Ga^{3+} as a Functional Substitute for Fe^{3+}: Preparation and Characterization of the $Ga^{3+}Fe^{2+}$ and $Ga^{3+}Zn^{2+}$ Forms of Bovine Spleen Purple Acid Phosphatase

Maarten Merkx and Bruce A. Averill*

E.C. Slater Institute, Biocentrum Amsterdam, University of Amsterdam, Plantage Muidergracht 12, 1018 TV, Amsterdam, The Netherlands

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ABSTRACT: A general method has been developed that allows the specific substitution of both iron atoms in the enzyme bovine spleen purple acid phosphatase (BSPAP), which possesses a dinuclear iron center at the active site. The approach is demonstrated by the preparation and characterization (atomic absorption spectrometry, enzyme kinetics, optical spectroscopy, and electron paramagnetic resonance spectroscopy) of two metal-substituted forms in which the ferric iron has been replaced by Ga^{3+}: $Ga^{3+}Fe^{2+}$-BSPAP and $Ga^{3+}Zn^{2+}$-BSPAP. Both forms are colorless but exhibit enzymatic activity comparable to that of the native $Fe^{3+}Fe^{2+}$-BSPAP. Small but consistent changes in kinetics parameters and pH profiles were detected both upon substitution of Fe^{3+} by Ga^{3+} and upon substitution of Fe^{2+} by Zn^{2+}. These results constitute the first evidence that the diamagnetic Ga^{3+} ion can serve as a *functional* analogue of Fe^{3+} in an enzyme, and suggest a novel approach for the study of the role of Fe^{3+} in other iron enzymes.

Purple acid phosphatases (PAPs)[1] are acid phosphatases that contain a dinuclear $Fe^{3+}M^{2+}$ center in their active site, with M = Fe^{2+} or Zn^{2+} (*1*, *2*). Their purple color originates from a tyrosinate-to-Fe^{3+} charge-transfer band (*3*−*5*). The best characterized members are the mammalian enzymes uteroferrin (Uf) and bovine spleen purple acid phosphatase (BSPAP) and a plant enzyme from red kidney beans (KBPAP). The mammalian enzymes contain a dinuclear $Fe^{3+}Fe^{2+}$ center, while the KBPAP has an $Fe^{3+}Zn^{2+}$ center. The mammalian enzymes can be oxidized to the Fe^{3+}-Fe^{3+} state, which is inactive. Reduction by dithionite to the $Fe^{2+}Fe^{2+}$ state results in the destruction of the metal center and the release of Fe^{2+}. KBPAP is the only PAP whose X-ray structure has been determined (*6*, *7*). The recently reported X-ray structures of two Ser/Thr-specific protein phosphatases (PP's), calcineurin (or PP2B) (*8*, *9*) and PP1 (*10*, *11*), revealed the presence of a dinuclear metal center that is similar to the KBPAP active site. A sequence motif incorporating most of the metal-coordinating amino acids found in the PAP and PP structures has now been identified in a large group of phosphoesterases, including other phosphomonoesterases, diadenosine tetraphosphatase, exonucleases, and 5′-nucleotidases (*12*−*14*). Although there is still some debate about the exact nature and oxidation state of the metals in the various protein phosphatases (*15*−*18*), it is clear that a dinuclear metal center of general formula Fe^{3+}-M^{2+} (Figure 1) is used by a large and important group of phosphatases (*19*). Among these phosphoesterases, the metal center and the catalytic mechanism are probably best characterized for the PAPs.

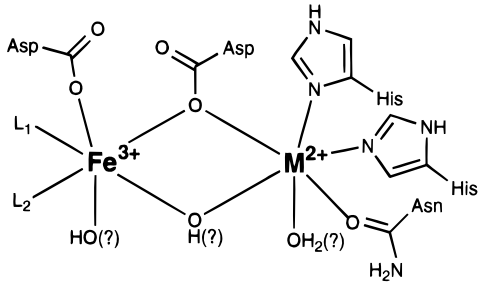

FIGURE 1: General structure of the dinuclear metal center found in purple acid phosphatases and protein phosphatase 1 and 2B. Purple acid phosphatase: L_1 = His, L_2 = Tyr, M^{2+} = Fe^{2+} or Zn^{2+}. Protein phosphatases: L_1 = $OH_{(2)}$, L_2 = His, M^{2+} = Zn^{2+}, Mn^{2+} (or possibly another metal).

Although both metals seem to be essential for catalysis, their individual roles remain unresolved. A classical approach for investigating the role of a metal in enzyme catalysis is substitution by another metal and characterization of the resulting perturbation in spectroscopic and/or enzymatic properties. In PAP, this approach has thus far been applied only to the divalent metal site. Thus, in uteroferrin and BSPAP, the replacement of the ferrous iron[2] by Zn^{2+} has been reported to occur without significant effects on kinetics parameters and inhibition constants (*20*−*23*). Likewise, the Zn^{2+} found in KBPAP and calcineurin can be

* To whom correspondence should be addressed. 31-20-5255045 (phone); 31-20-5255124 (fax); BAA@chem.uva.nl (e-mail).

[1] Abbreviations: AAS, atomic absorption spectrometry; BSA, bovine serum albumin; BSPAP, bovine spleen purple acid phosphatase; EPR, electron paramagnetic resonance; FeZn-BSPAP, BSPAP with iron at the ferric site and zinc at the ferrous site; FeZn-Uf, uteroferrin with iron at the ferric site and zinc at the ferrous site; GaFe-BSPAP, BSPAP with gallium at the ferric site and iron at the ferrous site; GaZn-BSPAP, BSPAP with gallium at the ferric site and zinc at the ferrous site; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; KBPAP, purple acid phosphatase from red kidney beans; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAP, purple acid phosphatase; *p*-NPP, disodium salt of *p*-nitrophenyl phosphate; PP, protein phosphatase; PP1, protein phosphatase 1; PP2B, protein phosphatase 2B; $P_{1/2}$, power at half-saturation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Uf, uteroferrin.

