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Investigation of Cobalt(II) Substituted Carboxypeptidase A Interacting with Azide and Cyanate Ions

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Abstract

Cobalt(II)-substituted carboxypeptidase A has been found to reversibly bind N_3^- and NCO^-, but not NCS^-, in the pH range 5–10, thus including the pH range of activity of the enzyme. The pH dependence of the anion binding constant is affected by two ionizations, which are assigned as those regulating k_{cat} and K_M. The electronic and ^1H NMR spectra are consistent with a substantially pseudotetrahedral geometry of the anion derivatives.

Introduction

Bovine carboxypeptidase A (CPA hereafter) is among the most studied zinc enzymes [1]. Its X-ray structure [2], refined up to 1.75 Å of resolution, has shown that the native zinc(II) ion is coordinated to two histidine (His-69 and -196) and one glutamic acid (Glu-72) residues and to a water molecule. Cobalt(II) can substitute the native zinc(II) ion [3], yielding a derivative which is more active than the native enzyme [4]. X-ray data have shown [5] that the zinc and cobalt derivatives are structurally extremely similar.

CPA dissolves in sizeable amounts only in high ionic strength solutions, such as 1 M NaCl. It has been reported [6] that anionic ligands do not bind the metal ion in the cobalt(II) substituted enzyme, while an apparent affinity constant around 10 M^{-1} at pH 7.5 has been calculated for the fluoride ions towards the manganese(II) substituted enzyme, through ^{19}F NMR relaxation measurements [7]. Very recently, Vallee *et al.* [8] have reported that some anionic ligands interact with cobalt(II) in cobalt-substituted CPA (CoCPA hereafter) at pH values below 6, *i.e.* in a pH range in which the enzyme is not active. Vallee *et al.* suggested that the affinity of anions for CoCPA drops upon deprotonation of the group with pK of 5.3 responsible for the pH dependence of k_{cat} [9]. Further investigation of anion binding to the metal in CPA and its dependence on the various acidic groups present in the cavity appeared interesting, for comparison purposes with carbonic anhydrase. In several respects, CPA and carbonic anhydrase are similar and the investigation of anion binding to the cobalt(II) substituted derivative of the latter enzyme [10] has been instructive as far as the chemical properties of the cavity are concerned. A comparison of the two enzymes is in our opinion by itself chemically and biochemically relevant. With this in mind we have investigated the interaction of NCO^-, N_3^- and NCS^- with the cobalt(II)-substituted derivative of CPA.

Experimental

All the chemicals used were of analytical grade. Carboxypeptidase A, prepared by the Cox method [11], was purchased from the Sigma Chemical Company as crystalline suspension and was used without further purification. Apocarboxypeptidase was prepared according to a modification of the procedure previously reported [6] by dissolving the crystals in 10% LiCl solution and dialyzing the solution for three days against four changes of a buffer solution containing 1 M NaCl, 0.1 M Tris-HCl, 2×10^{-3} M *o*-phenantroline at pH 6. This was followed by four–five changes of a dithizone extracted solution of the same buffer without *o*-phenantroline at pH 6.8, to remove the excess of the chelating agent. The apoenzyme solution thus obtained was exhaustively dialyzed against a 1 M NaCl solution extracted with dithizone to remove the Tris buffer, and then reacted with stoichiometric amounts of unbuffered metal chloride solutions in order to reconstitute the zinc enzyme or to generate the cobalt(II) derivative. Alternatively, the apoenzyme solution was dialyzed against two changes in two days of a 1 M NaCl, 2×10^{-4} M Tris-HCl, 1×10^{-3} M $CoSO_4$ buffer at pH 6.8 and finally against two changes in a day of a 2.5×10^{-3} M NaCl, 3×10^{-4} M Tris-HCl and 2×10^{-4} M $CoSO_4$

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buffered at pH 6.8 to crystallize the CoCPA thus obtained. All the dialysis procedures were carried out at 4 °C. The crystals were then dissolved in unbuffered 1 M NaCl solutions either in D_2O or in H_2O. The resulting pH of the solutions was around 6.8 (the pH of D_2O solutions (pH*) is always reported as an uncorrected pH-meter reading). The solutions obtained with the two above procedures were undistinguishable except for the rate of N_3^- binding (see later).

The enzyme concentrations were determined from the electronic spectra in the UV region using the molar absorbance at 278 nm of 6.42×10^4 M^{-1} cm^{-1} [12]; the cobalt(II) content was checked from the electronic spectra in the visible region, using a molar absorbance at 575 nm of 150 M^{-1} cm^{-1} [4, 6]. The cobalt(II) content was always over 90% with respect to the protein concentration.

