# Notes

## Interaction of Carboxypeptidase A with Anions: Crystal Structure of the Complex with the $HPO_4^{2-}$ Anion

**S. Mangani,*,† M. Ferraroni,‡ and P. Orioli‡**

Departments of Chemistry, University of Siena, 53100 Siena, Italy, University of Florence, 50121 Florence, Italy

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The interactions of small inorganic anions such as $Cl^-$, $N_3^-$, and $NCO^-$ with carboxypeptidase A (EC 3.4.17.1, CPA) have been the object of extensive research in the last few years.[1-3] Most of these studies take advantage of the spectral and magnetic properties of the cobalt-substituted derivative, which displays an activity toward peptide substrate hydrolysis even higher than the native zinc enzyme.[4] These studies have shown that the CPA active site becomes accessible to anions at low pH[5] or after chemical modification of the residue Glu-270,[6] which forms a strong hydrogen bond with the zinc-bound water molecule in the native enzyme.[7] These findings suggested that the breaking of the Glu-270–Wat-571 hydrogen bond was responsible for the activation of the zinc-bound water toward anion replacement. Indeed the crystal structures of the binary complexes of CPA with the D-phenylalanine and D-tyrosine[8] inhibitors and of the ternary complex with azide and L-phenylalanine[9] have provided the structural evidence for the above hypothesis. In both structures a strong hydrogen bond is formed between Glu-270 and the amino group of the amino acid inhibitor with consequent breaking of the former hydrogen bond with Wat-571, allowing, in the ternary complex, its replacement by an azide anion.

The above rationalization cannot hold in the case of the phosphate and pyrophosphate anions which have been shown by NMR and spectral studies on the cobalt–CPA derivative to bind directly to the metal atom at pH above 6 also in absence of carboxylate or amino acid inhibitors bound to the enzyme.[10]

In order to find an explanation for the behavior of the two anions, we have undertaken an X-ray diffraction investigation on the binary complex formed by phosphate and CPA.

### Experimental Section

**Protein Crystallization and Crystal Soaking.** Bovine carboxypeptidase A prepared by the method of Cox[11] was purchased from Sigma Chemical Co. and used without further purification. CPA crystallized in the space group $P2_1$ ($a$ = 51.52 Å, $b$ = 60.20 Å, $c$ = 47.34 Å, $\beta$ = 97.4°) by dialysis of an enzyme solution in 1.2 M LiCl and 0.02 M Tris–HCl at pH 7.5 against a similar solution 0.15 M in LiCl. Crystals of approximate dimensions 0.8 × 0.4 × 0.4 mm were obtained after several days. The crystals were then crosslinked using a 0.02 M Hepes buffer solution, 0.15 M in LiCl at pH 7.5 containing 0.02% (v/v) glutaraldehyde. The crystals were kept in the crosslinking solution for 30 min and then transferred to a soaking solution of the same buffer and salt composition containing 0.05 M $Na_2HPO_4$ and stored for 2 weeks in a cold room (280 K) before data collection.