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exchanged for Fe^{2+} without major effects on catalysis (*15, 24, 25*). Metal substitutions with other divalent metals such as Co^{2+} and Cd^{2+} have also been reported for KBPAP (*25*) and Uf (*21, 26*), resulting in enzyme derivatives that exhibit activities varying from 20 to 100% of the native activity. The substitution of the invariant ferric site iron by another metal has not been reported (with the exception of ^{57}Fe). The Fe^{3+} has been proposed to act as a Lewis acid that can generate a coordinated hydroxide nucleophile at low pH. These findings have been incorporated into a model in which the phosphate ester coordinates to the divalent metal in a rapid binding step, followed by direct attack on the ester group by an Fe^{3+}-ligated hydroxide (*27*−*29*).

With the ultimate goal of resolving the roles played by both metals in phosphate ester hydrolysis, we have developed a general method for the preparation of metal-substituted forms of BSPAP in which either the ferric site or the ferrous site iron (or both) is specifically replaced by other metals. Our initial efforts have focused upon substitution of the ferric site iron by gallium. The use of Ga^{3+} as an analogue of Fe^{3+} is well documented, both in inorganic chemistry and in protein chemistry. Ga^{3+} and Fe^{3+} have similar ionic radii (0.62 Å vs 0.65 Å), charges, and coordination preferences (*30*). Complexes of Fe^{3+} and Ga^{3+} also show similar ligand exchange rates and pK_a values for coordinated water, which is important when studying catalytic reactions involving ligand exchange on the metal. Unlike Fe^{3+}, Ga^{3+} is a main group element and always diamagnetic. Finally, Ga(III) is the only biologically accessible redox state for gallium, whereas Fe(II), Fe(III), and Fe(IV) have all been detected in biological systems (*31*). Gallium has been used as a structural analogue of Fe^{3+} in iron-binding proteins such as transferrin (*32*−*34*), lactoferrin (*35*), and ovotransferrin (*36, 37*) and in model complexes of binuclear iron proteins (*38, 39*). This approach has recently been extended to electron transport proteins with the report of the preparation of a Ga_2S_2 analogue of ferredoxin (*40*) and the preparation of a rubredoxin-like mononuclear gallium center after reconstitution of apo-ferredoxin with gallium (*41, 42*). The Ga-substituted ferredoxin is no longer functional, however, since Ga^{3+} is the only biochemically accessible redox state. For those enzymes in which Fe^{3+} acts solely as a Lewis acid and does not change its oxidation state during catalysis, Ga^{3+} might well be expected to be a good functional analogue.

We report the preparation and characterization of two catalytically active metal-substituted forms of BSPAP in which the ferric iron has been replaced by Ga^{3+}, $Ga^{3+}Fe^{2+}$-BSPAP, and $Ga^{3+}Zn^{2+}$-BSPAP. These results constitute the first evidence that Ga^{3+} can serve as a *functional* analogue of Fe^{3+} in an enzyme system. The availability of four different BSPAP forms, in which either the Fe^{3+} or the Fe^{2+} or both have been specifically substituted, namely, FeFe-BSPAP (native), FeZn-BSPAP, GaFe-BSPAP, and GaZn-BSPAP, enabled us to ascribe the observed changes in kinetics constants and pH profiles to effects of the metal substitution itself, rather than to artifacts introduced by their preparation. The Fe^{2+}-to-Zn^{2+} substitution could be studied both in the FeFe/FeZn pair and in the GaFe/GaZn pair, and the effect of the Fe^{3+}-to-Ga^{3+} substitution could be studied both in the FeFe/GaFe pair and in the FeZn/GaZn pair.

[2] Throughout this article, the 'ferric site' is the metal binding site that contains the ferric iron in the mixed valent Fe^{3+}-Fe^{2+} oxidation state, while the 'ferrous site' is the metal binding site that contains the ferrous iron in the mixed valent oxidation state.

EXPERIMENTAL PROCEDURES

Methods and Materials. Unless stated otherwise, protein solutions contained 40 mM sodium acetate, 1.6 M KCl, and 20% (v/v) glycerol pH 5.0. $Na_2S_2O_4$ was added from a 100 mM stock solution in 100 mM Tris, pH 9.0, which was prepared freshly each day. For all experiments involving (half) apo forms of BSPAP, plastic disposable labware was boiled in 5% HCl and rinsed with Millipore water, while glassware was stored in 10% HCl and rinsed with Millipore water just before use. Sephadex G-25 columns (Pharmacia) were washed with 2−3 volumes of 2 mM 1,10-phenanthroline, followed by several volumes of Millipore water and buffer. Buffers were treated with Chelex-100 (BioRad) to remove metal impurities.

Bovine spleen purple acid phosphatase was isolated essentially as previously described (*43*). Occasionally, after the phenyl-Sepharose step, the buffer was exchanged for 50 mM sodium acetate, 0.15 M KCl, pH 5.0, and the protein was loaded onto a small (2.5 × 3 cm) hydroxylapatite column (Bio-Gel HTP−Gel, BioRad) in the same buffer. After washing with 1 column volume of the 0.15 M KCl−acetate buffer, the purple band at the top of the column was eluted from the column with the same buffer containing 0.5 M KCl. The KCl concentration was then raised again to 2 M KCl to restore the high-salt conditions. Finally the protein was exposed to buffer containing 40 mM sodium acetate, 1.6 M KCl, 100 mM ascorbic acid, and 5 mM $Fe(NH_4)_2(SO_4)_2$, pH 5.0, for 5 min before applying it to a gel filtration column [Sephadex G-75 or Sephacryl S-200 (Pharmacia), 50 mM acetate and 2 M KCl, pH 5.0]. This procedure converts all BSPAP to the Fe^{3+}-Fe^{2+} form and also removes any phosphate that bound to BSPAP during the hydroxylapatite column step. This procedure (with or without the hydroxylapatite column) yields the so-called high-salt form of the enzyme, which is characterized by a λ_{max} of 536 nm for the $Fe^{3+}Fe^{2+}$ oxidation state. Preparations were homogeneous based on Coomassie-stained SDS−PAGE gels and had A_{280}/A_{536} ratios of ∼15. Enzyme that was used to prepare metal-substituted forms was passed through a small Chelex-100 column before use to remove any loosely bound metal ions. Protein determination was done by measuring the absorbance at 536 nm due to the tyrosinate-to-Fe^{3+} charge-transfer band (ϵ = 4080 $M^{-1}\cdot cm^{-1}$) for the native enzyme and FeZn-BSPAP and the absorbance at 280 nm (ϵ = 60 000 $M^{-1}\cdot cm^{-1}$) for the apo enzyme and the Ga-substituted forms of BSPAP.

