann, 1963).

Prior to NMR analysis D_2O was added and the pH was checked and adjusted with small aliquots of HCl or KOH. pH values quoted are meter readings unadjusted for the effect of D_2O on the glass electrode. During pH titration experiments, the solutions of pepsinogen were kept above pH 5 in order to minimize autocatalytic degradation to pepsin. A standard solution of *O*-phospho-L-serine (12.5 mM) was titrated under identical conditions. The pK_a and Hill coefficient were calculated from the titration data by a computer program written by Dr. J. R. Brisson.

Denaturation of Pepsinogen. To a concentrated solution of pepsinogen in 10 mM MES and 20 mM Tris-HCl (pH 8) were added SDS and 2-mercaptoethanol both to 1%, EDTA to 1 mM, and D_2O to 14%. The final concentration of pepsinogen was 10.8 mg/mL. Chelex resin was added to the mixture to prevent metal ion contamination from the added SDS, and the protein was incubated under these denaturing conditions at 30 °C for 6 h. Pepsinogen was also denatured by dialyzing against 8 M urea, 25 mM DTT, 1 mM EDTA, and 50 mM Tris-HCl, pH 8, for 24 h at room temperature. D_2O was added and the pH adjusted before NMR analysis.

Dephosphorylation of Pepsinogen. To 3 mL of pepsinogen in 10 mM MES, 20 mM Tris-HCl, and 5 mM $MgCl_2$ at either pH 8.0 or pH 5.8 was added the required alkaline or acid phosphatase. Dephosphorylation was allowed to proceed for 5–10 h at 30 °C. D_2O was added and the pH adjusted prior to NMR analysis. The final concentration of pepsinogen was 3 mg/mL.

[1] Abbreviations: ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

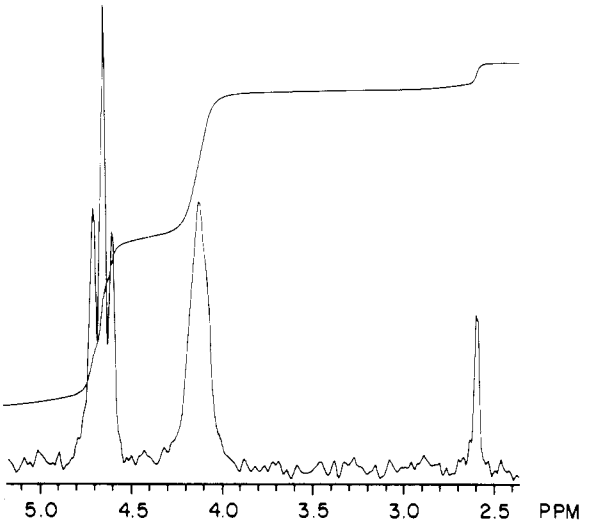

FIGURE 1: ^{31}P NMR spectrum of pepsinogen (4.12 ppm) and standard phosphoserine (4.67 ppm) in 10 mM MOPS, 10 mM Tris-HCl, 1 mM EDTA, and D_2O, pH 7.1. The spectrum was recorded during an experiment to calculate the T_1 by progressive saturation and represents a delay time of 7 s. The computer integral is also shown. The minor peak at 2.6 ppm was due to inorganic phosphate. See text for details of NMR parameters.

The activities of the phosphatases were determined before and after NMR analysis. Activities were measured spectrophotometrically at 410 nm with 1 mM *p*-nitrophenyl phosphate at pH 8 or pH 5.8 at 21 °C.

^{31}P NMR Spectroscopy. ^{31}P NMR was performed on a Nicolet NT 300 WB spectrometer at 121.5 MHz. The temperature was 21 ± 1 °C. Samples of approximately 4 mL were placed in 8 × 12 mm flat-bottom NMR tubes (Wilmad) fitted with a Vortex supressor. Spectra were acquired as described previously (Williams et al., 1985) and are presented with an additional line broadening of 5 Hz. Chemical shifts are referenced to 85% H_3PO_4.

The concentration of endogenous phosphate in pepsinogen was calculated by determining the T_1 (spin–lattice) relaxation times of both phosphoserine and pepsinogen under identical experimental conditions. To a solution of pepsinogen (final concentration 0.36 mM) in 10 mM MES, 20 mM Tris HCl, and 1 mM EDTA, pH 7.4, were added phosphoserine to 0.405 mM and D_2O to 12% (v/v). The final pH was 7.1.