**X-ray Data Collection, Solution and Refinement of the Structure.** X-ray data were collected on a Philips PW1100 automatic four circle diffractometer using graphite monochromated Cu K$\alpha$ radiation to a maximum resolution of 2.0 Å. The integrated intensities were collected by the $\omega$-scan technique and corrected for Lorentz and polarization effects. A linear decay correction (11% maximum), based on the intensities of three control reflections, was applied. A total of 22594 independent reflections were measured on one crystal in two sets. After scaling and merging ($R_{merg} = \sum|I - \langle I\rangle|/\sum I = 0.062$) 14865 reflections with $I > 1.5\,\sigma(I)$ were obtained and used for the structure refinement. Fourier difference maps were calculated with the set of programs PROTEIN.[12] Model building was performed on an Evans & Sutherland PS390 system with the graphic software FRODO[13] running on a Vax Station 3200 computer. The molecular model was refined by the restrained least-squares method of TNT set of programs.[14] Initial phases and structure factors were calculated from the atomic coordinates of native CPA from entry 5CPA of the Brookhaven Protein Data Bank.[15] The initial crystallographic agreement factor $R_{cryst}$ ($R_{cryst} = \sum|F_o - F_c|/\sum|F_o|$) was 0.30 for 10.0–2.0 Å data. The first difference Fourier map, calculated with $|F_{complex\text{-}obsd}| - |F_{nat\text{-}calcd}|$ coefficients and phases from the native structure, clearly showed a tetrahedrally shaped electron density peak in the place of the water molecule (Wat-571) bound to the zinc atom. In this map the density corresponding to the phosphate anion was between 6.0$\sigma$ and 3.0$\sigma$. A least-squares refinement of the structure was undertaken without the contribution of the water molecules of the active site cavity and of those residues most probably affected by the anion binding (Glu-270, His-69, His-196, Glu-72, Arg-127, Arg-71, Arg-145, Tyr-248, Ser-197, Ser-254, Thr-246, and $Zn^{2+}$). After few cycles a new difference map was calculated, and the water molecules and the above-mentioned residues were remodeled on the experimental electron density peaks. Further refinement of this model gave a final difference map, where the phosphate anion was clearly visible and could be modeled quite accurately. The final cycles of refinement included all the atoms of the protein and of the anion and converged smoothly to an $R$ factor of 0.146 for data between 10.0- and 2.0-Å resolution. The highest peaks in the final $\Delta F$ map were below 2.0$\sigma$. Root-mean-square deviations from ideal values of stereochemical parameters were 0.02 Å for bond lengths and 3.1° for bond angles, 0.02 Å for planarity of trigonal atoms, and 0.04 Å for other planes. The root-mean-squares deviations of the atomic positions were 0.15 Å, as estimated from a Luzzati plot.[16] The final model includes 193 water molecules. The refined atomic B factors for the atoms of the phosphate group vary from 25.2 to 40.7 Å$^2$ and average to 32.1 Å$^2$. This value compared to the mean $B$ factor for the protein molecule of 14.5 Å$^2$ indicates that the occupancy of the phosphate group in the crystal complex is about 50%.

### Results and Discussion

Figure 1 shows the difference electron density map at the metal site of the CPA–phosphate complex calculated with coefficients $|F_o| - |F_c|$ and phases from the refined model. Figure 2 shows the position of the phosphate ion bound to the active site cavity as obtained from the previous map and the hydrogen-bonding

† University of Siena.
‡ University of Florence.

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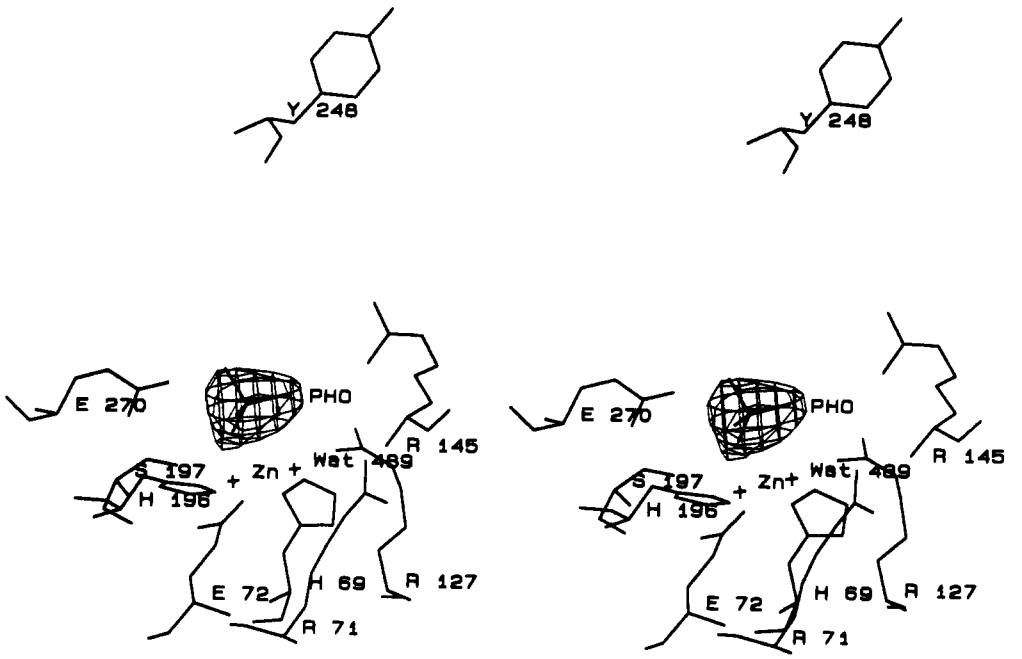


**Figure 1.** Stereoview of a difference Fourier map of the CPA–phosphate complex calculated with coefficients $|F_{\text{complex-obsd}}| - |F_{\text{complex-calcd}}|$ and phases from the refined model of the complex. The positive electron density is contoured at the level of $3.0\sigma$. The coordinates of the final model are superimposed.