The electronic spectra in the UV–Vis and near IR regions were recorded at room temperature on a Cary 17D spectrophotometer on unbuffered H_2O or D_2O solutions, either on reconstituted enzyme or on dissolved crystal preparations. N_3^- and NCO^- were added as unbuffered concentrated solutions whose pH's were previously adjusted to the desired value with concentrated HCl or NaOH solutions. The spectral variation in the visible region observed upon addition of increasing amounts of anion solutions was used to calculate the K_{app} values through a two parameter best fitting procedure. The dilution of the enzyme solutions was taken into account in the calculations; final dilutions did not exceed 20%.

^1H NMR measurements were performed at 15 °C using an instrument based on a Bruker CXP 100 consol and a Varian DA 60 1.41 T electromagnet, equipped with an external lock circuit granting a ±1 Hz long term stability. The spectra were recorded in quadrature detection mode on a 100 kHz spectral width, using the following pulse sequence (modified DEFT [13]):

$$90^\circ_x - \tau - 180^\circ_x - \tau - 90^\circ_x - \text{acquisition}$$

This sequence was further phase alternated (according to the Bruker standard PAPS program) to minimize build up of coherent noise [14]. The 90°_x pulse was adjusted for each sample and was always found to be around 3 μs. Recycle times and τ values were around 50 and 30 ms respectively, to allow suppression of the slow-relaxing signals [13]. The T_1 measurements of the 4-H histidine proton signals were performed with the same pulse sequence, using variable τ values, and measuring the signal heights M_τ as a function of τ. The data were best fitted to the following equation [13]:

$$M_\tau = M_\infty(1 - 2e^{-\tau/T_1} + e^{-2\tau/T_1})$$

with a non-linear two parameter best fitting program to obtain M_∞ and T_1 values. The estimated error is less than 10%. ^1H NMR water proton relaxation measurements were performed at 4 and 60 MHz at room temperature using the inversion recovery method.

Results

Addition of N_3^- and NCO^- ions to CPA solutions changes the electronic spectrum of the cobalt(II) chromophore (Fig. 1). In particular, there is a two-

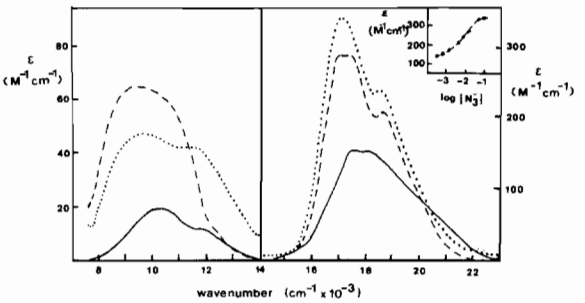

Fig. 1. Room temperature electronic absorption spectra of CoCPA (——) at pH 7.0 and in the presence of 1 M NaCl, and of its adducts with N_3^- (· · ·) and NCO^- (– – –). The near IR spectra were obtained in D_2O solutions at pH* 7.0. The inset represents the maximum molar absorbance at 17.1 $cm^{-1} \times 10^{-3}$ as a function of N_3^- concentration (log scale).

three fold increase in the intensity of the spectrum of the anion-containing species both in the visible and near infrared regions, together with a shift of the absorption maxima, especially in the near infrared region, towards lower energies. NCS^- does not affect the spectrum of CoCPA at pH 6.8 in 1 M NaCl up to a concentration of about 1 M, indicating that the upper limit of the apparent affinity constant (K_{app}) of this anion for the equilibrium:

$$\text{Enzyme} + \text{Anion} \rightleftharpoons \text{Enzyme–Anion}$$

is about 0.1 M^{-1}. By fitting the changes of the electronic spectra as a function of N_3^- and NCO^- concentrations the values of the apparent affinity constants were 110 ± 10 M^{-1} for N_3^- and 100 ± 15 M^{-1} for NCO^-, at pH 7 and in the presence of 1 M NaCl. The apparent affinity constants measured in the pH range from 6 to 10.5 are shown in Fig. 2. In the high pH region the affinity of both anions drops dramatically. The enzyme solutions are stable in 1 M NaCl down to pH around 6; at lower pH values the solutions rapidly become turbid. In order to extend measurements at pH values lower than 6, the chloride concentration of the enzyme solution had to be raised to 3 M. Under these conditions the K_{app} values of the two anions decrease from pH

