Spectroscopic Studies of the Interaction of Nickel(II) Carboxypeptidase with Phosphate and Pyrophosphate

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The interaction between nickel(II)-substituted carboxypeptidase A and the inhibitors phosphate and pyrophosphate has been investigated at pH 7 by electronic absorption and ^{1}H, ^{31}P, and ^{13}C NMR spectroscopies. Upon binding to the nickel enzyme, the longitudinal relaxation rates T_1^{-1} of the ^{31}P nucleus of these inhibitors are enhanced significantly compared to the diamagnetic zinc enzyme. The $Ni^{II} \cdots {}^{31}P$ distances indicate that both inhibitors bind directly to the metal ion under rapid-exchange conditions. From the temperature dependence of the isotropic shifts and the molar absorbance values of the 1:1 adducts formed, a pseudo-octahedral co-ordination for the metal ion is suggested. Proton NMR titrations of nickel(II) carboxypeptidase with phosphate in the presence and absence of D-phenylalanine (D-Phe) indicate that the occupancy of the S'_1 subsite of the enzyme does not substantially modify the affinity of the phosphate anion for the metal ion. Carbon-13 NMR T_2^{-1} measurements on $^{13}CO_2$-labelled D-Phe show that phosphate or pyrophosphate do not compete for binding to Arg-145. These results are discussed in terms of the general model of anion interaction with carboxypeptidase.

Carboxypeptidase A (CPA) is a zinc exopeptidase (M = 34 742) which catalyses the hydrolysis of the C-terminal amino acid of a polypeptide chain.[1] Its X-ray structure has been refined at 1.54 Å of resolution and the catalytic zinc ion is bound to two imidazole groups from His-69 and His-196, to a carboxylate bidentate group from Glu-72 and to solvent.[2]

It is known that C-terminal products of peptide or ester hydrolysis can interact with groups present in the active site cavity, possibly with Arg-145.[3,4] Indeed the X-ray structures of several inhibitors or substrate analogues invariably show that the terminal carboxylate binds Arg-145.[5–7] At neutral pH inorganic anions such as N_3^-, NCO^- or Cl^- show low affinity for the metal ion of CPA. However, the binding of anions to CPA is synergistically promoted by the presence of an amino acid in the S'_1 subsite of the enzyme.[8–10]

It is reported that phosphate is a competitive or partially competitive inhibitor of both the peptidase and esterase activity of CPA.[11] Pyrophosphate has also been suggested to act as an inhibitor.[9] We have recently investigated the interaction of these inhibitors with the cobalt-substituted carboxypeptidase (CoCPA).[12] Both inhibitors bind directly to the metal ion and their affinity is not affected by the presence of an amino acid in the S'_1 subsite of the enzyme.

The nickel(II)-substituted carboxypeptidase (NiCPA) retains practically completely the enzymatic activity [13] and its structure has been determined by X-ray crystallography.[14] The metal ion is co-ordinated to the same residues as in the native enzyme, and the ligand stereochemistry is close to square pyramidal. We recently studied this metalloderivative and its interaction with the amino acids L- and D-phenylalanine as well as the formation of ternary complexes with these amino acids and azide.[15]

In the present work, we have investigated the interaction of NiCPA with phosphate and pyrophosphate as well as the effect on this interaction of the binding of an amino acid in the non-metallic S'_1 site, using UV/VIS, ^{1}H and ^{31}P NMR spectroscopies. Since Arg-145 has been proposed as the high-affinity non-metallic binding site for anions,[8,9,16] we performed ^{13}C NMR studies of $^{13}CO_2$-labelled D-Phe in order to check the possibility of another binding site for phosphate and pyrophosphate.

