Catalytic Metal Ion Binding in Enolase: The Crystal Structure of an Enolase–Mn^{2+}–Phosphonoacetohydroxamate Complex at 2.4-Å Resolution[†,‡]

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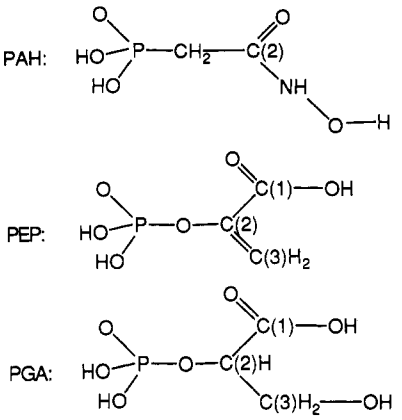
ABSTRACT: Enolase, a glycolytic enzyme that catalyzes the dehydration of 2-phospho-D-glycerate (PGA) to form phosphoenolpyruvate (PEP), requires two divalent metal ions per active site for activity. The first metal ion, traditionally referred to as "conformational", binds in a high-affinity site I. The second metal ion, "catalytic", binds in site II only in the presence of a substrate or substrate analogue and with much lower affinity for the physiological cofactor Mg^{2+}. While the high-affinity site has been well characterized, the position of the lower affinity site has not been established so far. Here, we report the structure of the quaternary complex between enolase, the transition-state analogue phosphonoacetohydroxamate (PhAH), and two Mn^{2+} ions. The structure has been refined by using 16 561 reflections with $F/\sigma(F) \geq 3$ to an $R = 0.165$ with average deviations of bond lengths and bond angles from ideal values of 0.013 Å and 3.1°, respectively. The "catalytic" metal ion is coordinated to two oxygen atoms of the phosphono moiety of PhAH and to the carbonyl oxygen of Gly37. Most likely, disordered water molecules complement its coordination sphere. The interaction with the site II metal ion must stabilize negative charge on the phosphate group and produce electron withdrawal from carbon 2 of the substrate, facilitating proton abstraction from carbon 2, the rate-limiting step in the catalytic process. The Gly37 residue is located in the flexible loop Ser36–His43, which assumes an "open" conformation in the absence of substrate and a "closed" conformation in the presence of a substrate. The metal ion binding in site II must stabilize the "closed" conformation and the substrate/product binding. Thus the inhibitory effect of higher Mg^{2+} concentrations on enolase activity may be explained by the sequential reaction mechanism in which the site II metal ion must leave before the product is released from the enzyme.

Enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) is a "metal-activated metalloenzyme" (Brewer, 1981) that catalyzes the dehydration of 2-phospho-D-glycerate (PGA)[1] to phosphoenolpyruvate (PEP) in the glycolytic pathway and the reverse reaction, the hydration of PEP to PGA, in gluconeogenesis (Wold, 1971) (see Chart 1 for structures). The enzyme is a dimer having two identical subunits (Brewer & Weber, 1968; Brewer et al., 1970) of molecular mass 46 650 daltons (Brewer, 1981; Chin et al., 1981). Enolase has an absolute requirement for certain divalent metal ions for activity. The natural cofactor is Mg^{2+} which gives the highest activity (Wold & Ballou, 1957; Brewer, 1985). The enzyme can be deionized, and Mg^{2+} can be substituted by many other divalent metal ions such as Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}, which give lower activity than Mg^{2+}, and Ca^{2+}, and Sm^{3+}, which do not induce measurable activity (Brewer, 1985).

Three metal ion binding sites per subunit were found in enolase. Metal binding in site I, traditionally called "conformational", induces a conformational change in the active site and enables binding of substrate or substrate analogues (Hanlon & Westhead, 1969; Brewer, 1971; Faller & Johnson, 1974). The metal ion binding at this site is the tightest among the three sites. Following binding of a substrate or a substrate analogue, the second metal ion, called "catalytic", can bind in site II, and then the catalytic reaction occurs (Faller et al., 1977). If the concentration of metal ions is higher, the third metal ion, called "inhibitory", can bind. This metal ion binding inhibits enzymatic activity (Elliott & Brewer, 1980; Brewer et al., 1983; Faller et al., 1977). While different metal ions will bind to a given site with different activities, in the case of any particular one, of Mg^{2+} and the first-row transition metal divalent cations, metal ion binding in the "catalytic" site is much weaker when compared with that in the "conformational" site (Faller et al., 1977). Binding affinity

Chart 1



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‡ The atomic coordinates for the structure of enolase–Mn^{2+}–phosphonoacetohydroxamate–Mn^{2+} have been deposited to the Brookhaven Protein Data Bank as entry 1ELS.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; PGA, 2-phospho-D-glycerate; PhAH, phosphonoacetohydroxamate.