Release of Fe^{2+} from $Fe^{2+}Fe^{2+}$-BSPAP. The release of Fe^{2+} was monitored by measuring the absorbance of the $[Fe(phen)_3]^{2+}$ complex at 510 nm (ϵ = 11.1 $mM^{-1}\cdot cm^{-1}$). Reactions were performed with 10 μM BSPAP, 5 mM $Na_2S_2O_4$, and 1 mM 1,10-phenanthroline. The release of Fe^{2+} in the absence of 1,10-phenanthroline was measured by adding 1 mM 1,10-phenanthroline at various times after the addition of dithionite. The immediate increase in absorbance at 510 nm was ascribed to the reaction of free Fe^{2+} with 1,10-phenanthroline.

Preparation of Apo-BSPAP. Apo-BSPAP was prepared by adding sodium dithionite (final concentration ∼5 mM)

to ~80 μM BSPAP in the presence of 1 mM 1,10-phenanthroline. The release of Fe^{2+} was monitored by measuring the absorption of the $[Fe(phen)_3]^{2+}$ complex at 510 nm. Approximately 3 h after the addition of dithionite, the apo-enzyme was separated from the reagents using a Sephadex G-25 column. Fractions containing apo-enzyme were identified by their absorption at 280 nm and pooled.

Preparation of GaFe-BSPAP. 30−90 μM BSPAP was reduced by adding sodium dithionite to a final concentration of 5 mM. After 15 min reaction at room temperature, $GaCl_3$ was added to a final concentration of ~0.2 mM. After another 15 min, 1,10-phenanthroline was added (1 mM) to complex free Fe^{2+}, and the reaction mixture was applied to a Sephadex G-25 column (1.5 cm × 30 cm). Fractions containing GaFe-BSPAP were identified by their absorption at 280 nm and their phosphatase activity.

Preparation of GaZn-BSPAP. $GaCl_3$ (~5-fold excess) and $Zn(OAc)_2$ (~20-fold excess) were added to a solution of apo-BSPAP (~30 μM). Within 10 min, the phosphatase activity was restored. The solution was concentrated using a Centricon-30 concentrator (Amicon) and passed through a Sephadex G-25 column to remove excess metal ions. Fractions containing BaZn-BSPAP were identified by their absorption at 280 nm and their phosphatase activity.

Preparation of FeZn-BSPAP. FeZn-BSPAP was prepared in a way similar to GaZn-BSPAP. $FeCl_3$ (~200 μM) and $Zn(OAc)_2$ (~400 μM) were added to apo-BSPAP (15−30 μM), and the sample was incubated at 37 °C. When the phosphatase activity no longer increased (after 4−5 h), the sample was concentrated/diluted several times with buffer using a Centricon-30 concentrator. The $Fe(OH)_3$ precipitate that was formed was removed by centrifugation. Since metal analysis showed approximately 2 Fe atoms per protein even after chromatography on a Sephadex G-25 column and a Chelex-100-column, the enzyme was treated with sodium ascorbate (50 mM) and 1 mM 1,10-phenanthroline (1.5 h, room temperature), followed by chromatography on a Sephadex G-25 column. This treatment did not affect the phosphatase activity of the sample but removed adventiously but strongly bound Fe^{3+}.

Metal Analyses. Metal analyses were performed on a Hitachi180-80 polarized Zeeman atomic absorption spectrometer equipped with a graphite furnace. Adventitious metal ions were removed from protein and buffer by passage through a Chelex-100 column.

Spectroscopy. Optical spectra were measured on an HP8452A diode array spectrophotometer. X-band EPR spectra (9.4 GHz) were obtained on a Bruker ECS106 EPR spectrometer, equipped with an Oxford Instruments ESR900 helium-flow cryostat with an ITC4 temperature controller. The magnetic field was calibrated with an AEG Magnetic Field Meter. The frequency was measured with an HP 5350B Microwave Frequency Counter.

Kinetic Measurements. Enzyme assays were performed by monitoring the formation of *p*-nitrophenolate at 410 nm. At several times after enzyme addition, 250−500 μL aliquots were taken and quenched by mixing with 1.5 mL of 0.5 M NaOH to convert all product to the phenolate form ($\epsilon_{410\ nm}$ = 16.6 $mM^{-1}\cdot cm^{-1}$). Since BSPAP is partly inactivated in dilute solutions (*44*), assay solutions contained 0.5 mg/mL BSA to prevent inactivation. Specific activities were measured in a buffer containing 100 mM NaMES, 200 mM KCl, and 10 mM *p*-NPP, pH 6.00. To prevent oxidation, $Fe(NH_4)_2(SO_4)_2$ (0.2 mM) and ascorbate (15 mM) were added (for $Fe^{3+}Fe^{2+}$- and $Ga^{3+}Fe^{2+}$-BSPAP). Since the activity of BSPAP is quite sensitive to ionic strength, KCl was added to suppress the effect of small differences in ionic strength that result from the addition of various amounts of enzyme in buffer containing 2 M KCl. The high KCl concentration also prevents the substrate inhibition previously reported (*27, 45*), since normal Michaelis−Menten kinetics were observed up to 50 mM *p*-NPP. pH profiles were measured in 100 mM buffer (NaOAc, NaMES, or Na-HEPES), 200 mM KCl, and 50 mM *p*-NPP. pH values were measured immediately at the end of the assay (2 min after the addition of enzyme). For each determination of K_M and V_{max}, the hydrolysis rate was measured at nine different *p*-NPP concentrations between 0.3 and 50 mM. Values of K_M and V_{max} were obtained by a nonlinear fit of the Michaelis−Menten equation using the program Enzyme-Kinetics (Trinity Software).

RESULTS

From previous work on metal substitution reactions in various purple acid phosphatases, it was known that the fully reduced Fe^{2+}-Fe^{2+} form is not stable (*20, 46*). One of the iron atoms is released rapidly after addition of the strong reducing agent sodium dithionite (within several minutes), while the second iron is released more slowly in the presence of the Fe^{2+}-chelator 1,10-phenanthroline. This biphasic release of the Fe ions has been used in the preparation of FeZn-Uf (*20*) and FeZn-BSPAP (*23*). These FeZn-PAP forms were prepared by incubating the enzyme for only a few minutes with dithionite, yielding a one-iron, "half-apo" PAP, followed by a gel filtration step and the addition of Zn^{2+} under aerobic conditions. Initial experiments showed that a similar biphasic Fe release also occurs with fully reduced BSPAP. The release of the second Fe was, however, accompanied by severe protein precipitation. This problem was solved by the addition of 20% glycerol, which postpones the precipitation of apo-BSPAP, allowing the separation of apo-BSPAP from the $[Fe(phen)_3]^{2+}$ and dithionite. The same phenomenon has also been observed for apo-KBPAP (*47*). Metal analysis showed 0.05 residual Fe and a specific activity that was less than 0.1% of the native enzyme.