Experimental

Bovine carboxypeptidase A, prepared by the method of Cox *et al.*,[17] was purchased from Sigma Chemical Co. and further purified through affinity chromatography on CABS-Sepharose to remove protease contaminants.[18,19] Removal of the zinc ion was performed from crystalline enzyme according to the reported procedure.[20] Nickel reconstitution was accomplished by incubation of a very diluted apo-CPA solution ($\approx 10^{-5}$ mol dm^{-3}) containing an excess of the metal ion ($\approx 10^{-4}$ mol dm^{-3}) during 24 h at 2–4 °C.[21] Under these concentration and temperature conditions the NiCPA was stable for several weeks. The enzyme concentration was determined at 278 nm by using a molar absorption coefficient of 6.4×10^4 dm^3 mol^{-1} cm^{-1}.[22] The formation of NiCPA could be monitored by electronic and ^{1}H NMR spectroscopies. The compounds $NiSO_4 \cdot 6H_2O$, Na_2HPO_4, $Na_4P_2O_7 \cdot 10H_2O$, D-Phe, and all the other chemicals were Merck analytical grade reagents. The 99% $^{13}CO_2^-$-enriched D-Phe was obtained from MSD isotopes and the 99.7% D_2O from Fluka.

Optical spectra were registered on a UV/VIS/NIR Perkin-Elmer Lambda 9 spectrophotometer, using microcells with an optical path length of 10 mm and the NiCPA concentrations were approximately 0.5×10^{-3} mol dm^{-3}. The spectra were recorded using as reference a solution of the native enzyme under the same conditions. The estimated error in the ε values is about 10%. The pH values of all the solutions were measured on a Crison digit-501 pH meter provided with an Ingold combined microelectrode.

The samples for NMR measurements were concentrated to $(0.5–2) \times 10^{-3}$ mol dm^{-3} protein by ultrafiltration at 2–4 °C using a Centricon microconcentrator (Amicon) with a molecular weight cut-off of 10 000. For long NMR experiments several samples with the same NiCPA concentration were used due to the reduced stability of the metal derivative. The ^{1}H NMR spectra were recorded on a Bruker AC-200 MHz spectrometer at 20 and 10 °C [1 mol dm^{-3} NaCl, 50×10^{-3} mol dm^{-3} *N'*-(2-hydroxyethyl)piperazine-*N*-ethane-2-sulfonic acid (hepes) pH 7] using the SUPERWEFT[23] multipulse sequence, 180°–τ–90°–acquisition + delay, with τ values of about 90 and 83 ms recycle time. The use of such a sequence enables us selectively to reduce the intensity of signals having

longitudinal relaxation times longer than these signals of interest. Spectra typically consisted of ≈16 000 scans with 8k data points and a spectral width of 50 kHz. Chemical shifts were measured from the H_2O or HDO signals and referenced to $SiMe_4$ assumed at −4.8 ppm from the water signal. A 20 Hz line-broadening function was applied to improve the signal-to-noise ratio.

The ^{31}P NMR measurements were performed on a Bruker AC-200 MHz instrument at 20 and 10 °C, using a spectral width of 5 kHz with 8k data points and a 4 Hz line-broadening function. Longitudinal relaxation times T_1 were measured with the inversion-recovery method by using an appropriate non-linear least-squares-fitting program. The ^{13}C NMR transverse relaxation times, T_2, were obtained from the linewidth at half-height through the relation $T_2^{-1} = \pi\Delta\nu_{\frac{1}{2}}$.

In the presence of a paramagnetic centre, and under rapid-exchange conditions, the total relaxation rate of the ^{31}P nucleus changes as in equation (1),[24] where T_1^{-1} is the intrinsic

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

diamagnetic relaxation rate of the ^{31}P nucleus, T_{1M}^{-1} the rate enhancement introduced by the nearby paramagnetic centre and f_m the molar fraction of the bound ^{31}P species. Expression (2) can be written, where $[L]_T$ is the total ligand concentration,

$$T_{1p}^{-1} = T_1^{-1} - T_{1d}^{-1} = T_{1M}^{-1} \frac{[E]_T}{K^{-1} + [L]_T} \quad (2)$$

$[E]_T$ 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}.[24]