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to the "inhibitory" site varies with the metal ion and conditions (Elliott & Brewer, 1980) but is generally weaker than binding to the "conformational" site. The metal ion site's nomenclature is derived from the macroscopic effects of metal ion binding at these sites on enzymatic activity, but it does not well reflect the metal ion function at atomic resolution.

A number of compounds that appear to be analogues of the aci form of the normal carbanion intermediate and are good inhibitors of enolase have been obtained and studied by Cleland and co-workers (Anderson et al., 1984). One of them, phosphonoacetohydroxamate (PhAH), has K_i = 15 pM (as the trianion, with saturating Mg^{2+}) and is the strongest known inhibitor of enolase (Anderson et al., 1984).

Recently, the crystal structure of yeast apoenolase was determined (Lebioda et al., 1989) and refined at 2.25-Å resolution (Stec & Lebioda, 1990). The structure of the binary complex enolase–Zn^{2+} was refined by the restrained least-squares method to an R = 14.9% at 1.9-Å resolution (Lebioda & Stec, 1989). There are five ligands coordinated to the "conformational" Zn^{2+} which form an almost regular trigonal bipyramid with two monodentate carboxylic groups of Asp246 and Asp320 in the axial positions while two water molecules and the monodentate carboxylate groups of Glu295 are in the equatorial positions. The same coordination geometry was found later in the structure of the precatalytic ternary complex enolase–Mg^{2+}–PGA/PEP, with the hydroxyl oxygen of PGA replacing one water molecule (Lebioda & Stec, 1991). On the other hand, in the inhibitory ternary complex enolase–Ca^{2+}–PGA (Lebioda et al., 1991) the metal ion coordination was octahedral, with three carboxylic, two water, and the hydroxyl ligands. The other important difference between the two ternary complexes is that in the precatalytic complex the loops Ser36–His43, Val153–Phe169, and Asp255–Asn266 are closer to the active site, assuming the "closed" conformation as opposed to the "open" conformation found in the native enolase and the inhibitory complexes. On the basis of these crystal structures, the mechanism of enolase activity was proposed (Lebioda & Stec, 1991).

While the position of the "catalytic" metal ion has not been yet determined using crystallography, other experimental methods suggested a possible binding site of the "catalytic" metal ion. Brewer and Ellis (1983) used ^{31}P-NMR and proposed that the most probable binding site for the "catalytic" metal ion is at the phosphate group of substrate. Lee and Nowak (1992a,b) used EPR and NMR spectroscopy to study Mn^{2+} binding to enolase in the presence or absence of Mg^{2+} and estimated that the distance between the "conformational" and "catalytic" metal ions is at least 12-Å. Another study of metal ions binding to enolase carried out with EPR techniques by Poyner and Reed (1992) used Mn^{2+} ions as activators and PhAH as the substrate analogue. They proposed that both metal ions are chelated by the PhAH molecule and form a μ-O bridge through its carbonyl oxygen. These were thought to lie 3.2–3.8 Å apart. In addition to the carbonyl oxygen, the "conformational" Mn^{2+} binds the hydroxyl group while the "catalytic" Mn^{2+} binds one of the phosphate oxygen atoms. Earlier EPR studies (Chien & Westhead, 1971) also showed a Mn^{2+}–Mn^{2+} spin exchange coupling.