Release of the second iron was found to occur only in the presence of 1,10-phenanthroline, while release of the first iron was found to be independent of the presence of this Fe^{2+} chelator. As demonstrated in Figure 2, addition of sodium dithionite in the presence of 1,10-phenanthroline results in a biphasic release of both irons (solid line). The closed circles represent experiments in which BSPAP was reduced by the addition of sodium dithionite in the absence of 1,10-phenanthroline. At several times after the dithionite addition, 1,10-phenanthroline was then added with rapid mixing, and the immediate increase in the absorption at 510 nm was measured. This was assumed to originate from the reaction of 1,10-phenanthroline and Fe^{2+} that was no longer bound to the protein. Figure 2 shows that the rapid release of the first Fe^{2+} also takes place in the absence of 1,10-phenanthroline, but the second iron stays bound to the enzyme even 90 min after the addition of dithionite. The treatment of BSPAP with dithionite in the absence of 1,10-

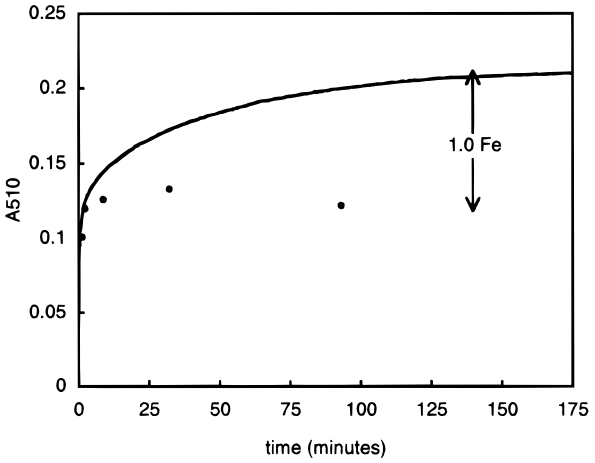

FIGURE 2: Time dependence of Fe^{2+} release after reduction of BSPAP by dithionite as followed by the formation of the $[Fe(phen)_3]^{2+}$ complex at 510 nm. The continuous line represents an experiment where BSPAP was reduced in the presence of 1 mM 1,10-phenanthroline. The closed circles represent the immediate increase in absorption when BSPAP was reduced in the absence of 1,10-phenanthroline, and 1,10-phenanthroline was subsequently added at several times after dithionite addition.

Table 1: Metal Contents of Metal-Substituted Forms of BSPAP[a]

enzyme	Ga	Fe	Zn
Ga−Fe	1.12 (0.08)	0.86 (0.07)	0.15 (0.03)
Ga−Zn	1.03 (0.15)	<0.1	0.93 (0.08)
Fe−Zn	nd	1.05 (0.09)	0.96 (0.03)

[a] Metal analyses were performed on an Hitachi 180−80 polarized atomic absorption spectrophotometer. Numbers in parentheses are standard deviation values.

phenanthroline thus yields the half-apo form, which still contains one Fe^{2+}.

After having established procedures to prepare both the half-apo and apo forms of BSPAP, the preparation of the GaFe and GaZn forms of BSPAP was explored. GaFe-BSPAP was prepared by the addition of Ga^{3+} to a solution of half-apo BSPAP. Addition of Ga^{3+} to the inactive half-apo BSPAP resulted in a rapid increase in phosphatase activity (complete within 5−10 min at room temperature). This Ga-containing form of BSPAP was separated from the other reagents (dithionite, excess metals, phenanthroline) by a Sephadex G-25 gel filtration column. As shown in Table 1, this protein contained stoichiometric amounts of gallium and iron as well as a substoichiometric amount of zinc. Its optical spectrum (Figure 3) no longer showed the tyrosinate-to-Fe^{3+} charge-transfer band at ∼550 nm, which is consistent with the replacement of Fe^{3+} by Ga^{3+}. The shoulder at 310 nm in the optical spectra of FeFe-BSPAP and FeZn-BSPAP was also absent in the GaFe-BSPAP spectrum. The specific activity of GaFe-BSPAP was comparable to that of native FeFe-BSPAP (Figure 4). In contrast to FeFe-BSPAP and FeZn-BSPAP, which are inactivated by the addition of dithionite, GaFe-BSPAP was fully active after dithionite addition, providing strong evidence for the selective replacement of Fe^{3+} by Ga^{3+}. Enzyme assayed aerobically in the absence of the mild reducing agents Fe^{2+}/ascorbate exhibited only 20% of the activity observed in the presence of the mild reductant, indicating that the active form is $Ga^{3+}Fe^{2+}$. The greater activity in the presence of Fe^{2+}/ascorbate did not result from the binding of Fe^{2+} by GaFe-BSPAP, since

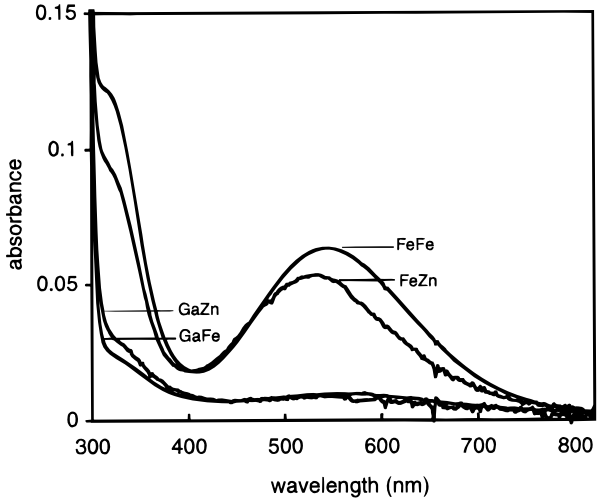

FIGURE 3: Optical spectra of native BSPAP, FeZn-BSPAP, GaFe-BSPAP, and GaZn-BSPAP in 40 mM acetate, 1.6 M KCl, and 20% glycerol (v/v), pH 5.0. Spectra were normalized to give the same protein concentration.