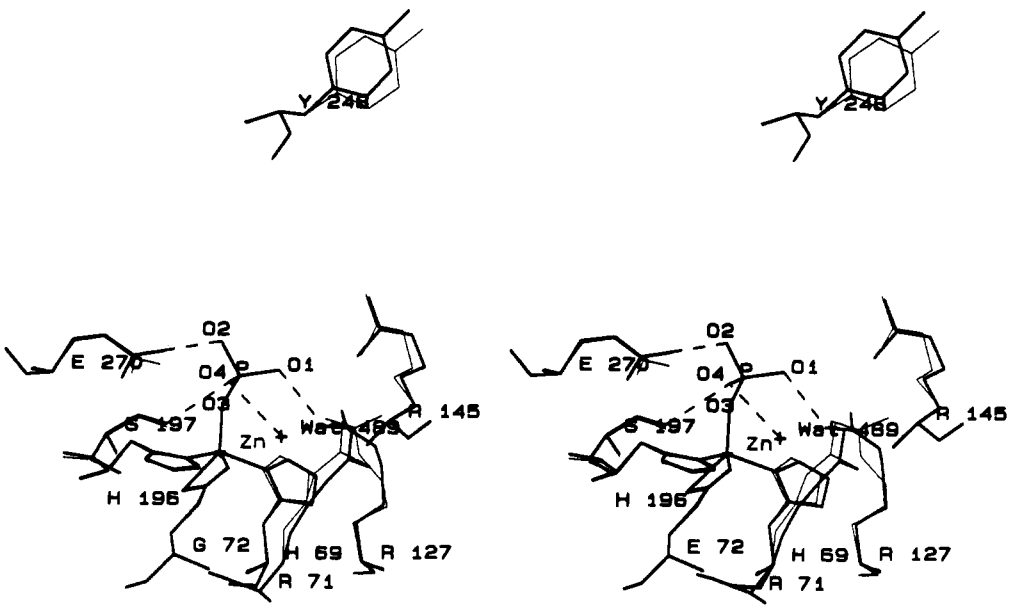


**Figure 2.** Stereoview of the binding of the phosphate anion to CPA. The coordinates from the refined model (thick bonds) are superimposed on those of native CPA[7] (thin bonds).

network formed with the other residues superimposed with the native enzyme model (5CPA). Table 1 reports the distances and angles around the zinc atom in the complex. The anion is probably bound as $HPO_4^{2-}$, which is the prevalent species present at pH 7.5. This protonation state is consistent with the observed hydrogen bonding pattern, but $H_2PO_4^-$ cannot be excluded in terms of the structural results. However the generic term phosphate has been used throughout the text to indicate the bound anion independently from its protonation.

Only one phosphate anion appears to be bound to the enzyme in the crystal structure. The inorganic anion has replaced the water molecule Wat-571 in the zinc coordination, and it is bound to zinc in a monodentate fashion. Inspection of the distances and angles around the zinc ion shows that its stereochemistry is unaltered after replacement of the water molecule by an oxygen of the phosphate anion. This stereochemistry has been described in terms of a distorted trigonal bipyramid or, alternatively, by combining the two carboxylate oxygen atoms of Glu-72 into an hypotetical atom located midway between the two, in terms of a distorted tetrahedron.[7] The phosphate ion forms four hydrogen bonds with the residues Glu-270, Arg-127, and Ser-197 and with Wat-489 (see Figure 2 and Table 2). Upon the formation of the complex, the side chains of the residues Arg-127 and Glu-270 change their native orientation in order to bind the anion (Figure 2). It is interesting to notice that Glu-270 is not hydrogen-bonded to the metal bound phosphate oxygen O3. This behaviour appears to be a characteristic of the monohydrogen (or dihydrogen) phosphate anion. Actually, the best CPA inhibitors known so far have a phosphonate group with only two oxygens available to act simultaneously as zinc ligands and as hydrogen bonding donors/acceptors toward the Glu-270 and Arg-127 side chains.[17] The