We have carried out numerous experiments to localize the "catalytic" metal ion binding site by soaking the native crystals in artificial mother liquors containing metal ion and the substrate. Even in 10 mM PGA, 100 mM Mg^{2+}, and acetate buffer, pH 6.0, or in 5 mM PGA, 40 mM Zn^{2+}, and acetate buffer, pH 6.0, the "catalytic" metal ion binding site could not be localized, presumably because of the high ionic strength of the crystallization solution, 3 M ammonium sulfate. Since PhAH is such a strong inhibitor and a transition-state analogue, we thought it should increase binding of the "catalytic" metal ion to such occupancy that we will be able to find its site. This approach was successful, and we describe here the position of the "catalytic" metal ion binding site and discuss its role in enolase catalysis.

MATERIALS AND METHODS

Materials. Dowex 1-Cl resin, phosphonoacetic acid, NH_2-OH-HCl, LiCl, LiOH were purchased from Sigma. Ethanol, methanol, acetone, H_2SO_4, NaOH, and HCl were reagent grade. Deionized water was used as solvent.

Synthesis of Phosphonoacetohydroxamate. Phosphonoacetic acid was converted to phosphonoacetohydroxamate by the method of Anderson et al. (1984) and applied to a 2.5 × 25 cm column of Dowex 1-Cl. The column was washed with 100 mL of water twice and eluted with a total volume of 1000 mL of a 0–1.0 M LiCl gradient in 20 mM HCl. Fractions with PhAH were collected at about 70–80 mM LiCl according to its characteristic UV spectrum and visible spectrum (Anderson et al., 1984). To obtain purer product, only those fractions in the central range were used. They were titrated to pH = 8.0 with LiOH. The lithium salt of PhAH was recovered by lyophilization and removal of the LiCl by washing twice with dry methanol–acetone (1:4), yield 52%. The final product was stored at −20 °C over P_2O_5. The proton NMR (in D_2O) showed J_{PCH} = 18.9 Hz and δ = 2.5, which were within experimental error to the results obtained by Anderson et al. (1984). Our lower yield was expected because part of the eluted fractions had been discarded in order to keep the product at a higher purity.

Preparation of the Enolase–Mn^{2+}–PhAH–Mn^{2+} Complex and Crystal Data Collection. Crystals of yeast enolase suitable for X-ray diffraction studies were obtained by the seeding technique. Old crystals grown previously (Lebioda & Brewer, 1984) were broken into seeds, and some seeds were transferred into a preequilibrated solution. This solution included equal volumes of 3% enolase, 2 mM Mg^{2+}, 1 mM EDTA, 0.3 mM glutathione, 50 mM citric buffer (pH = 5.0), and 53% saturated ammonium sulfate which had been equilibrated against a 53% saturated ammonium sulfate for 2 days. The visible crystals appeared after 5 days, and crystal growth was completed within 3 weeks. Although some of the crystals had different morphology than crystals grown previously, they had the same unit cell dimensions and belonged to the same space group. The crystals were dropped into capillaries which were filled with artificial mother liquor solution made with 70% saturated ammonium sulfate at pH = 6.0. Then, certain amounts of the inhibitor, PhAH, and Mn^{2+} were added to the top of the capillaries, forming a concentration gradient of the ligands. Through the gradient, the ligands diffused slowly into the crystal (Lebioda & Zhang, 1992). This method decreases the probability of crystal cracking during the soaking process. After equilibrium was reached, the final concentrations of PhAH and Mn^{2+} were 2 and 10 mM, respectively. The crystals were resistant to the mother liquor changes; the unit cell dimensions $a = b$ = 124.1 Å, c = 66.9 Å were the same, within experimental error, as those for native crystals at pH = 5, binary crystals at pH = 6.0, and all inhibitory complexes except the enolase–Mg^{2+}–F^-–P_i complex. The data were collected with Rigaku R-AXIS II system and processed with the standard software.