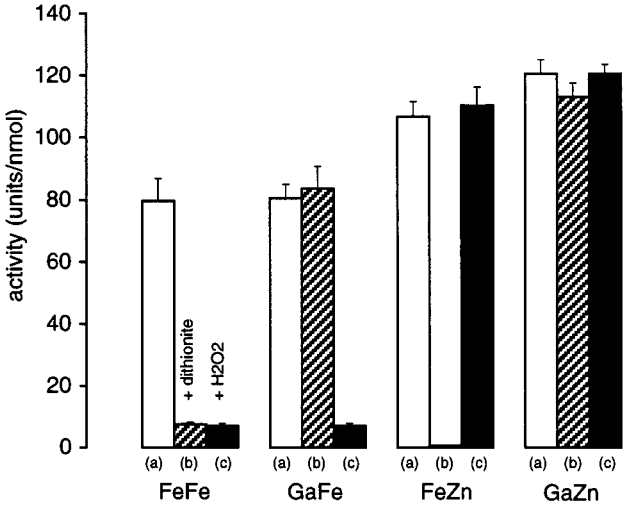

FIGURE 4: Bar graph illustrating the specific activities of FeFe, GaFe, FeZn, and GaZn forms of BSPAP under various conditions: (a) without treatment; (b) after incubation with sodium dithionite (5 mM; 10 min at 0 °C); and (c) after incubation with H_2O_2 (2 mM; 10 min at 0 °C). Assays were performed at 22 °C and pH 6.0 with 10 mM *p*-NPP. Assays for (a) and (b, FeFe/GaFe) were performed in a buffer containing 100 mM NaMES, 200 mM KCl, 15 mM ascorbate, and 0.2 mM $Fe(NH)_2(SO_4)_2$, pH 6.0. Assays for (b, FeZn/GaZn) and (c) were performed in 100 mM NaMES and 200 mM KCl, pH 6.0.

activity assays which were done anaerobically in the presence of dithionite but in the absence of Fe^{2+} gave similar results. GaFe-BSPAP was apparently more easily oxidized by oxygen (yielding the inactive $Ga^{3+}Fe^{3+}$ form) than the native enzyme, which in the absence of phosphate is relatively stable in the $Fe^{3+}Fe^{2+}$ oxidation state. The ∼15% residual activity that was found even after the treatment with H_2O_2 is probably due to a GaZn-BSPAP impurity, which is consistent with the ∼0.15 Zn found in these GaFe-BSPAP preparations.[3] Upon standing at room temperature, GaFe-BSPAP showed a tendency to slowly disproportionate, which

[3] The native enzyme also shows some residual activity (5−10%) after treatment with hydrogen peroxide. We have found that this activity results from an 'impurity' of FeZn-BSPAP in native BSPAP (M. Merkx and B. A. Averill, unpublished results).

Table 2: Kinetics Parameters for the Hydrolysis of *p*-NPP by Various Forms of Bovine Spleen Purple Acid Phosphatase[a]

enzyme	k_{cat} ·10^{-3} (s^{-1})	K_M (mM)
Fe^{3+}-Fe^{2+} [b]	1.76 (0.09)	1.18 (0.22)
Ga^{3+}-Fe^{2+} [b]	1.41 (0.13)	3.25 (0.30)
Fe^{3+}-Zn^{2+} [c]	2.84 (0.18)	3.25 (0.39)
Ga^{3+}-Zn^{2+} [c]	3.09 (0.44)	5.49 (0.36)

[a] Numbers in parentheses are standard deviation values. [b] Assays were performed at 22 °C and pH 6.0 in a buffer containing 100 mM NaMES, 200 mM KCl, 15 mM ascorbate, and 0.2 or 2 mM $Fe(NH_4)_2(SO_4)_2$. [c] Assays were performed at 22 °C and pH 6.0 in a buffer containing 100 mM NaMES and 200 mM KCl.

resulted in the re-formation of some native enzyme, detected as a dithionite-sensitive phosphatase activity and a purple color.

GaZn-BSPAP was obtained by the addition of Ga^{3+} and Zn^{2+} to apo-BSPAP. Addition of both metals to inactive apo-BSPAP resulted in a rapid increase in phosphatase activity, which was complete within 10 min at room temperature. The addition of either Ga^{3+} or Zn^{2+} alone did not result in this increase in phosphatase activity. The putative GaZn-BSPAP was separated from excess metal ions by a Sephadex G-25 gel filtration column. Metal analysis showed the presence of stoichiometric amounts of gallium and zinc but very little iron (Table 1). Like GaFe-BSPAP, GaZn-BSPAP also lacked the characteristic purple color of the native FeFe enzyme (Figure 3). Its specific activity as measured at 10 mM *p*-NPP and pH 6.0 was even higher than that of the native enzyme. This activity was insensitive both to reduction by dithionite, indicating the replacement of Fe^{3+} by Ga^{3+}, and to oxidation by hydrogen peroxide, indicating the replacement of Fe^{2+} by Zn^{2+} (Figure 4).

A new method was used for the preparation of FeZn-BSPAP. Rather than isolating the half-apo BSPAP form, followed by the addition of Zn^{2+} [the method previously used by us and others (*20*−*23*)], FeZn-BSPAP was obtained by the addition of Fe^{3+} and Zn^{2+} to apo-BSPAP. Complete formation of FeZn-BSPAP takes ∼4 h at 37 °C, which is much slower than the formation of GaZn-BSPAP. Metal analysis of this FeZn-BSPAP form showed the presence of stoichiometric amounts of Fe and Zn (Table 1). In addition, the FeZn-BSPAP showed a tyrosinate-to-Fe^{3+} charge-transfer band at ∼530 nm, with an extinction coefficient only slightly lower than the 4080 M^{-1}·cm^{-1} of the native enzyme (Figure 3), and a specific activity that was higher than that of the native enzyme, but comparable to the specific activity of GaZn-BSPAP. Like native BSPAP, treatment with dithionite eliminated the phosphatase activity (Figure 4). The activity was, however, not affected by hydrogen peroxide, which is consistent with the replacement of the ferrous iron by zinc. A more extensive characterization of FeZn-BSPAP (including EPR spectra of various anion complexes) will be published elsewhere (*48*). Here we report only its kinetics properties for comparison with those of native BSPAP, GaFe-BSPAP, and GaZn-BSPAP. Typical overall yields (relative to the amount of native enzyme used) for these metal-substituted forms were ∼60% for GaFe-BSPAP and ∼50% for BaZn-BSPAP and FeZn-BSPAP.