Results and Discussion

NiCPA(phosphate) *Complex*.—The ^1H NMR spectrum of an aqueous solution of 2.5 × 10^{-3} mol dm^{-3} NiCPA displays three resolved isotropically shifted signals, in the downfield region, situated at δ 57.3 (a), 53.4 (c) and 49 (d) [Fig. 1(*a*)]. These signals are assigned to the HNε2 and HCδ2 protons of the two co-ordinated histidines, His-69 and 196 (Scheme 1). Only signal a disappears when the spectrum is registered in D_2O, and it is assigned as the HNε2 exchangeable proton of a co-ordinated histidine. The second NH histidine is missing in the ^1H NMR spectrum, as occurs for the cobalt(II) derivative, and it has been suggested that it exchanges rapidly with bulk water on the NMR time-scale.[8] Similarly, the ^1H NMR spectrum of the adduct with phosphate shows three resolved signals at δ 58.5, 56.9 and 53.4 [Fig. 1(*b*)]. When the spectrum of the adduct is recorded in D_2O only the signal at δ 58.5 disappears, indicating the presence of one exchangeable NH proton and two HCδ2 protons. The correlation between the signals of NiCPA and the phosphate complex can be easily obtained because the inhibitor binds the metal ion in a rapid-exchange regime. Therefore, the chemical shift of each signal depends on the molar fraction of bound enzyme. This allowed the use of a titration technique to follow the changes in the isotropically shifted proton signals with increasing phosphate concentration. The changes in shifts of the HNε2 and HCδ2 signals as a function of the anion concentration are shown in Fig. 1. The variation is essentially complete at 1 mol dm^{-3} phosphate concentration, and the sigmoidal curve obtained can be fitted by a simple equilibrium of the type NiCPA + phosphate $\rightleftharpoons$ NiCPA(phosphate) with an affinity constant K_{app} = 12 ± 1 dm^3 mol^{-1} (pH 7, 293 K).

The temperature dependence of the isotropically shifted resonances of the NiCPA(phosphate) complex has been investigated in the temperature range 3–30 °C. All the signals follow a Curie-like behaviour, *i.e.* their isotropic shifts decrease with increasing temperature, and the linewidths become slightly narrower. In Fig. 2 the observed isotropic shifts of the HNε2 and HCδ2 protons are plotted *vs.* T^{-1}. The intercept values at infinite temperature were within the diamagnetic region, except for signal d. This signal exhibits only small changes (≈2 ppm) in the studied temperature range and it becomes broader when the temperature is decreased. These results indicate that the shifts for the phosphate complex are essentially contact in origin as expected for an orbitally non-degenerate ground state with excited levels far higher in energy. This is the case in pseudo-octahedral nickel(II) co-ordination.

When phosphate is added to a solution of NiCPA (pH 7, 50 × 10^{-3} mol dm^{-3} hepes) the visible spectrum of the nickel enzyme is slightly modified. Thus, the intensity of the d–d transitions decreases ($\varepsilon_{685} \approx 7$ dm^3 mol^{-1} cm^{-1}) and, in addition, the band at 695 nm is displaced to 685 nm. The position and molar absorbance of the absorption bands is consistent with a pseudo-octahedral co-ordination of the metal ion.

^{31}P NMR spectroscopy may shed further light on the interaction of NiCPA and phosphate. The longitudinal relaxation rate, T_1^{-1}, for the ^{31}P nucleus is drastically increased when bound to the paramagnetic nickel derivative as compared to the diamagnetic native enzyme. The relaxation rate enhancement can be attributed to dipolar coupling of the ^{31}P nucleus with the paramagnetic nickel ion, and thus can be used to calculate the M ••• ^{31}P distance in the adduct by using the Solomon equation [25] (3). In this equation μ_o is the permeability

$$T_{1M}^{-1} = (2/15)(\mu_o/4\pi)^2 \cdot \frac{\gamma_n^2 g_e^2 \mu_B^2 S(S+1)}{r^6} \left(\frac{7\tau_c}{1+\omega_s^2\tau_c^2} + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} \right) \quad (3)$$

