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Interaction of Phosphate and Pyrophosphate with Cobalt(II) Carboxypeptidase

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The mode of interaction between cobalt(II) carboxypeptidase A and the inhibitors phosphate and pyrophosphate has been investigated at pH 7.7 through electronic spectroscopy and ^1H, ^{31}P, and ^{13}C NMR spectroscopies. Analysis of the paramagnetic effect on the relaxation rates of the ^{31}P nucleus at different inhibitor concentrations clearly indicates direct coordination of both phosphate and pyrophosphate to the metal center under fast exchange conditions. This view is consistent with ^1H NMR and electronic spectroscopy results. The cobalt(II) chromophore is slightly perturbed upon phosphate binding and remains essentially pentacoordinated; on the other hand, binding of pyrophosphate causes a decrease in the intensity of the electronic spectra that may be indicative of a switch of cobalt(II) environment from penta- to hexacoordinated possibly through chelation by the inhibitor. The affinity constants of both phosphate and pyrophosphate for the metal are relatively low under the used solution conditions (1 M sodium chloride). In the case of phosphate, the inhibition constant for peptide hydrolysis has been determined under the same conditions and found to be in agreement with the spectroscopic affinity constant. Neither phosphate nor pyrophosphate compete with L-Phe for binding to Arg-145. ^{13}C NMR line-width measurements on ^{13}C-enriched L-Phe show that the amino acid binds to Arg-145 both in the presence and in the absence of phosphate and pyrophosphate. The results are analyzed and interpreted on the basis of the general model for the interaction between carboxypeptidase and anions.

Introduction

Carboxypeptidase A (CPD) is a zinc metalloenzyme that catalyzes the hydrolysis of the carbonyl terminal residue from peptide or ester substrates by cleavage of the peptide or ester bond.[1] The cobalt(II)-substituted derivative (Co(II)-CPD) has been shown to display an even higher catalytic activity with respect to the native system; owing to its favorable electronic properties, it has been extensively used as a spectroscopic probe for the investigation of the active-site structure and reactivity of CPD.[1]

It is known that phosphate (P) is a competitive or partially competitive inhibitor of both peptidase and esterase activity of CPD.[2] Pyrophosphate (PP) has also been reported to act as an inhibitor.[3] Inhibition could occur both at the metal site, which is proposed to coordinate the carbonyl group of substrates, or at Arg-145, which is responsible for the recognition and binding of the terminal carboxylate group of the substrate.

In order to shed light on the interaction of the enzyme with both inhibitors and to understand the structural features of the resulting adducts, we have undertaken an extensive investigation of the system through electronic spectroscopy and both ^1H and ^{31}P NMR spectroscopy.

^{13}C NMR studies have been also performed on samples containing ^{13}C-enriched L-Phe—an inhibitor that is known to bind to Arg-145[3]—in order to check whether there is competition between L-Phe and phosphate inhibitors for the Arg-145 site.

Experimental Section

Bovine carboxypeptidase prepared by the method of Cox et al.[4] was purchased from Sigma Chemical Co. and further purified according to standard procedures.[5] All reagents used were of analytical grade. The 90% ^{13}C enriched L-Phe was obtained from MSD isotopes.

Metal removal and cobalt(II) replacement were performed as previously described.[3] Protein crystals were dissolved in 0.05 M Hepes buffer, pH 7.7, in the presence of 1 M NaCl. Formation of the cobalt(II)-substituted enzyme was monitored through electronic absorption spectroscopy.

Inhibition of the peptidase activity by phosphate was measured spectrophotometrically with the substrate hippuryl-L-phenylalanine (Merck) as described by Folk and Schirmer.[6] The experiments were run in 1 M sodium chloride, 25 mM Tris-HCl buffer, pH 7.7, at 25 °C.

Electronic absorption spectra were run at room temperature on a double-beam Cary 17D and on a Perkin-Elmer Lambda 9 spectrophotometers.