The relaxation times were determined by the progressive saturation method (Freeman & Hill, 1969). A pulse angle of 90° was applied, and after an acquisition period plus a variable delay time, the next 90° pulse was applied. Seven delay times ranging from 0.4 to 7.0 s were used. The acquisition time was 0.25 s, the spectral width was ±2000 Hz, and 5000 scans were acquired per experiment. The relative peak areas were determined from peak height at half-width (for phosphoserine, the height of the central peak was used) and also by computer integration.

Results

The ^{31}P NMR spectrum shown in Figure 1 was obtained during an experiment to calculate the T_1 by progressive saturation and represents a delay time of 7 s (see Materials and Methods for details). The endogenous phosphoserine of pepsinogen can be seen at 4.12 ppm, while standard phosphoserine has a resonance of 4.67 ppm. The small peak at 2.59 ppm was assigned to contaminating inorganic phosphate. The T_1 relaxation times of the phosphate moiety of pepsinogen and of phosphoserine under identical experimental conditions were 2.8 and 3.3 s, respectively. From a comparison of the peak

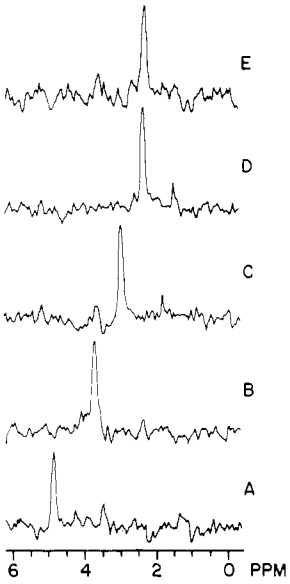

FIGURE 2: ^{31}P NMR spectra of pepsinogen (0.38 mM) in 20 mM MOPS, 20 mM Tris-HCl, 1 mM EDTA, and 12.5% D_2O: A, pH 8.23; B, pH 6.97; C, pH 6.57; D, pH 6.23. E: Pepsinogen (0.138 mM) denatured in 8 M urea, 25 mM DTT, 50 mM Tris-HCl, 1 mM EDTA, and 12.5% D_2O, pH 6.24. 2000 scans each. See text for details.

areas at maximum resonance intensity with the known concentrations of both pepsinogen and phosphoserine, a value of 0.98 phosphate group per molecule of pepsinogen was obtained. This is in agreement with the earlier observations of Northrop (1939), which were based on elemental analysis of pepsinogen.

It has been reported that pepsinogen can be dephosphorylated by both acid phosphatases (Perlmann, 1952; Clement et al., 1970) and intestinal alkaline phosphatase (Perlmann, 1952). We have therefore used ^{31}P NMR to monitor the effect of a variety of phosphatases on the phosphoserine group of pepsinogen. Pepsinogen was incubated as described in Materials and Methods with alkaline phosphatases from *Escherichia coli* (25.7 units), pig intestinal mucosa (3.2 units), and beef liver (0.24 units). We were unable to detect any loss of phosphoserine after incubation for 5 h with either of the mammalian phosphatases. After 10-h incubation with *E. coli* enzyme, over 70% of the phosphoserine remained intact. In contrast, incubation of pepsinogen with a potato acid phosphatase (0.83 unit) for 5 h at pH 5.8 completely removed its phosphoserine resonance with the concomitant production of a signal at 1.4 ppm which, from its chemical shift at this pH value and line width, was assigned to free inorganic phosphate (data not shown).

The endogenous phosphoserine group was titratable over the range pH 8.23–5.35. Representative spectra are shown in Figure 2. Over this pH range the line width (minus the additional line-broadening value of 5 Hz) was 10.6 ± 1.3 Hz (n = 6). Denaturation of pepsinogen in 8 M urea and 25 mM DTT plus 1 mM EDTA did not affect either the line width or the chemical shift of the resonance with respect to undenatured pepsinogen at either pH 6.24 (Figure 2E) or pH 7.44 (not shown). Over this pH the chemical shift followed closely that of free phosphoserine (Figure 3). The pK_{a_2} and Hill coefficients were calculated from the titration data of undenatured pepsinogen. The pK_{a_2} for pepsinogen was found to be 6.70 ± 0.01 and indicates that the phosphate group is present in the dianionic form at physiological pH. The pK_a for phosphoserine under identical conditions was 5.82. As noted by Vogel and Bridger (1983), a high pK_a value (i.e., higher than that of free phosphoserine) is a property frequently seen with other phosphoproteins, for example, ovalbumin