of a vacuum, γ_n is the nuclear magnetogyric ratio, g_e is the electron g factor, ω_I is the nuclear Larmor frequency, ω_s is the electronic Larmor frequency and τ_c is the correlation time. From the T_1 ^{31}P NMR measurements and through the use of equation (2), the full paramagnetic effect T_{1M}^{-1} was calculated as 2330 s^{-1}. By using equation (3), with a τ_s value of 5 × 10^{-11} s,[26,27] a Ni^{II} ••• ^{31}P distance of 3.0 ± 0.2 Å was determined. This distance is consistent with direct co-ordination of the phosphate group to the metal.

We have also performed a ^1H NMR titration of 2.1 × 10^{-3} mol dm^{-3} NiCPA with phosphate in the presence of 1.4 × 10^{-3} mol dm^{-3} D-Phe. The changes in shifts of the HNε2 and HCδ2 signals as a function of the phosphate concentration are similar to those described above in the absence of D-Phe. Thus, no measurable difference in K_{app} for phosphate occurs in presence of the amino acid.

In order to check the possibility of an additional non-metallic binding site having higher affinity for phosphate, we have made ^{13}C NMR T_2^{-1} measurements for ^{13}C-enriched D-Phe in the presence of NiCPA. It has been established that D-Phe binds to an Arg residue inside the cavity, probably Arg-145.[3] Recent crystallographic studies on the binary complex CPA–D-Phe [28] support this hypothesis as well as ^1H and ^{13}C NMR results on CoCPA.[3,8] Carbon-13 NMR T_2^{-1} measurements of a solution of 3 × 10^{-3} mol dm^{-3} labelled D-Phe$^{13}CO_2$ in the presence of 10^{-3} mol dm^{-3} NiCPA and increasing amounts of phosphate show that the ^{13}C linewidth remains essentially constant for phosphate concentrations in the range (1–300) × 10^{-3} mol dm^{-3}. These results indicate that under these conditions phosphate does not compete with D-Phe at Arg-145.

NiCPA(pyrophosphate) *Complex*.—The visible spectrum of NiCPA is practically not affected by pyrophosphate, and only a small decrease in the intensity of the d–d transitions is observed ($\varepsilon_{695} \approx 4$ dm^3 mol^{-1} cm^{-1}). Similarly to the phosphate complex, the intensity and position of the bands is consistent with pseudo-octahedal co-ordination. Probably, the