^1H NMR samples were usually 1 mM. ^1H NMR measurements were performed at 90 MHz on a Bruker CXP 90 instrument at 300 K using the modified driven equilibrium Fourier transform (MODEFT) pulse sequence for water suppression.[7]

It has to be noted that addition of phosphate and pyrophosphate at high concentrations causes opalescence of the samples and slight protein denaturation. At very high anion concentrations (in particular in the case of pyrophosphate) protein precipitation is relevant. This is a severe limitation for absorption spectroscopy; the limitation is less severe for ^1H NMR measurements due to the intrinsic broadness of the line widths of paramagnetically shifted signals and to the much lower sensitivity of the relevant NMR parameter (the chemical shift) to changes in protein concentration.

^{31}P NMR measurements were performed on a Bruker AC 200-MHz instrument at 20 °C. Longitudinal relaxation times T_1 were measured with the inversion recovery method by using an appropriate nonlinear least-squares-fitting program. Transverse relaxation times T_2 were obtained from the line width at half-height through the relation $T_2^{-1} = \pi\Delta\nu$.

^{13}C T_2^{-1} NMR measurements were obtained on a Bruker AC 200-MHz instrument at 25 °C.

In the presence of a paramagnetic center, the total longitudinal relaxation rate of the $H^{31}PO_4^{2-}$ group changes according to[8]

$$T_1^{-1} = f_m T_{1M}^{-1} + T_{1dia}^{-1} \quad (1)$$

where T_{1dia}^{-1} is the intrinsic diamagnetic relaxation rate of the ^{31}P nucleus, T_{1M}^{-1} is the rate enhancement introduced by the nearby paramagnetic center, and f_m is the molar fraction of the bound ^{31}P species. It can be shown that

$$f_m = \frac{[E_T]}{\frac{1}{K} + [L_T]} \quad (2)$$

where $[L_T]$ is the total ligand concentration, $[E_T]$ is the total enzyme concentration, and K is the affinity constant for ligand binding. Titration of T_{1p}^{-1} as a function of $[L_T]$ can therefore be used to determine the affinity constant and T_{1M}^{-1}.[8]

$$T_{1p}^{-1} = T_{1M}^{-1}\left(\frac{[E_T]}{\frac{1}{K} + [L_T]}\right) \quad (3)$$

Results

Spectral Studies on Phosphate Binding to the Metal. ^1H NMR spectroscopy has been previously shown to be a powerful tool for the investigation of the active site of cobalt(II)-substituted CPD;[9,10]

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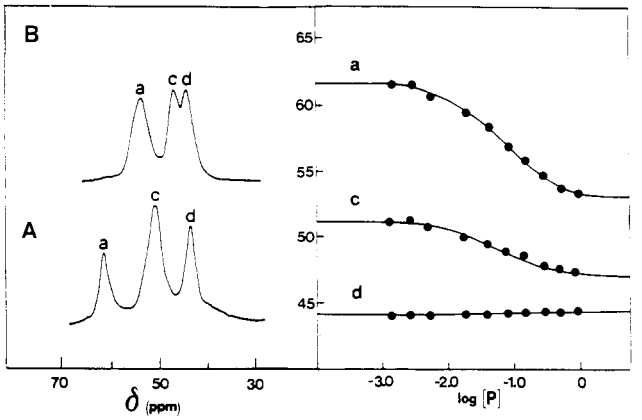

Figure 1. ^{1}H NMR spectra of Co(II)–CPD alone (a) and in the presence of 0.8 M P (b). Variations of chemical shifts of signals *a*, *c*, and *d* versus phosphate concentration together with the best fitting curve. Solution conditions are 1 mM Co(II)–CPD, 50 mM HEPES buffer, 1 M NaCl, pH 7.7.