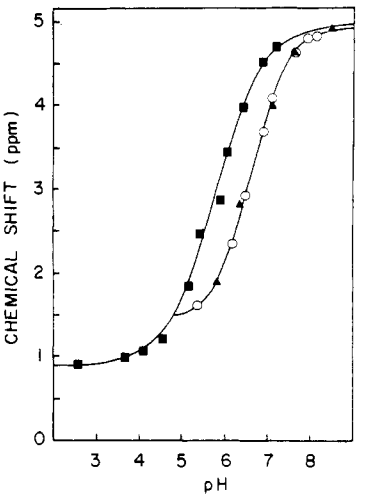

FIGURE 3: pH titration curves calculated from ^{31}P NMR chemical shifts of pepsinogen (○), phosphoserine (■), and pepsinogen denatured in 1% SDS and 1% 2-mercaptoethanol (▲). All titrations were performed in the presence of 1 mM EDTA. See text for details and NMR parameters.

(Vogel & Bridger, 1982), tropomyosin (Vogel & Bridger, 1983), human salivary proteins (Bennick et al., 1981), and phosvitin (Vogel, 1983). The Hill coefficient for pepsinogen was 0.981, which signifies that there is no major interaction with charged groups in the vicinity of the phosphoserine. Denaturation in urea (above) or in SDS under reducing conditions (Figure 3) did not affect the titration behavior of pepsinogen.

Part of the electron density map with the refined molecular model or porcine pepsinogen (James & Sielecki, 1986) is shown in Figure 4. Serine-68 has the phosphoryl group covalently attached to O^{γ}; the electron density of this phosphoryl group is weak, indicating a high degree of disorder. This map also shows that the side chain of glutamate-70 is poorly ordered. The side chains of both residues point to a large solvent cavity in the crystals and make no intermolecular or intramolecular contacts or hydrogen bonds. The Asx-type turn (Baker & Hubbard, 1984) involving the side chain of Thr-67 and the main-chain NH of Glu-69 ensures that the path of the polypeptide chain changes direction with the result that Ser-68 points toward the solvent. Phosphorylated Ser-68 would therefore be expected to titrate, to be freely mobile, and to be accessible to enzymic modification. This confirms the conclusions inferred from the NMR results.

Discussion

During the least-squares refinement of the structure of porcine pepsinogen with the X-ray crystallographic data, it was not possible to establish conclusively that the side chain of serine-68 was phosphorylated (Sielecki et al., unpublished data). The isotropic thermal motion parameters refined to unacceptably large values such that the contribution to the X-ray scattering from the modeled phosphate was less than that from a solvent water molecule. The relatively weak and diffuse electron density on the final $2|F_o| - |F_c|$ map is shown in Figure 4. The electron density at the oxygen atoms falls off to near noise levels, and the phosphoryl oxygen atoms were positioned primarily on acceptable staggered conformation relative to the other atoms of serine-68.

Using ^{31}P NMR, we were able to show that the phosphoserine group was present at full occupancy on pepsinogen and that it exhibited a number of characteristics, in particular, unrestrained mobility, which could explain the crystallographic