A total of 36 445 observations with $F \geq 3\sigma(F)$ was merged and averaged, yielding 16 561 symmetry-independent reflec-

Table 1: Summary of Parameters and Results of Restrained Refinement

	deviations	
	σ	final rms
distances (Å)		
bond length (1–2 neighbors)	0.020	0.013
bond angles (1–3 neighbors)	0.040	0.048
planes (1–4 neighbors)	0.050	0.051
planar groups (Å)	0.020	0.009
chiral volume (Å^3)	0.150	0.153
nonbonded contacts (Å)		
single-torsion contacts	0.450	0.237
multiple-torsion contacts	0.450	0.286
possible H-bonding contacts	0.450	0.315
torsion angles (deg)		
peptide plane (ω)	3.0	
1.6		
staggered (±60°, 180°)	12.0	19.4
orthonormal (±90°)	16.0	32.3
weighting parameters for diffraction data A,B $[S = A - B(\sin\theta/\lambda - 1/6]$	8, −25	
final R (%)	16.5	
no. of water molecules in the model	342	

Table 2: Number of Reflections with $F > 3\sigma(F)$ and R Factors as a Function of Resolution for the Enolase–Mn^{2+}–Phosphonoacetohydroxamate Complex

D_{min} (Å)	8–4.5	3.5	3.0	2.8	2.6	rest	total
no. of reflections	2471	3344	3508	1997	2444	2176	15 940
completeness (%)	89.9	92.8	88.2	80.3	75.0		86.0
R (%)	16.8	15.0	15.3	15.6	16.0	16.5	16.5

tions with an R merge of 5.6%. The reflections constituted 83.3% of all possible reflections to 2.45-Å resolution. These reflections were used in the structure refinement.

The structure of the precatalytic ternary complex enolase–Mg^{2+}–PGA, which was determined at 2.2-Å resolution and refined to R = 16.9% (Lebioda & Stec, 1991), was used as the starting model. It gave R = 21% at the beginning of the refinement, indicating that this model was a very good starting point. All ligands coordinated to the metal ions: side chains, the inhibitor molecule, and water molecules were fitted to omit difference Fourier maps. The dictionary for the PhAH molecule was constructed using the bond lengths and bond angles found in the crystal structures of trichostatin A (Eng-Wilmot & Helms, 1981) and [diethyl (*N,N*-diethylcarbamoyl)methylenephosphonate]thorium nitrate (Bowen et al., 1982). No restraints on the torsion angles of the PhAH molecule were applied. The structure refinement by restrained least squares was carried out with the PROLSQ program (Hendrickson & Konnert, 1980). The final values of the refinement parameters are summarized in Table 1 and the distributions of R values in Table 2. A plot of average isotropic temperature factors as a function of residue numbers is represented in Figure 1.

RESULTS AND DISCUSSION

Structure. The refined structure is very similar to that of the native enolase, and the hydrogen-bonding pattern and the Ramachandran plot are very similar to those presented previously (Stec & Lebioda, 1990). The exception to that is the position of the movable loop Ser36–His43, which assumes, at least partially, the "closed" conformation found in the precatalytic ternary complex, enolase–Mg^{2+}–PGA/PEP (Lebioda & Stec, 1991). The electron density for Ser36–Gly37 is very strong (Figure 2), but the density for residues Ala38–

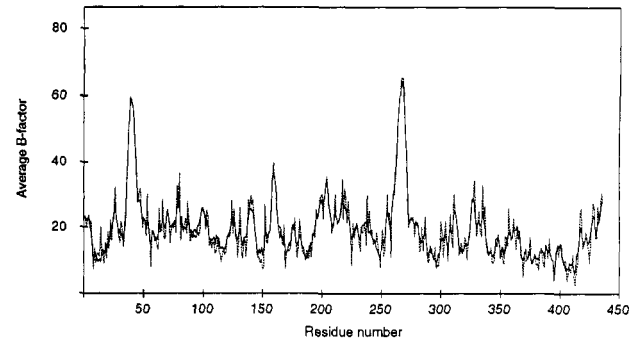

FIGURE 1: Average temperature factor for main-chain atoms (solid line) and side-chain atoms (dotted line) plotted against the residue number.

Val42 is very weak, and this part of the loop appears to be disordered. There is also some density corresponding to a fragment of the "open" conformation, indicating population of both the "closed" and "open" conformational states. Also, the unit cell dimensions for the inhibitory quaternary complex enolase–Mn^{2+}–PhAH–Mn^{2+} are closer to those of the native crystals rather than to those of the precatalytic complex. The ordered solvent is very similar to that in the precatalytic complex structure except for the active site area where three additional water molecules were localized, two coordinated to the "conformational" Mn^{2+} and the third in contact with the phosphonate group. The disordered part of the enolase molecule, residues Lys263–Lys271, still indicated overlapping multiple conformations and could not be resolved. The environments of the PhAH molecule, "conformational" Mn^{2+}, and "catalytic" Mn^{2+} are described below in more detail.