Table 2 compares the kinetics parameters of the various metal-substituted forms of BSPAP for the hydrolysis of *p*-NPP at 22 °C and pH 6.00. Overall, the kinetics parameters

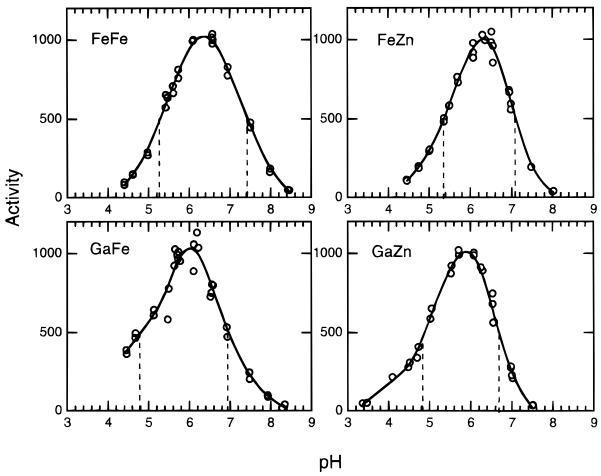

FIGURE 5: pH dependencies of FeFe-BSPAP, FeZn-BSPAP, GaFe-BSPAP, and GaZn-BSPAP at 50 mM *p*-NPP and 22 °C. Assays for FeFe-BSPAP and GaFe-BSPAP were performed in 100 mM buffer, 200 mM KCl, 15 mM ascorbate, and 0.2 mM $Fe(NH_4)_2(SO_4)_2$. Assays for FeZn-BSPAP and GaZn-BSPAP were performed in 100 mM buffer and 200 mM KCl. Activities are in arbitrary units and were scaled to obtain the same maximal activities for all BSPAP forms.

are surprisingly similar, but not identical. The replacement of Fe^{2+} by Zn^{2+} resulted in a small (2−3-fold) increase in K_M and a nearly 2-fold increase in k_{cat}. The replacement of Fe^{3+} by Ga^{3+} also resulted in a ∼2-fold increase in K_M, but did not affect k_{cat}. Importantly, the availability of four different metal-containing forms, in which either the Fe^{3+} or the Fe^{2+} or both have been specifically replaced, allows us with some confidence to ascribe the relatively small changes in kinetics parameters to the metal substitution itself and not to some artifact introduced by their preparation. The replacement of Fe^{2+} by Zn^{2+} has the same effect on k_{cat} and K_M when going from FeFe-BSPAP to FeZn-BSPAP and when going from GaFe-BSPAP to GaZn-BSPAP. The same is true for the Fe^{3+}-to-Ga^{3+} substitution in FeFe-BSPAP vs GaFe-BSPAP and in FeZn-BSPAP vs GaZn-BSPAP.

Figure 5 shows the pH dependencies of the hydrolysis of *p*-NPP at saturating amounts of *p*-NPP (50 mM) for native FeFe-, FeZn-, GaFe-, and GaZn-BSPAP. The pH profiles for all four BSPAP forms are similar, but again small and consistent differences were observed. The Fe^{3+} to Ga^{3+} substitution resulted in a shift to lower pH of ∼0.5 for both the acidic and the basic limb of the bell-shaped pH profile. The Fe^{2+} to Zn^{2+} substitution resulted in a decrease of ∼0.3 pH unit for the basic limb, but did not affect the acidic limb of the pH profile. The effects of the Fe^{3+} replacement by Ga^{3+} and the Fe^{2+} replacement by Zn^{2+} were additive: thus, the acidic limb of the GaZn profile coincided with the acidic limb of the GaFe profile, and both were ∼0.5 pH unit lower than those of FeFe-BSPAP and FeZn-BSPAP. The acidic limb of the GaZn profile was ∼0.8 pH unit (0.5 + 0.3) lower relative to the pH profile of the native enzyme.

The mammalian purple acid phosphatases show a rhombic EPR spectrum at $g < 2$, typical of an antiferromagnetically coupled Fe^{3+}-Fe^{2+} cluster with an $S = {}^1/_2$ groundstate (*1*, *2*). Antiferromagnetic coupling results in a net spin of $S = 0$ for the $Fe^{3+}Fe^{3+}$ state, which makes this state EPR-silent. A potentially attractive property of the GaFe species is that in the oxidized $Ga^{3+}Fe^{3+}$ state it should show an EPR signal

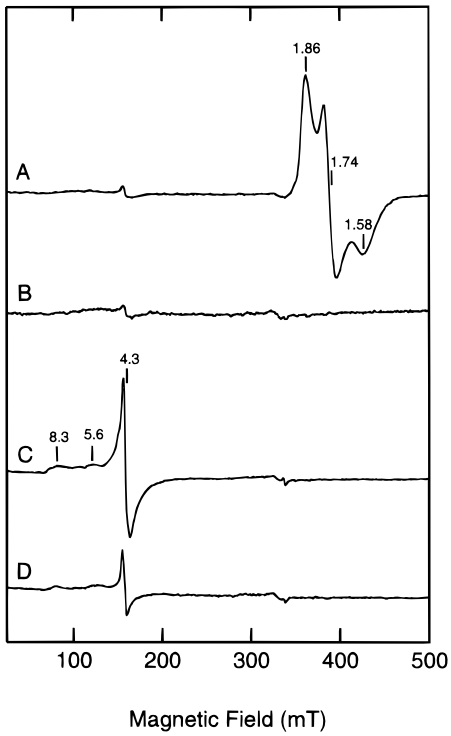

FIGURE 6: EPR spectra of (a) FeFe-BSPAP in the presence of 50 mM ascorbate; (b) GaZn-BSPAP in the presence of 50 mM ascorbate; (c) GaFe-BSPAP in the presence of 2 mM H_2O_2; and (d) GaFe-BSPAP in the presence of 50 mM ascorbate. EPR conditions: microwave power, 2 mW; microwave frequency, 9.426 GHz; modulation, 12.7 G at 100 kHz; temperature, 5 K. Buffer: 40 mM acetate, 1.6 M KCl, 20% glycerol (v/v). Amplitudes were corrected for differences in gain, temperature, and protein concentration.