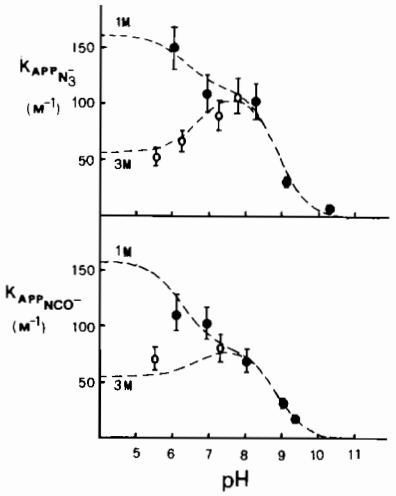

Fig. 2. pH dependence of K_{app} for N_3^- and NCO^- anions, measured through spectrophotometric titrations, in unbuffered solutions of CoCPA containing 1 M (filled symbols) or 3 M (open symbols) NaCl. The curves are best fit curves calculated through simultaneous fitting of the data to the following equation:

$$K_{app} = \frac{K_{a1}K_{(X)2} + K_{(X)1}[H^+]}{[H^+] + K_{a1} + K_{(Cl^-)1}[H^+][Cl^-]} \left(\frac{1}{1 + \frac{K_{a2}}{[H^+]}} \right)$$

where $pK_{a1} = 5.3$ and $pK_{a2} = 8.9$. The best fit values for the other parameters are: $K_{(Cl^-)1} = 9.6$; $K_{(N_3^-)1} = 1670$; $K_{(N_3^-)2} = 114$; $K_{(NCO^-)1} = 1640$; $K_{(NCO^-)2} = 81$.

7.5 to 5.5 (Fig. 2). On the other hand, the K_{app} of both anions at pH 6.1 in the presence of 1 M NaCl is larger than that at pH 7. The recent results of Vallee *et al.* [8] indicate that the data at low pH can be rationalized by assuming competition between N_3^- and NCO^- on one side and Cl^- on the other.

The ^1H NMR spectrum of the azide derivative of CoCPA was recorded with the aim of detecting the proton signals which experience paramagnetic isotropic shift. the ^1H NMR spectrum of a D_2O solution of the N_3^- adduct of CoCPA at pH* 6.8, 15 °C and in the presence of 1 M NaCl is reported in Fig. 3, together with the assignment of the signals and their T_1^{-1} values. The assignment is proposed on the basis of the previously reported spectrum of the unligated enzyme [15] and on the assumption that the T_1^{-1} values are grossly dependent on the reciprocal of the sixth power of the distance of the nucleus from the paramagnetic center. The two signals at 56.5 and 43 ppm downfield have been assigned to the 4-H's of the two coordinated histidine residues (His-69 and -196) which are therefore shown to remain coordinated. Their T_1^{-1} values are considerably larger (240 and 360 s^{-1}, respectively) than in the unligated enzyme (140 s^{-1}) [15]. While the T_1^{-1} values of the histidine 4-H protons in the unligated enzyme are smaller than in the azide adduct, their linewidths are much larger. This was attributed [15] to the existence of different, slowly interconverting conformers of the unligated enzyme in solution; accordingly, binding of N_3^- would drive the protein to equilibrate rapidly between the different conformers or to assume a single dominant conformation.

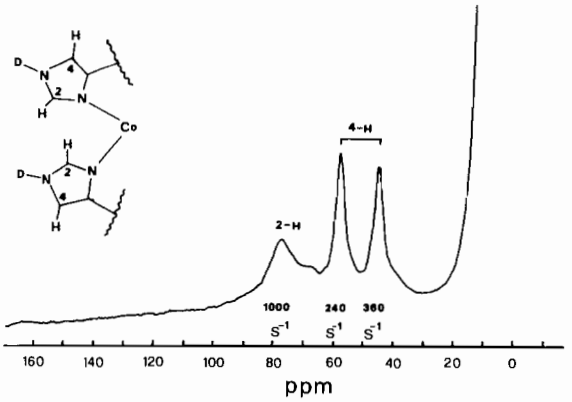

Fig. 3. 60 MHz ^1H NMR spectrum at 15 °C of unbuffered solution in D_2O at pH* 6.8 and 1 M NaCl of the N_3^- adduct of CoCPA. The proposed assignment and the T_1^{-1} values of the signals are also reported.

Measurement of water proton relaxation rates on solutions of the N_3^- adduct at 4 and 60 MHz show that the water T_1^{-1} values are not measurably different from those obtained [15] on solutions of the native zinc enzyme, and are substantially lower than those of the unligated enzyme [15]. This suggests the absence of water in the metal coordination sphere of the N_3^- adduct.