PhAH Molecule Environment. The omit electron density for the inhibitor molecule is shown in Figure 3. It consists of two connected lobes of density: one fairly globular, with maximum 9σ, which was assigned to the phosphonate moiety, and the second elongated, with maximum 5σ, assigned to the organic part of the molecule. The connecting density is weaker but visible at about 3σ level. In our previous studies of several phosphorylated ligands we also have observed weaker electron density in the regions between the phosphate and organic parts of the ligands. This is probably due to the series termination effect and a negative ripple around the strong phosphate density. Otherwise, the map is in very good agreement with the expected structure of the ligand.

It is apparent that the PhAH molecule has its hydroxamate oxygen atom coordinated to the "conformational" Mn^{2+} as proposed previously (Poyner & Reed, 1992). The PhAH molecule does not have a carboxylic group as does the PGA molecule, and this functionality is replaced by a carbonyl oxygen. So, the ionic interactions between the carboxylic group of PGA and residues His373 and Lys396 observed in the precatalytic complex are not present here. However, the side chain of Lys396 forms an H-bond (3.0 Å) to the carbonyl oxygen. There are no contacts between the carbonyl oxygen and the metal ions proposed by Poyner and Reed (1992), who reported a μ-O bridge, between the two Mn^{2+} ions, formed by the carbonyl oxygen. The electron density for the phosphonate group is strong but fairly spherical, and the positions of the oxygen atoms are to some extent tentative. We think that two oxygen atoms, O3P and O4P, are coordinated to the "catalytic" Mn^{2+} and O2P is H-bonded (3.1 -Å) to the OG atom of Ser39. There also is a contact, 3.0 Å, between the O3P and NH2 of Arg374.

There is no obvious explanation why PhAH binding to enolase is so much stronger than the binding of the substrates,

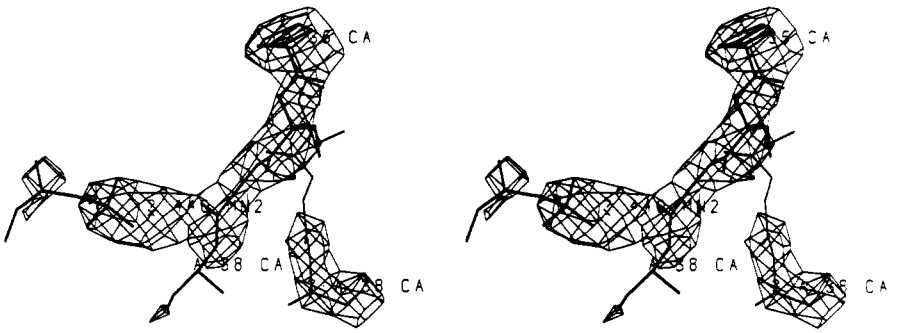

FIGURE 2: Electron density, $2F_o - F_c$, contoured at the 4.0σ level for the movable loop Ser36–His43 in the vicinity of the "catalytic" metal ion. The model in the "closed" conformation is drawn in heavy lines and corresponds to that in the precatalytic complex (Lebioda & Stec, 1991); the "open" conformation drawn for comparison, in lighter lines, is that from the enolase–Zn^{2+} complex (Lebioda & Stec, 1989). The electron density indicates that both conformations are populated.

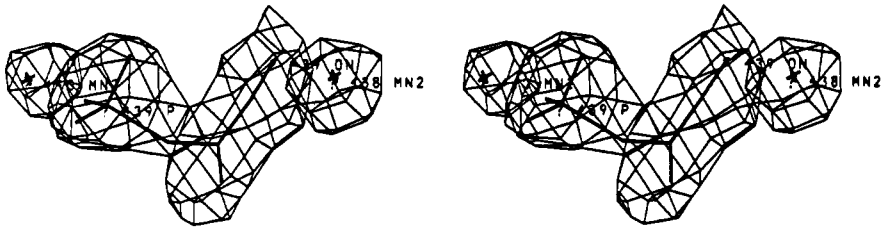

FIGURE 3: The environment of PhAH with two metal ions bonded in the active site. The electron densities, contoured at the 3.0σ level for PhAH and the 6.0σ level for metal ions, are from omit difference Fourier maps.