characteristic of high-spin Fe^{3+} ($S = {}^5/_2$), allowing the coordination properties of this redox state to be studied by EPR. The reduced $Ga^{3+}Fe^{2+}$ state with an $S = 2$ is a non-Kramer system (*49*) that will not be easily detectable in the normal perpendicular measurement mode. Figure 6 shows EPR spectra of native $Fe^{3+}Fe^{2+}$-BSPAP, GaZn-BSPAP, $Ga^{3+}Fe^{3+}$-BSPAP, and $Ga^{3+}Fe^{2+}$-BSPAP. EPR conditions (microwave power, temperature) were identical for all species and optimal for the native enzyme. A single rhombic species with $g = 1.86$, 1.74, and 1.58 was observed for the native FeFe enzyme at pH 5.0 in 20% glycerol. Previous experiments in the absence of glycerol showed two rhombic species with $g < 2$ with variable intensities (*50*). The effect of glycerol suggests that this might have been a freezing artifact, although a solvent-induced change in pK_a can also not be ruled out. These spectra show the total absence of native Fe^{3+}-Fe^{2+}-BSPAP in the metal-substituted BSPAP forms. The EPR spectra of as-isolated GaFe-PAP and GaFe-BSPAP treated with 2 mM hydrogen peroxide were similar, both their form and their intensity, indicating that GaFe-BSPAP is easily oxidized on air to $Ga^{3+}Fe^{3+}$. They show a rather broad peak around $g = 4.3$ along with a very broad absorption over the entire range from $g = 10$ to $g = 5$. The $g = 4.3$ signal originates from a high-spin Fe^{3+} in a rhombic environment ($E/D \sim 0.3$). The signals between $g = 10$ and $g = 5$ probably also originate from high-spin Fe^{3+} with various, more axial rhombicities. The combined effects of E/D strain, g-strain, and multiple species may be responsible for the broadness of this band. Under the conditions shown in Figure 6, the $g = 4.3$ signal was saturated, since the $P_{1/2}$ at 5 K was determined to be at ~1 mW. Unfortunately, the addition of 50 mM phosphate, the product of the reaction and an inhibitor of BSPAP, did not lead to significant spectral changes. The addition of ascorbate (50 mM), which activates GaFe-BSPAP, resulted in the disappearance of most of the high-spin Fe^{3+} signals. The remaining signals might be due to an impurity that contains Fe^{3+} in the ferric site and also gives rise to the small residual band around 550 nm that can be detected in the optical spectra.

DISCUSSION

Preparation of GaFe-BSPAP and GaZn-BSPAP. In this paper we report the successful substitution of the ferric site iron of BSPAP by gallium, allowing the preparation of GaFe-BSPAP and GaZn-BSPAP. Evidence for this substitution includes the following: (1) metal analyses show the presence of equimolar amounts of Ga and Fe for GaFe-BSPAP and equimolar amounts of Ga and Zn for GaZn-BSPAP; (2) the activities of the gallium-containing BSPAP forms are not affected by sodium dithionite; (3) both gallium-containing BSPAP forms lack the purple color characteristic of the tyrosinate-to-Fe^{3+} charge-transfer band; (4) the characteristic EPR signal of $Fe^{3+}Fe^{2+}$-BSPAP is totally absent in the gallium-containing BSPAP forms; and (5) both kinetics parameters and pH optima are different for all four BSPAP species (FeFe, GaFe, FeZn, and GaZn). GaFe-BSPAP was prepared by addition of Ga^{3+} to the BSPAP species obtained after reduction by dithionite in the absence of 1,10-phenanthroline, which was shown to yield the half-apo form of the protein. FeZn-PAP forms have been prepared previously by the reduction of PAP with dithionite for 2 min in the presence of 1,10-phenanthroline, followed by gel filtration and the addition of Zn^{2+} (*20*, *23*). The fact that a substantial portion of the second iron (although possibly not all) is still bound to the protein after this gel filtration step indicates a reasonably strong binding to the protein (*21*), and is in agreement with our finding that half-apo BSPAP is quite stable in the absence of 1,10-phenanthroline. The observation of Buhi et al. that the amount of Fe^{2+} released after reduction of Uf in the presence of 2,2-bipyridine is only 60% compared to the amount of Fe^{2+} released in the presence of 1,10-phenanthroline is also in accordance with our conclusion that 1,10-phenanthroline is necessary to abstract the second iron from PAP (*46*).

It is not known where the residual ferrous iron binds in half-apo BSPAP, but previous metal substitution studies have shown that this iron is quite mobile. Reconstitution of the half-apo form with $^{57}Fe^{2+}$ was shown to occur with a nearly equal distribution of ^{57}Fe over both metal sites for Uf (*51*), while a ~2:1 ratio for the two sites was observed in BSPAP (*52*). This explains why half-apo BSPAP can be used to prepare both the FeZn (iron in ferric site) and GaFe-BSPAP (iron in ferrous site). In the presence of excess Ga^{3+}, Ga^{3+} binds at the ferric site and Fe^{2+} occupies the remaining ferrous site. In the presence of excess Zn^{2+}, Zn^{2+} binds at the ferrous site and the Fe^{2+} occupies the ferric site and is subsequently oxidized. FeZn-BSPAP and FeZn-Uf prepared via the half-apo PAP forms were reported to have extinction coefficients of 2100 $M^{-1}\cdot cm^{-1}$ for BSPAP (*23*) and 2000 $M^{-1}\cdot cm^{-1}$ (*22*) or 3654 $M^{-1}\cdot cm^{-1}$ (*21*) or 4000 $M^{-1}\cdot cm^{-1}$ (*53*) for Uf. Our present FeZn-BSPAP preparation shows

an extinction coefficient of ~3500 $M^{-1}\cdot cm^{-1}$. A likely explanation for these discrepancies is that the preparations with ϵ ~2000 $M^{-1}\cdot cm^{-1}$ contained a mixture of dinuclear metal sites, in which part of the iron and zinc were not bound at the proper metal site. This explanation is supported by our finding that the specific activity of our FeZn-BSPAP with the native-like extinction coefficient is 150% compared to the native enzyme (when assayed at 10 mM *p*-NPP at pH 6.0) while Davis and Averill (*23*) and David and Que (*22*) reported specific activities of 80−100% compared to the native activity.