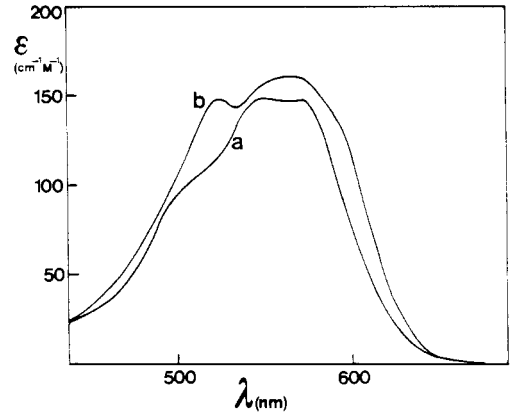

Figure 2. Electronic spectra in the visible region of (a) Co(II)–CPD alone and (b) Co(II)–CPD in the presence of 0.8 M P. Protein concentration is 0.4 mM; other conditions are as in Figure 1.

in particular the ^{1}H NMR spectra of Co(II)–CPD allow the observation of three relatively narrow, isotropically shifted signals in the downfield region which are assigned to the NH of His-69, and to the δ-CH protons of His-69 and His-196.

Co(II)–CPD was reacted with increasing amounts of HPO_4^{2-} at pH 7.7 in the presence of 1 M NaCl and the process followed through ^{1}H NMR spectroscopy (Figure 1). The ^{1}H NMR spectra are not sensitive to the addition of phosphate up to concentrations as large as 5 mM. For higher phosphate concentrations, signals *a* and *c* start moving few parts per million upfield. The process, which is fast on the NMR time scale, reaches completion at 0.8 M phosphate concentration and can be fitted to a simple equilibrium of the type Co(II)–CPD + P ⇄ Co(II)–CPD*P with an affinity constant of 16 ± 2 M^{-1} (Figure 1).

It can be observed that only signals *a* and *c* assigned to His-69[10] change their chemical shift upon binding of phosphate whereas the position of signal *d* assigned to His-196 remains virtually unchanged; this could mean that His-69 slightly changes its spatial arrangement whereas His-196 remains in the same orientation.

The electronic spectrum of Co(II)–CPD, in the presence of a saturating concentration of phosphate, under the same experimental conditions, is reported in Figure 2 together with that of native Co(II)–CPD. Inspection of the spectra suggests that binding of phosphate causes marked changes of the spectral shape but does not affect the molar extinction coefficient, which remains substantially unaltered. This suggests that phosphate modifies the metal center, probably binding directly to it, but does not alter its overall geometry. It should be remembered that the metal is

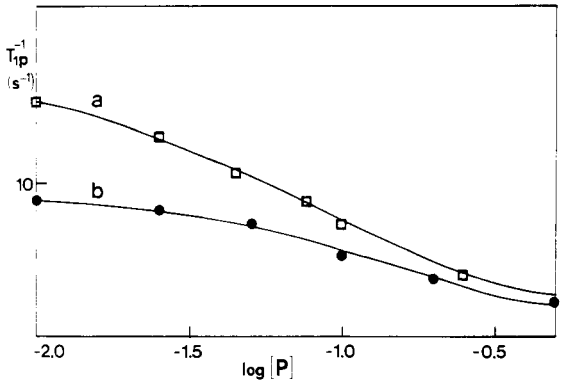

Figure 3. Dependence of T_{1p}^{-1} values versus phosphate concentration for (a) Co(II)–CPD alone and (b) Co(II)–CPD plus 10 mM L-Phe. Protein concentration is 0.5 mM; other conditions are as in Figure 1.

bound to two histidines and to a bidentate glutamate; the pentacoordinated geometry is completed by a water molecule.[11] Therefore, it can be suggested that phosphate binds to the metal, displacing the coordinated water molecule.

In order to check whether the spectrally determined affinity constants reflect inhibition of the enzyme, we have measured the peptidase activity of the native enzyme under the same solution conditions at increasing phosphate concentrations. Peptidase activity has been measured with the hippuryl-L-phenylalanine substrate monitoring the absorbance increase at 254 nm. Marked inhibition of the activity is observed only for phosphate concentrations higher than 10 mM (at saturating phosphate concentration residual activity is about 30%); best-fit analysis of the experimental data gives a K_i value of 105 ± 15 mM, which is in good agreement with the spectroscpic results.