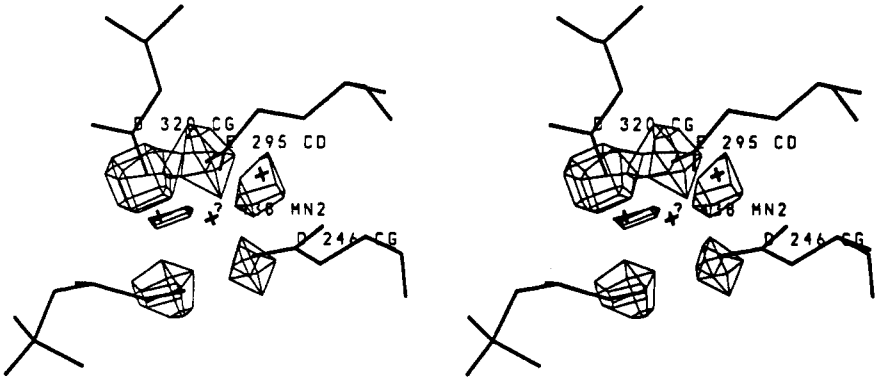

FIGURE 4: Coordination of the Mn^{2+} ion in the "conformational" site. The electron densities, contoured at the 6.0σ level for the side chains of residues and the 3.0σ level for other ligands, are from omit difference Fourier maps. The ligands form an almost regular octahedron.

PGA and PEP. It can be speculated that some of the energy of interaction between the carboxylic group of PGA and the side chains of Lys396 and His373 is used to distort the PGA molecule to the coplanar conformation (Lebioda & Stec, 1991). The distortion of the enzyme-bound substrate/product is corroborated by a variety of evidence (Brewer & Ellis, 1983; Brewer & Collins, 1980) and can consume a significant portion of the binding energy. The other substrate/product, PEP, which is planar, does not provide the hydroxyl group–metal ion interaction provided by PhAH.

"Conformational" Mn^{2+} Coordination. The environment for the "conformational" Mn^{2+} is shown in Figure 4. The positions of the three unidentate carboxylic ligands from the protein molecule to the "conformational" metal ion, Asp246, Glu295, and Asp320, are very close to those observed before. The ligands have good electron density in $2F_o - F_c$ maps. The oxygen atom of the hydroxamate group of PhAH is coordinated to the metal ion as discussed above. Two peaks were found at the "conformational" Mn^{2+} in a $F_o - F_c$ map at the 2σ contour level. The size and the distances to the metal ion indicate that they correspond to two water molecules. So, the coordination of the "conformational" Mn^{2+} is octahedral rather than the trigonal bipyramidal observed for Mg^{2+} and Zn^{2+} complexes. In each case, the structure about the conformational metal ion is well-defined. Consequently, the larger coordination number observed can be due either to the larger ionic radius of Mn^{2+}, 0.80 Å, versus 0.65 Å for Mg^{2+} and 0.74 Å for Zn^{2+} ions, or to the nature of the PhAH ligand. Nowak et al. (1973) reported two freely exchanging water molecules at the Mn^{2+} ion in the enolase–Mn^{2+} complex. This would suggest 5-fold coordination in the binary complex, unless there is a water molecule that does not exchange on the NMR time scale.

"Catalytic" Mn^{2+} Binding Site. An initial difference Fourier map calculated with the ligand, PGA, omitted from the model showed a strong, elongated electron density at the phosphate binding site. When the PhAH molecule was fitted to its position, there was still left an unassigned large density next to the phosphonate group. We hoped that this is the binding site for the "catalytic" Mn^{2+} ion, but to proceed cautiously, we assigned a water molecule to this position in the initial refinement. After a few cycles, the temperature factor for this water refined to 2 $Å^2$, and only software constraints prevented it from refining to a negative value. This indicated that a stronger scatterer must be bound in this position. Therefore, the water molecule was replaced with an

Mn^{2+} ion, and we proceeded with the refinement. The final temperature factor for this Mn^{2+} ion was 45 Å^2 while for the "conformational" Mn^{2+} ion the corresponding value was 26 Å^2. A difference Fourier map calculated for the final model with both metal ions omitted showed electron density peaks of 10σ in the "catalytic" site and 13σ in the "conformational" site. The distance between the metal ions was 8 Å.