$Ga^{3+}Fe^{2+}$-BSPAP seems to be more easily oxidized by air than $Fe^{3+}Fe^{2+}$-BSPAP. A similar observation was reported by Borovik and Que in a study of model complexes for dinuclear iron proteins. They found that the redox potential of the $Ga^{3+}Fe^{2+}/Ga^{3+}Fe^{3+}$ couple in their GaFe complex was substantially lower than the redox potential of the corresponding FeFe complex (+587 mV vs +692 mV) (*39*).

Kinetics Properties of GaFe-BSPAP and GaZn-BSPAP. One of the goals of metal substitution studies is to compare the enzymatic properties of the native enzyme and the metal-substituted forms with the known properties of the metals. The fact that the substitution of Fe^{3+} by Ga^{3+} results in enzymes with activities and kinetic parameters that are similar to those of the native enzyme confirms the hypothesis that the role of the ferric iron in catalysis is to act as a Lewis acid that can generate, even under acidic conditions, the hydroxide nucleophile that attacks the phosphate ester. The finding that the acidic limb of the pH optimum of both Ga-substituted enzymes is lowered by approximately 0.5 pH unit compared to their Fe^{3+}-containing counterparts may indicate a slightly lower pK_a of this coordinated water molecule for the Ga enzymes. The pK_a values for the first deprotonation of the hexa-aqua complexes of Ga^{3+} and Fe^{3+} are 3.1 and 2.7 respectively, so at least in the aqua complexes the situation is reversed and Fe^{3+} is slightly more acidic than Ga^{3+} (*30, 54*). Such trends are known to be only valid for similar types of complexes, however, so the relative acidities of Fe^{3+} and Ga^{3+} may very well differ in the PAP active site, especially given the small pK_a differences observed both for the aqua complexes and for the various metal-substituted BSPAP forms. Of course, we can also not exclude the possibility that the observed shift in pH dependence results from the Ga insertion via other, more secondary effects: e.g., the slightly smaller ionic radius of Ga^{3+} may affect the structure of the active site and result in different pK_a values for amino acids in the PAP active site.

In contrast to earlier studies, which found no clear effects of the Fe^{2+} to Zn^{2+} substitution, we do find small but consistent effects both on k_{cat} and on K_M. The nearly 2-fold higher k_{cat} values for the zinc-containing BSPAP forms indicate the involvement of this divalent metal in the rate-determining step, and may be the result of the greater Lewis acidity of Zn^{2+} compared to Fe^{2+}. These interpretations are, however, still speculative and hindered by the lack of (detailed) knowledge about the mechanism of the PAP's (i.e., the rate-determining step is still unknown).

This study shows that purple acid phosphatase is an attractive enzyme for site specific metal substitution studies, as it contains two clearly differentiated metal sites: a ferric site with a high affinity for trivalent metals (mainly the result of the tyrosinate ligand), and a ferrous site with a high affinity for divalent metals. Ga^{3+} and Fe^{3+} are probably too similar to elicit drastic changes in the activity of BSPAP, so the perturbation may have been too subtle. Further studies will therefore concentrate on preparing other metal-substituted forms of BSPAP of the general formula M^{3+}-Zn^{2+}, using trivalent metals that are more different from Fe^{3+} than Ga^{3+}.

Implications: Gallium as a Substitute for Ferric Iron. Substitution of Fe^{3+} by Ga^{3+} has been reported for the iron-binding proteins transferrin (*32*−*34*), lactoferrin (*35*), and ovotransferrin (*36, 37*) and, very recently, also for the Fe_2S_2 cluster-containing ferredoxins (*40*). These gallium analogues have been used to study the protein structure near the metal site by high-resolution NMR techniques. In the native enzymes, such studies are severely hampered by the paramagnetism of the iron which gives rise to line broadening. GaZn-BSPAP is also diamagnetic (and fully active), and is therefore an attractive form to study the structure and dynamics of BSPAP (or other enzymes with a similar active site metal center) by means of high-resolution NMR techniques.

Another potential use of Fe^{3+} substitution by Ga^{3+} is in those systems in which the iron is part of a larger spin-coupled complex (as in BSPAP), in that it allows the magnetic and spectroscopic properties of the other metals to be probed in the absence of a spin coupling interaction with the ferric iron. A similar strategy has been used to characterize the ferric site iron of Uf by studying the FeZn form by EPR, resonance Raman (*22*), EXAFS (*53*), and Mössbauer (*51*) spectroscopies. In BSPAP, the replacement of the ferric iron by gallium enabled us to study the fully oxidized state of the enzyme by EPR, which is not possible for the native oxidized $Fe^{3+}Fe^{3+}$ state because of the strong antiferromagnetic coupling. Unfortunately, the EPR spectrum of $Ga^{3+}Fe^{3+}$ seems to consist of a mixture of high-spin Fe^{3+} species with various rhombicities, and no clear effect of phosphate binding could be observed. It is highly unlikely that phosphate does not bind to $Ga^{3+}Fe^{3+}$-BSPAP, since all other enzymatic properties [including the inhibition constant for phosphate (*43, 48*)] are similar for $Ga^{3+}Fe^{2+}$-BSPAP and $Fe^{3+}Fe^{2+}$-BSPAP.

In general, Ga^{3+} might be expected to be a good functional substitute for Fe^{3+} in those enzymes where the metal acts solely as a Lewis acid and does not change its oxidation state during catalysis. This study presents the first example of an enzyme in which iron has been replaced by gallium, yielding catalytically active forms. Other enzymes which are thought to use the Lewis acidity of Fe^{3+} in catalysis (and where substitution of Fe^{3+} by Ga^{3+} may yield functional and sometimes diamagnetic analogues) include the other phosphate ester hydrolyzing enzymes having the same sequence motif as the purple acid phosphatase (notably the serine /threonine-specific protein phosphatases) (*19, 55*), nitrile hydratase (*56*), aconitase (*57*), and intradiol dioxygenase (*58, 59*). For the intradiol dioxygenases, it is still not clear whether the iron undergoes a change in oxidation state during the catalytic cycle. Such a question might be answered by substitution of the native Fe^{3+} by Ga^{3+}. If the gallium-substituted enzyme shows an activity similar to the native enzyme, this would provide clear evidence against any change in oxidation state during the catalytic cycle.

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