Further insight into the interaction of phosphate with CPD comes from ^{31}P NMR spectroscopy. The binding of phosphate to Co(II)–CPD was followed by analyzing the changes in the nuclear relaxation rates of the ^{31}P NMR nucleus of phosphate. ^{31}P NMR T_1 and T_2 values were measured at protein concentrations of 0.5 mM and phosphate concentrations ranging from 1×10^{-2} to 1 M. In all instances, the T_1^{-1} and T_2^{-1} values were drastically increased with respect to analogous solutions containing Zn(II)–CPD. It can be easily deduced that the increase in nuclear relaxation rates is entirely due to the interaction of phosphate with the paramagnetic cobalt(II) center in the active site.

The paramagnetic contributions to the longitudinal relaxation rates (T_{1p}^{-1}) obtained by subtracting from the experimental data of paramagnetic Co(II)–CPD solutions those of the corresponding diamagnetic Zn(II)–CPD solutions at increasing phosphate concentrations are shown in Figure 3. The T_{2p}^{-1} values are about 30 times larger than T_{1p}^{-1} and follow the same pattern. Nevertheless, we prefer to work on T_1^{-1} in order to avoid possible exchange-controlled line broadening. A cause of divergence between T_2 and T_1 is the contact contribution, which is larger for the former.

The relaxation data (T_{1p}^{-1}) are concentration dependent and show a sigmoidal behavior if reported on a logarithmic scale indicating the presence of a single binding site for phosphate; therefore, they can be used to estimate the affinity constant of phosphate for the protein, assuming that the exchange rate between bound and free ligand is fast on the NMR time scale. The best fit of the experimental results to eq 3, taking both T_{1M}^{-1} and K as unknown, gave $T_{1M}^{-1} = 2.6 \times 10^3$ s^{-1} and $K = 13 \pm 2$ M^{-1}. The latter value is nicely consistent with that found through ^{1}H NMR spectroscopy, indicating that both the variations in ^{31}P NMR relaxation rates and the changes of ^{1}H NMR chemical shifts reflect the same chemical event, which is fast on the NMR time scale.

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The T_{1M}^{-1} value can be related to the metal–phosphorus distance through the Solomon equation:[12]

$$T_{1M}^{-1} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left(\frac{7\tau_c}{1+\omega_S^2\tau_c^2} + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right) \quad (4)$$

where μ_0 is the permeability of vacuum, r is the distance between the ^{31}P nucleus of phosphate and the cobalt(II) ion, γ_N is the nuclear magnetogyric ratio, g_e is the electron g factor, μ_B is the Bohr magneton, S is the spin quantum number, and ω_I and ω_s are the nuclear and electronic Larmor frequencies. If we use $\tau_c = 3 \times 10^{-12}$ s, as was previously estimated for Co(II)–CPD,[13,14] we obtain a very short metal–phosphorus distance (2.4 Å) that is indicative of direct coordination of the phosphate group to the metal. Indeed, the obtained value is markedly lower than expected for a two-bond distance (3.2 Å) and can be accounted for by assuming that ligand-centered dipolar effects are operative and important. In other words, the unpaired electron is not just on the metal but delocalizes onto phosphate itself.[8,15,16] A similar behavior has already been observed in the case of the interaction of phosphate with cobalt(II) carbonic anhydrase[17] and for some metal complexes.[18]

Phosphate is the only anion so far which directly binds to the metal at pH above 6. Other anions like azide and cyanate have been found to bind only in the presence of a carboxylate anion, or an amino acid at the S_1' site, typically L-Phe. The affinity of azide for Co(II)–CPD in the presence of L-Phe under saturating conditions (0.1 M) at pH 7.7 is 300 M^{-1}. We have repeated the ^{31}P NMR studies in the presence of 20 mM L-Phe and found that the new value of the affinity constant of phosphate for Co(II)–CPD is 7 ± 1 M^{-1}.