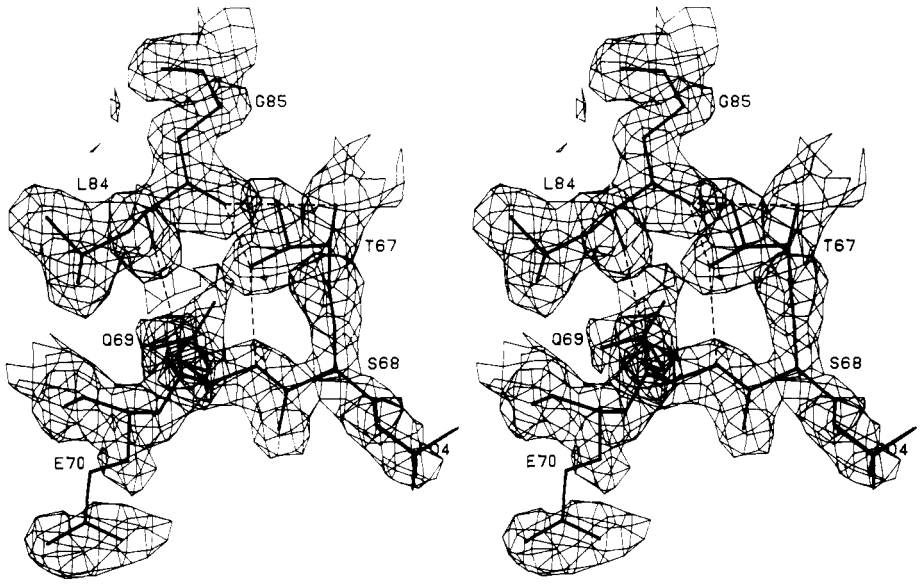

FIGURE 4: Stereoscopic view of two strands of polypeptide chain from porcine pepsinogen in the vicinity of Ser-68. Electron density contours are from the 1.8-Å resolution [$2|F_o| - |F_c|$, α_c map] and are drawn at +0.40 e Å^{-3} ($|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes; α_c are the calculated phase angles based on the refined molecular model of pepsinogen). The phosphoryl group on Ser-68 has a weak associated electron density; it points toward a large solvent cavity in this crystal form. Hydrogen-bonding interactions are denoted by dashed lines. The polypeptide chain changes direction at Ser-68 partly as a result of an Asx-type turn involving $O^{\gamma 1}$ of threonine-67 and the main-chain NH of glutamine-69. In addition, Thr-67 $O^{\gamma 1}$ donates a hydrogen bond to the main-chain carbonyl oxygen atom of leucine-84. The side-chain density of glutamate-70 is discontinuous at C^{γ}, indicating relatively high thermal motion for this residue.

anomalies mentioned above. In addition, the titratable behavior, Hill coefficient, and narrow line width are fully consistent with the three-dimensional environment in the vicinity of serine-68, as established by crystallographic means. These results therefore demonstrate that two independent, powerful physical techniques can converge to enhance our understanding of protein structure and dynamics at a molecular level.

The results are also in keeping with observations on phosphoserine residues serving a noncatalytic function in other proteins, e.g., ovalbumin (Vogel & Bridger, 1982) and ATP-citrate lyase (Williams et al., 1985), and other phosphoproteins [reviewed by Vogel (1984)]. However, the chemical shift of protein-bound phosphoserine does not necessarily mirror exactly that of free phosphoserine, even, as shown here, after denaturation. This indicates that the protein backbone may exert some effect on the phosphoryl moiety. However, the Hill coefficient calculated here for pepsinogen was close to unity. Moreover, the crystal structure determination confirms that there are no positively charged groups near the phosphoserine moiety. Therefore, the exact nature of such an interaction is unclear.

The primary sequence surrounding the phosphoserine group of pepsinogen is Glu-Ala-Thr-Ser(P)-Gln-Glu-Leu (Tang et al., 1973). As noted by Vogel and Bridger (1982), the Ser-(P)-X-Glu sequence is common to a number of other phosphoproteins, including the ovalbumins and α- and β-caseins. Using ^{31}P NMR, Vogel & Bridger (1982) showed that both phosphoserine groups of ovalbumin enjoy a high degree of mobility and exhibit characteristics consistent with their being located on the surface of the protein. However, of these two phosphoserine groups the phosphate of Ser-68 of ovalbumin was preferentially removed by an acid over an alkaline phosphatase. Also, it is this phosphoserine that shares the Ser-(P)-X-Glu sequence with pepsinogen. In contrast, the phosphate of Ser-334 in ovalbumin was readily removed by both alkaline and acid phosphatase and resides in a dissimilar polypeptide sequence. The results presented here, from both X-ray crystallography and NMR, show that the phosphate group of Ser-68 of pepsinogen is located on the surface of the molecule. One might therefore expect it to be an ideal candidate for removal by most phosphatases. Taken together, the results suggest that the Ser(P)-X-Glu may serve as a general recognition site for certain protein kinases and phosphatases.

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Registry No. L-Phosphoserine, 407-41-0; pepsinogen, 9001-10-9.

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Regulation of Rabbit Liver Fructose-1,6-bisphosphatase by Metals, Nucleotides, and Fructose 2,6-Bisphosphate As Determined from Fluorescence Studies†