The coordination of the "catalytic" Mn^{2+} ion could not be determined accurately besides the interactions with the phosphonate moiety of the inhibitor and the carbonyl oxygen of Gly37. We think, as discussed above, that the phosphonate group interacts with the ion in a bidentate mode; such an interaction mode was observed in the manganese–cytosine 5′-phosphate complex (Aoki, 1976). The peptide chain provides only one ligand to the "catalytic" metal ion, specifically, the carbonyl oxygen of Gly37 from the loop Ser36–His43. The remainder of the coordination sphere must be filled with disordered solvent, perhaps three water molecules, as there was no electron density that would indicate the positions of other ligands. The environment of the metal ion explains very well the observations that the "catalytic" metal ion binds only in the presence of substrates or substrate analogues and that the binding is not as strong as binding as to the "conformational" site. Binding of 2 equiv of metal ion/equiv of subunit in the absence of substrate or analogue must involve binding to the "conformational" and "inhibitory" sites (Elliot & Brewer, 1980).

Two functions of the "catalytic" metal ion in enolase activity are apparent. First, the presence of the positive charge at the phosphate moiety of the substrate should cause electron withdrawal from carbon 2 and weakening of the covalent bond between the carbon atom and the hydrogen atom (Brewer, 1985; Brewer & Ellis, 1983). Second, binding to Gly37, which is localized in the movable loop and available for binding only in the "closed" conformation of the enzyme, must stabilize this conformation.

It is possible that the product release from the enzyme takes place only in the "open" conformation. At high metal ion concentrations, the stabilization of the "closed" conformation slows down the rate of product release as opposed to the rate of substrate–product interconversion in the active site. This could be an explanation for part of the enolase inhibition observed at Mg^{2+} concentrations above 1 mM (Faller et al., 1977). Binding of Mg^{2+} to the "inhibitory" site, which has not been demonstrated as it has for Zn^{2+}, Co^{2+}, and Mn^{2+} (Faller et al., 1977; Elliott & Brewer, 1980; Brewer et al., 1983; Lee & Nowak, 1992b), would also be expected to contribute to inhibition.

CONCLUSIONS

On the basis of the exceptionally tight binding of PhAH to enolase it was postulated that the enolase–PhAH complex should closely resemble the transition-state complex (Anderson et al., 1984). The crystal structure reported here corroborates this hypothesis since the PhAH molecule binds in a manner analogous to that of the substrate molecule and induces the same conformational state of the enzyme. It should be emphasized that another competitive inhibitor, phosphoglycolate, does not induce the "closed" conformation (Lebioda et al., 1991). The presence of the "catalytic" metal ion in the transition-state analogue complex, which could not be located in the substrate complexes, appears to confirm the crucial role of this metal ion in the catalytic process. Our data confirm the conclusion by Poyner and Reed (1992) that the hydroxamate oxygen of PhAH coordinates to the "conformational" Mn^{2+} and an oxygen of the phosphonate group coordinates to the "catalytic" Mn^{2+}. Evidence from EPR measurements on Mn^{2+}–enolase complexes with substrate/product or with PhAH presented by Chien and Westhead (1971) and by Poyner and Reed (1992) indicates that "conformational" and "catalytic" Mn^{2+} are close enough (3.2–3.8 Å) for spin exchange to occur; Poyner and Reed (1992) also showed direct coordination between Mn^{2+} and the carbonyl oxygen of PhAH. On the other hand, Lee and Nowak (1992b) saw no spin exchange. PhAH is a slow-binding inhibitor (Anderson et al., 1984) and thus may bind to enolase in several ways. The X-ray data were obtained using crystals incubated with the inhibitor for days or weeks and give no hint of multiple modes of binding, show no unexpected changes in protein conformation, and are consistent with previously published structures. We are confident of the interpretation of our results presented here.

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