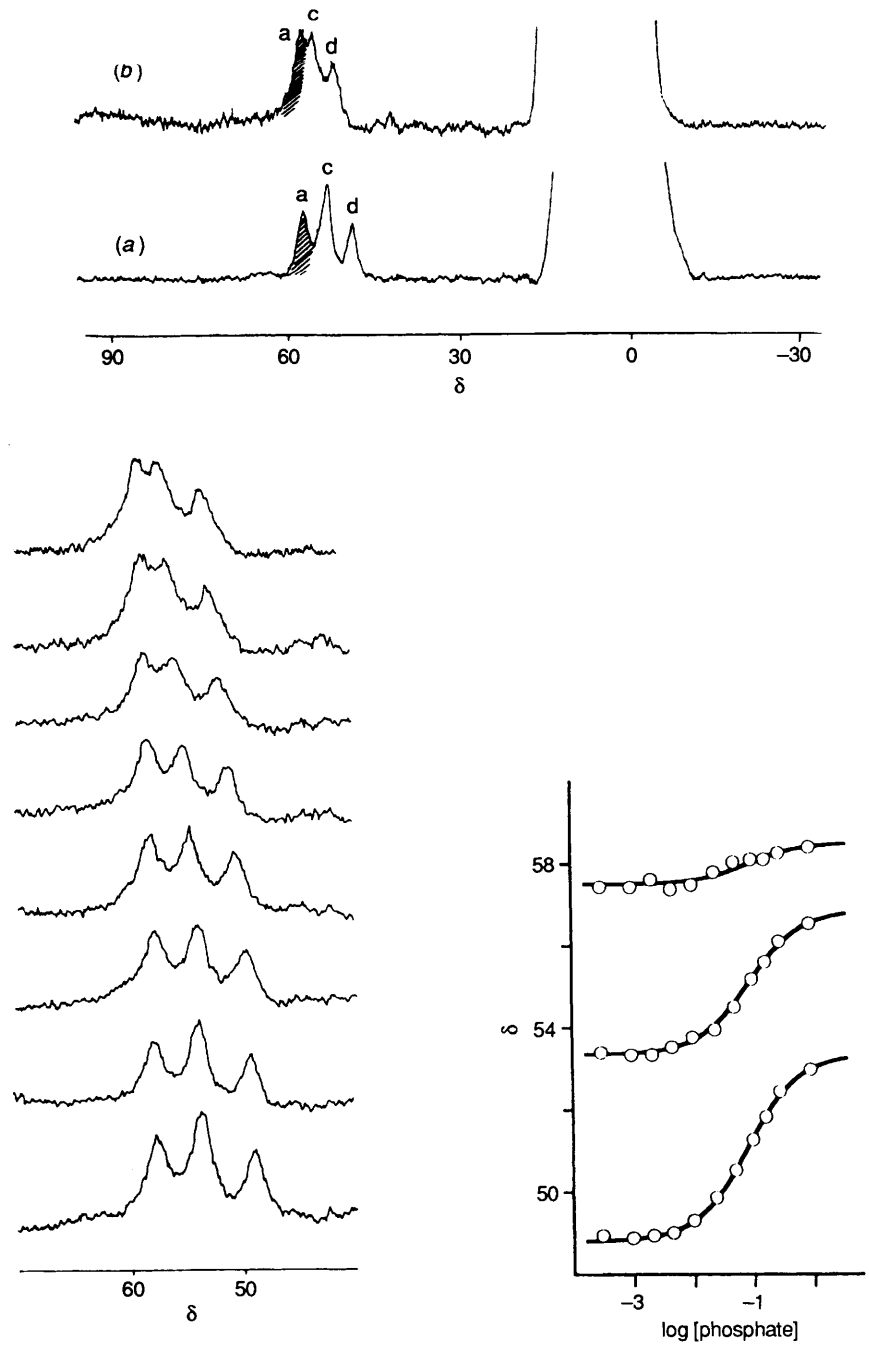


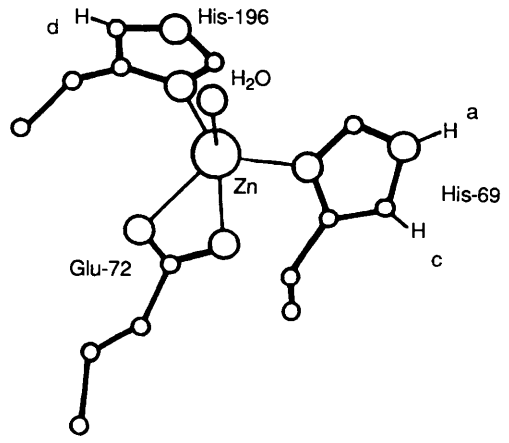
Fig. 1 200 MHz ^{1}H NMR spectra of NiCPA (*a*) and of its adduct with phosphate (*b*). The variations in chemical shifts of signals a, c and d with phosphate concentration are also shown with the best fitting curve. Solution conditions are 2.5 × 10^{-3} mol dm^{-3} NiCPA, pH 7 (50 × 10^{-3} mol dm^{-3} hepes, 1 mol dm^{-3} NaCl) and 293 K

pyrophosphate binds directly to the metal ion as a bidentate ligand displacing the co-ordinated water molecules.

Apparently, the ^{1}H NMR spectrum of NiCPA does not display any modification at low pyrophosphate concentrations. However, inhibitor concentrations higher than (6–10) × 10^{-3} mol dm^{-3} produce protein precipitation causing severe broadening of the NMR signals; so a quantitative study of the interaction is hindered.