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ABSTRACT: The fluorescent nucleotide analogue formycin 5′-monophosphate (FMP) inhibits rabbit liver fructose-1,6-bisphosphatase (I_{50} = 17 μM, Hill coefficient = 1.2), as does the natural regulator AMP (I_{50} = 13 μM, Hill coefficient = 2.3), but exhibits little or no cooperativity of inhibition. Binding of FMP to fructose-1,6-bisphosphatase can be monitored by the increased fluorescence emission intensity (a 2.7-fold enhancement) or the increased fluorescence polarization of the probe. A single dissociation constant for FMP binding of 6.6 μM (4 sites per tetramer) was determined by monitoring fluorescence intensity. AMP displaces FMP from the enzyme as evidenced by a decrease in FMP fluorescence and polarization. The substrates, fructose 6-phosphate and fructose 1,6-bisphosphate, and inhibitors, methyl α-D-fructofuranoside 1,6-bisphosphate and fructose 2,6-bisphosphate, all increase the maximal fluorescence of enzyme-bound FMP but have little or no effect on FMP binding. Weak metal binding sites on rabbit liver fructose-1,6-bisphosphatase have been detected by the effect of Zn^{2+}, Mn^{2+}, and Mg^{2+} in displacing FMP from the enzyme. This is observed as a decrease in FMP fluorescence intensity and polarization in the presence of enzyme as a function of divalent cation concentration. The order of binding by divalent cations is Zn^{2+} = Mn^{2+} > Mg^{2+}, and the K_d for Mn^{2+} displacement of FMP is 91 μM. Methyl α-D-fructofuranoside 1,6-bisphosphate, as well as fructose 6-phosphate and inorganic phosphate, enhances metal-mediated FMP displacement from rabbit liver fructose-1,6-bisphosphatase. In the presence of the α-methyl substrate analogue the K_d's for Mn^{2+} and Mg^{2+} are 14 μM and 0.8 mM, respectively. The K_d for Mn^{2+} binding to 4 enzyme sites per tetramer obtained by fluorescence experiments in the presence of methyl α-D-fructofuranoside 1,6-bisphosphate strongly indicates that divalent cation binding to the catalytic metal site (K_m for Mn^{2+} is 15 μM) is responsible for FMP displacement. It is concluded that catalytic metal and nucleotide binding is competitive, an observation sufficient to explain the effect of AMP on substrate turnover. Fructose 2,6-bisphosphate does not enhance metal ion mediated displacement of FMP. Mn^{2+} and Zn^{2+} concentrations sufficient to displace a significant proportion of enzyme-bound FMP had no effect on the fluorescence of the enzyme–FMP complex when saturating fructose 2,6-bisphosphate was present. Fructose 2,6-bisphosphate either blocks catalytic metal binding or permits concomitant binding of FMP and catalytic metal ion. In light of the results of other investigators, the former hypothesis is a more plausible interpretation. A model for the molecular regulation of fructose-1,6-bisphosphatase monomer by metal, AMP, and fructose 2,6-bisphosphate is presented.

Hydrolysis of D-fructose 1,6-bisphosphate (Fru-1,6-P_2)[1] to D-fructose 6-phosphate and inorganic phosphate is catalyzed by fructose-1,6-bisphosphatase (EC 3.1.3.11). The rabbit liver enzyme is a tetramer of M_r 140 000 (Traniello et al., 1971, 1972). Fructose-1,6-bisphosphatase from mammalian liver requires the presence of divalent metal ions such as Zn^{2+}, Mn^{2+}, Mg^{2+}, or Co^{2+} for catalytic activity (Frey et al., 1976; Tejwani et al., 1976; Pedrosa et al., 1977; Kirtley & Dix, 1971). The affinity of fructose-1,6-bisphosphatase for these divalent cations decreases in the following order: Zn^{2+} > Mn^{2+} > Mg^{2+} (Benkovic et al., 1978a,b). Because of the high affinity for Zn^{2+}, Benkovic et al. (1978b) have proposed that fructose-1,6-bisphosphatase may function in vivo as a zinc metalloenzyme. Rabbit liver fructose-1,6-bisphosphatase contains 8 divalent cation sites per molecule of enzyme (two per subunit) (Benkovic et al., 1978a,b; Libby et al., 1975). The high-affinity metal ion site on each subunit is referred to as the "structural"[2] metal ion site. The low-affinity sites have been dubbed "catalytic" because the dissociation constants for

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[1] Abbreviations: AMP, adenosine 5′-monophosphate; α-Me-Fru-1,6-P_2, methyl α-D-fructofuranoside 1,6-bisphosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; FMP, formycin A 5′-monophosphate; FBPase, fructose-1,6-bisphosphatase; PMSF, phenylmethanesulfonyl fluoride; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] While the terms "structural" and "catalytic" denote specific functions for the two metal ions associated with each fructose-1,6-bisphosphatase subunit, direct evidence for the participation of either metal ion in the catalytic mechanism is not available. However, we have chosen to use the usual convention of referring to the high-affinity metal sites as structural and the low-affinity metal sites, which require the presence of substrate for binding, as catalytic.

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