It has been proposed that L-Phe binds to an Arg residue inside the cavity, possibly Arg-145.[13] Indeed, a low-resolution X-ray report on a binary complex between L-Phe and CPD[19] and a recent crystallographic study on the ternary complex CPD/L-Phe/benzoyl-L-Phe[20] support this hypothesis as well as ^1H and ^{13}C NMR results on Co(II)–CPD.[10,13] ^{13}C NMR line widths for L-Phe ^{13}COO in the presence of Co(II)–CPD experience paramagnetic broadenings that are proportional to r^{-6} and to the mole fraction of the bound species.[13] A solution containing 1 mM Co(II)–CPD and 20 mM L-Phe has a ^{13}C NMR line width that remains essentially constant upon addition of increasing amounts of P. This means that under these conditions phosphate does not compete with L-Phe at Arg-145. The electronic spectrum is absolutely identical with that without L-Phe. The picture which results is that of a L-Phe molecule binding Arg-145 and simultaneously a phosphate molecule binding to the metal without any apparent interaction.

Finally, the possibility of formation of mixed ternary complexes between Co(II)–CPD and pseudohalides in the presence of phosphate has been checked. No such ternary complexes are formed.

Interaction between Co(II)–CPD and Pyrophosphate. Interaction between Co(II)–CPD and pyrophosphate has been investigated by performing parallel experiments such as those performed for phosphate. The electronic spectra of Co(II)–CPD are dramatically affected by pyrophosphate. The spectrum at a 0.11 M concentration of pyrophosphate is reported in Figure 4d. The intensity of the d–d transitions drastically decreases, and probably the reported spectrum is not the limit spectrum. This behavior can be rationalized by assuming that pyrophosphate binds to the metal, inducing a switch of its coordination number from penta- to hexacoordinated; pyrophosphate probably acts as a bidentate ligand. Since pyrophosphate induces some precipitation of the protein, the solution must be filtered before every measurement, and therefore, it is not possible to determine the affinity constant.

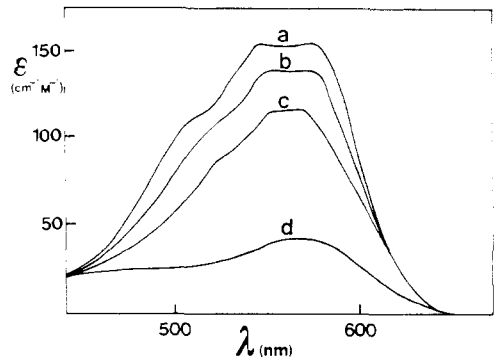

Figure 4. Electronic spectra of Co(II)–CPD in the visible region upon addition of increasing amounts of pyrophosphate. Pyrophosphate concentrations are, respectively, (a) 0, (b) 10, (c) 30, and (d) 110 mM. Protein concentration is 0.5 mM; other conditions are as in Figure 1.

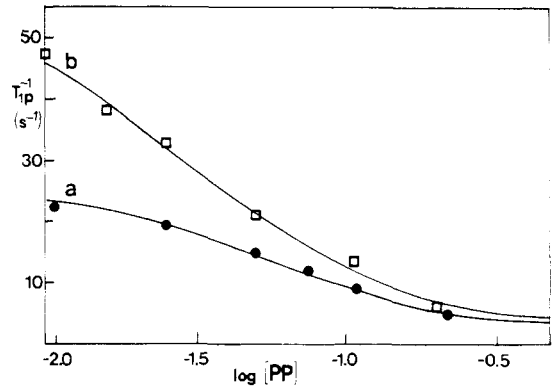

Figure 5. Dependence of T_{1p}^{-1} values versus pyrophosphate concentration for (a) Co(II)–CPD alone and (b) Co(II)–CPD plus 20 mM L-Phe. Protein concentration is 1.0 mM; other conditions are as in Figure 1.

The ^1H NMR spectra recorded at pH 7.7 do not show any perturbation in the low pyrophosphate concentration range; only for pyrophosphate concentrations higher than 10–20 mM do signals *a* and *c* start moving upfield under fast-exchange conditions. The effect is the same as for the phosphate case. Further increases in pyrophosphate concentrations cause sample precipitation and broadening of the NMR lines.