Fortunately, the ^{31}P NMR study shed more light on this interaction. The T_1 and T_2 values were measured at 0.4 × 10^{-3} mol dm^{-3} protein and pyrophosphate concentrations ranging from 2.5 to 60 × 10^{-3} mol dm^{-3}. Only one signal is observed, indicating that the two phosphorus atoms are equivalent under rapid-exchange conditions. Furthermore, the fact that T_{2p}^{-1} values are about one order of magnitude higher than the corresponding values for T_{1p}^{-1} ensures that the latter are in the rapid-exchange regime and thus can be used for calculations of M • • • ^{31}P distance. In Fig. 3 the ^{31}P NMR T_1^{-1} values are plotted against the logarithm of pyrophosphate concentration. The sigmoidal curve obtained indicates the presence of a single binding site for pyrophosphate. The best fit of the experimental values gave T_{1M}^{-1} = 1200 s^{-1} and K_{app} = 130 ± 10 dm^3 mol^{-1} (pH 7, 283 K). By using the Solomon equation,[25] with a τ_s value of 5 × 10^{-11} s,[26,27] a Ni^{II} • • • ^{31}P distance of 3.2 ± 0.2 Å is obtained, consistent with direct co-ordination of the pyrophosphate to the metal ion. A similar behaviour has been reported in the case of the cobalt enzyme.[12]

It is known that at alkaline pH pyrophosphate anion interacts with aqueous nickel(II) forming two complexes of stoichiometry 1:1 and 1:2.[29,30] The determined stability



Scheme 1

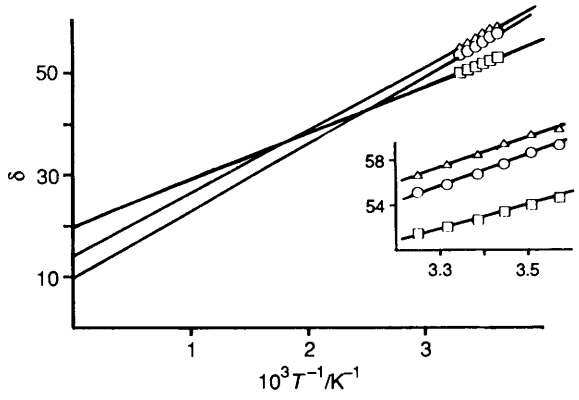


Fig. 2 Temperature dependence of the isotropic shifts for the NiCPA(phosphate) complex at pH 7; (△), a; (○), c; (□), d

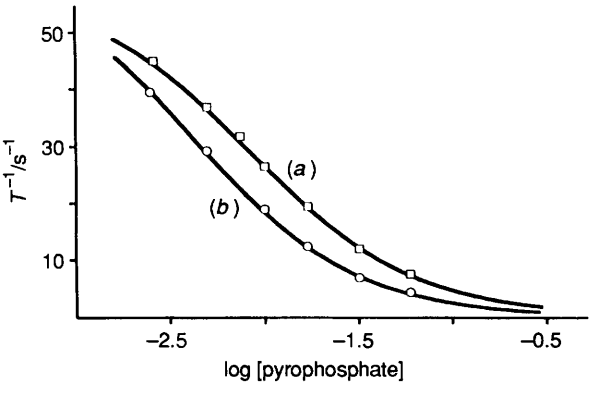


Fig. 3 Values of T_1^{-1} for the ^{31}P nucleus as a function of pyrophosphate concentration in the presence of (*a*) 0.5 × 10^{-3} mol dm^{-3} NiCPA, (*b*) 0.5 × 10^{-3} mol dm^{-3} Ni^{2+}(aq). Solution conditions are pH 7 (50 × 10^{-3} mol dm^{-3} hepes, 1 mol dm^{-3} NaCl) and 283 K