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**Table 1.** Zinc Coordination Distances and Angles in the CPA–Phosphate Complex and in Native CPA

| | CPA–phosphate | native CPA |
|---|---|---|
| | Distances (Å) | |
| Zn–Oε1 E72 | 2.1 | 2.2 |
| Zn–Oε2 E72 | 2.5 | 2.3 |
| Zn–Nδ1 H69 | 2.2 | 2.1 |
| Zn–Oε1 H196 | 2.2 | 2.1 |
| Zn–O3[a] | 1.9 | |
| Zn–O Wat571 | | 2.1 |
| | Angles (deg) | |
| Nδ1 H69–Zn–O3(Wat571) | 107.6 | 121.6 |
| Nδ1 H69–Zn–Nδ1 H196 | 96.2 | 98.8 |
| Nδ1 H196–Zn–O3(Wat571) | 102.0 | 95.6 |
| Nδ1 H69–Zn–Oε[b] E72 | 110.9 | 107.2 |
| Nδ1 H196–Zn–Oε E72 | 119.2 | 129.2 |
| Oε E72–Zn–O3 (Wat571) | 118.2 | 106.0 |

[a] O3 indicates the phosphate oxygen interacting with zinc. [b] Oε stands for a dummy atom positioned midway between the two carboxylate oxygens of the bidentate ligand Glu-72.

**Table 2.** Distances (Å) between CPA and the Phosphate Anion (Root-Mean-Square Deviation of the Atomic Positions ≅ 0.15 Å)[a]

| CPA | phosphate | distance |
|---|---|---|
| Nη1 R127 | O1 | 2.9* |
| Nη2 R127 | O1 | 3.3 |
| O Wat489 | O1 | 3.6 |
| Oε1 E270 | O2 | 2.4* |
| Nδ1 H69 | O3 | 3.3 |
| Oε1 E72 | O3 | 3.1 |
| Nδ1 H196 | O3 | 3.2 |
| Oε1 E270 | O3 | 3.1 |
| O Wat489 | O4 | 3.0* |
| Oε1 E72 | O4 | 3.4 |
| O S197 | O4 | 3.0* |

[a] Possible hydrogen bonds as judged from both distance and geometry criteria are indicated by an asterisk.

same is true for carboxylate-[18] and hydrated carbonyl-type[19] inhibitors. In all these cases the oxygen couple binds the zinc ion as a more or less symmetric bidentate ligand with the oxygen atoms engaged in interactions with the Glu-270 and Arg-127 residues.[20] The phosphate anion has the unique property to have three different oxygens available to satisfy the bonding requirements of the CPA metal site where, in order to be a strong inhibitor, a molecule must be able to bind the metal and, at the same time, to be hydrogen bonded with Arg-127 on one side and to Glu-270 on the other side. One of the oxygens must be protonated in order to interact with the negatively charged carboxylate side chain of Glu-270. This fact is reminiscent of the hydrogen bonding scheme found in the phosphate binding protein (PBP) where $10^5$-fold discrimination between monohydrogen phosphate and sulfate anions is obtained through the presence in the anion binding site of an aspartate side chain.[21]

The side chains of Arg-145 and of Tyr-248 remain both in the native positions as expected. These residues are involved in substrate binding and are "sensitive" only to carboxylate bearing aromatic inhibitors interacting with the S1′ hydrophobic pocket. The water molecules Wat-567 and Wat-587 bound in the native structure into the active site cavity near the zinc ion were not found in the structure of the present complex. A new water molecule, Wat-489, is observed instead, bound to one of the phosphate oxygens.

The phosphate and pyrophosphate ions are the only anions able to bind to the zinc at neutral pH and in absence of an amino acid or carboxylate group in the active site cavity of the enzyme. As we mentioned above, the binding of the phosphate ion to the metal ion has been predicted by a multinuclear NMR experiment in solution.[10] The perfect fit of the phosphate ion in the enzyme cavity, allowing the formation of a network of strong hydrogen bonds, provides a satisfactory explanation for this anomalous behaviour. The enthalpic contribution to the free energy of the phosphate binding from the four hydrogen bonds is the driving force of the complex formation and is able to explain the quite relevant inhibitory power of the phosphate ($K_i$ = 105 mM).[10] However it must be noticed that such constant has been evaluated in high ionic strength medium (NaCl 1 M). Williams and Auld[22] have determined the phosphate $K_i$ in absence of chloride and found a much lower $K_i$ = 0.40 mM at pH 7.5. In this case the authors have located a non-metal high-affinity site for the anion and have proposed Arg-145 for this role. Our experiment has been conducted at intermediate ionic strength (0.15 M LiCl), but no evidence whatsoever has been found for the proposed non-metallic binding site.

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