Measurements of the ^{31}P NMR relaxation rates at increasing pyrophosphate concentrations gave the pattern of T_{1p}^{-1} values reported in Figure 5; only one signal is observed, indicating that the two phosphorus atoms are equivalent under rapid exchange conditions. Analysis of the experimental data on the basis of the above reported treatment gives an affinity constant of pyrophosphate for Co(II)–CPD = 20 ± 3 M^{-1} and a metal–phosphorus distance equal to 2.3 Å. Again these results are consistent with direct binding of pyrophosphate to the metal. The affinity constant is qualitatively consistent with that deduced from the electronic spectra.

Pyrophosphate binds the metal ion also in the presence of L-Phe (20 mM) with an affinity constant of 50 ± 7 M^{-1} as estimated from the electronic and ^{31}P NMR spectra. The pattern is similar to that shown in the absence of L-Phe; the increase of the affinity constant is however small and does not lead to any suggestion in terms of structural properties. ^{13}C NMR measurements of 20 mM ^{13}C-enriched L-Phe ($^{13}COO^-$) in the presence of 0.5 mM

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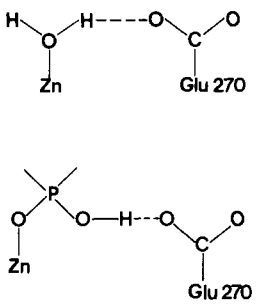
Chart I



Co(II)–CPD and increasing amounts of pyrophosphate have shown that the ^{13}C line width is independent of the concentration of pyrophosphate. Evidently, there is no competition between the two species.

Discussion

Joint application of different spectroscopic techniques has permitted extensive insight into the mechanistic details of the interaction of CPD with phosphate and pyrophosphate. For both anions, low-affinity direct binding to the metal center has been nicely documented by ^1H NMR, ^{31}P NMR and electronic spectroscopy. Whereas binding of phosphate to the metal causes only a distortion of the chromophore without alteration of the overall pentacoordinated geometry of the cobalt(II) center, binding of pyrophosphate causes the metal to switch from pentacoordinate to hexacoordinate.

The capacity of phosphate and pyrophosphate to bind to the metal, at variance with the behavior of other anions such as azide, cyanate, and thiocyanate, deserves some comment. It is believed that both the hydrogen bond between the metal-coordinated water and the carboxylate group of Glu-270 and the charge of the latter prevent anions from binding the metal and displacing the water. Indeed, methylation of Glu-270 makes zinc or cobalt available for binding.[21] Also, binding of carboxylates at Arg-145 allows the substitution of coordinated water by weakening the zinc–water–glu-270 network.[10] It is possible that the peculiar acid–base and steric properties of the two anions studied here allow direct water substitution by re-forming a zinc–P–Glu-270 bond net. This could be the main reason why phosphate and pyrophosphate behave differently from azide, cyanate, and thiocyanate.[3,10]

In agreement with Chart I, we may recall that phosphate has also been suggested to bind the alkaline form of other zinc enzymes like alkaline phosphatase[22] and carbonic anhydrase,[17] directly coordinating to the metal.

The inhibition properties of phosphate for native carboxypeptidase under the present ionic strength conditions have been determined; the K_i value of 105 ± 15 mM is in good agreement with the affinity constant values obtained from spectroscopy for the cobalt(II) derivative. This probably means that under the present experimental conditions binding of phosphate to the metal represents the actual mechanism for the inhibition of peptidase activity. A similar behavior may also hold for pyrophosphate.

It cannot be ruled out however that an additional nonmetallic binding site exists for phosphate, with higher affinity, which could be related to the inhibition of the catalytic activity at low chloride concentration. Indeed, the K_i value previously detected at low ionic strength (0.2 mM) is very low and cannot be reasonably explained on the ground of direct binding to the metal.[2] Competition between phosphate and chloride at the level of this nonmetallic site would account for the present results in the sense that at low ionic strength phosphate preferentially binds to the nonmetallic site whereas in 1 M sodium chloride it preferentially binds to the metal. A previous proposal had indicated Arg-145 as the probable nonmetallic site.[2]

In conclusion, we have shed light on the complex interaction between phosphate (and pyrophosphate) and CPD that pertains to the immediate neighbohorood of the metal ion and accounted for the mechanism of enzyme inhibition at high chloride concentration.

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