constant of the 1:1 complex is very high in this medium (≈6.5 × 10^5 dm^3 mol^{-1}). In order to compare the reactivity of the nickel inside the active cavity of CPA and aqueous nickel(II), we have performed a ^{31}P NMR study of the system Ni^{2+}(aq)–pyrophosphate at pH 7. The conditions described above for NiCPA were maintained. The measured ^{31}P T_1 values are plotted in Fig. 3. The data can be fitted by a sigmoidal curve with an affinity constant of K_{app} = 290 ± 20 dm^3 mol^{-1}. Furthermore, the addition of pyrophosphate to a solution of Ni^{2+}(aq) at pH 7 produces little changes in the optical spectrum. Thus, the position of the absorption bands at 392 (ε = 6) and 715 nm (ε = 3 dm^3 mol^{-1} cm^{-1}) are displaced to 405 (ε = 8) and 740 nm (ε = 3 dm^3 mol^{-1} cm^{-1}) respectively. From the absorption data as a function of the pyrophosphate concentration, an affinity constant of about 300 dm^3 mol^{-1} can be estimated, totally consistent with that obtained from ^{31}P NMR spectroscopy. These results indicate that the formation constant for the 1:1 complex at pH 7 is three orders of magnitude less than that at high pH, suggesting that the protonated forms of pyrophosphate show much lower affinity for Ni^{2+}(aq) than does the pyrophosphate anion $P_2O_7^{4-}$. Although the optical spectrum of NiCPA(pyrophosphate) shows some differences in the position of the signals compared to the Ni^{2+}(aq)–pyrophosphate complex, since different donor atoms complete the respective co-ordination spheres, the affinity constants of both complexes are of the same order of magnitude, which suggests a similar binding fashion of pyrophosphate in the two systems.

In addition, we have made ^{13}C NMR T_2^{-1} measurements of a solution of 3 × 10^{-3} mol dm^{-3} $^{13}CO_2^-$-labelled D-Phe in the presence of 1 × 10^{-3} mol dm^{-3} NiCPA and pyrophosphate concentrations ranging from 1 × 10^{-3}–100 × 10^{-3} mol dm^{-3}. As in the case of phosphate, the ^{13}C linewidth is independent of the pyrophosphate concentration. Thus, under the present experimental conditions there is no competition between the two species.

Conclusion

At neutral pH, nickel(II) carboxypeptidase forms 1:1 complexes with phosphate and pyrophosphate anions. These inhibitors bind directly to the metal ion of the enzyme, and the optical spectra and temperature dependence of the isotropic shifts are consistent with a pseudo-octahedral nickel co-ordination. The higher affinity of pyrophosphate is probably due to bidentate co-ordination of this ligand.

It is well established that inorganic anions, like azide or cyanate, only bind directly to the metal ion when the S'_1 subsite of the active cavity is occupied by an amino acid.[8–10] This behaviour has recently been explained on the basis of crystallographic studies on the binary complexes of CPA with D-Phe and D-Tyr.[28] Thus, the carboxylate of the bound amino acid salt links with Arg-145, and the α-amino group interacts with Glu-270, and as a consequence of these interactions the hydrogen bond between the Glu-270 and the co-ordinated water molecule is broken facilitating substitution of the latter. In this context, phosphate and pyrophosphate display a singular behaviour because they bind to the metal ion in the absence of amino acids. Furthermore, the occupancy of the S'_1 subsite by an amino acid does not produce any measurable modification of the affinity of these inhibitors. Probably, the particular acid–base and steric properties of these ligands allow direct water displacement by reconstituting a Glu–phosphate–zinc hydrogen net.

Finally, Arg-145 has been proposed as the non-metallic binding site for inorganic anions.[8,9,16] Although we have investigated this possibility by ^{13}C NMR spectroscopy for the two anions studied here, no evidence of this has been obtained. However, the possibility of such a binding site for phosphate, with higher affinity, which could be related to the inhibition of the catalytic activity of carboxypeptidase at low chloride concentration,[11] could not be excluded.

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