In order to rule out the possibility that irreversible phenomena could take place upon addition of the N_3^- or NCO^- anions to CoCPA solutions, the adduct solutions were dialyzed against a 1 M solution of NaCl at pH 7, and the electronic and ^1H NMR spectra of the unligated enzyme were again obtained. Furthermore, after addition of N_3^- to CoCPA in such amount that the limit electronic spectrum was developed, the stronger inhibitor β-phenylpropionate ($K_{app} > 10^3$ [6]) was added to the solution and the limit electronic spectrum of the β-phenylpropionate adduct was obtained. Both N_3^- and NCO^- bind instantaneously to CoCPA obtained by addition of stoichiometric cobalt(II) chloride to apoenzyme solutions. N_3^-, unlike NCO^-, develops affinity for CoCPA freshly dissolved from crystals only with time; the pseudo first order rate constant for adduct formation in 0.5 M N_3^- solutions measured through electronic spectroscopy is about 10^{-5} s^{-1}. In this case the binding of N_3^- is reversed by dialysis or addition of β-phenylpropionate. The electronic and NMR spectra of the N_3^- adduct are independent of the kind of starting solution. The slow binding of

N_3^- to CoCPA when dissolved from crystals is another example of the peculiar behavior of CPA, which is claimed to be remarkably different from solid state to solution [16]. A *cis–trans* interconversion, which is proposed to occur between apo and reconstituted protein, may explain the experimental data as the interconversion barrier is of the correct order of magnitude for the observed time [2, 17].

Discussion

Azide and cyanate anions are found to bind the cobalt(II) ion in CoCPA in the pH range of activity of the enzyme, with apparent affinity constants which depend on pH and, at low pH, also on chloride concentration.

The enzyme has two acidic groups in the cavity which have been found to affect the catalytic properties of the native and cobalt(II)-substituted enzymes [9]. One, with a pK of about 5.3, regulates the pH dependence of k_{cat} [9] and has been proposed to be the group governing the affinity of chloride for the metal ion [8]; the other acidic group affects the pH dependence of K_M, and has been found to affect also the electronic spectra of CoCPA, with a pK_a value of 9 [9] and 8.8 [6], respectively.

The possible equilibria involved in the present systems are therefore:

$$EH_2^{2+} \overset{K_{a1}}{\rightleftharpoons} EH^+ + H^+, \quad EH^+ \overset{K_{a2}}{\rightleftharpoons} E + H^+,$$

$$EH_2^{2+} + X^- \overset{K_{(X)1}}{\rightleftharpoons} EH_2X^+, \quad EH^+ + X^- \overset{K_{(X)2}}{\rightleftharpoons} EHX,$$

$$E + X^- \overset{K_{(X)3}}{\rightleftharpoons} EX^-$$

where X^- is Cl^-, N_3^- or NCO^-, and EH_2^{2+} and EH^+ are the diprotonated and the monoprotonated enzyme species, respectively. Chloride has always to be present in the system, together with the investigated anion, and the various enzyme species are simultaneously in equilibrium with both kinds of anions. The narrow range of possible chloride concentrations (1–3 M) [8] and the instability of the enzyme solutions below pH 5.5 do not result in sufficient significant experimental points needed to obtain all the unknown parameters through a best fitting procedure of all the data in Fig. 2. However, it can be reasonably assumed that chloride has negligible affinity for EH^+ and E [8] and, from inspection of the present data, that $K_{(X)3}$ for both N_3^- and NCO^- is not significantly different from zero. With these assumptions, and by taking $pK_{a1} = 5.3$ [9] and $pK_{a2} = 8.9$, *i.e.* equal to the average between the reported values of 9.0 [9] and 8.8 [6], the K_{app} data for both N_3^- and NCO^- can be simultaneously best fitted to obtain $K_{(N_3^-)1}$, $K_{(N_3^-)2}$, $K_{(NCO^-)1}$, $K_{(NCO^-)2}$ and $K_{(Cl^-)1}$.

The best fit curves are shown in Fig. 2, with the best fit values of the above parameters reported in the caption. While the values of $K_{(X)2}$ are quite reliable (estimated deviations within 10%), those of $K_{(X)1}$ for the three anions should only be taken as order of magnitude estimates. It appears that the affinity of both N_3^- and NCO^- is about one order of magnitude higher for EH_2^{2+} than for EH^+; the $K_{(Cl^-)1}$ value is consistent with that which can be estimated from ref. 8. A different choice of the pK_{a1} value affects the $K_{(X)1}$ values, but not their ratio nor the $K_{(X)2}$ values, and does not improve the fitting. Increasing or decreasing the pK_{a2} value makes the fitting substantially worse.

The K_{app} data are consistent with the idea that the two acidic groups regulating the pH dependence of anion binding to CoCPA are the same as those that regulate k_{cat} at low pH and K_M at high pH for the same enzyme. While there is general consensus that the acidic group with low pK_a is the Glu-270 residue [8], the high pH dissociating group has not been definitely identified. The Tyr-248 residue has been suggested [17] as a candidate, although the coordinated water cannot be ruled out [2, 17, 18].

The charge of the cavity seems to be determinant as far as the affinity constant of the anions is concerned. Whereas the apparent affinity constant of chloride is decreased below detectability above pK_{a2} [8], those of N_3^- and NCO^-, which are stronger ligands, drop to zero only above pK_{a2}. The latter behavior is somewhat similar to that experienced by carbonic anhydrase [10, 19] for which the pH dependence of the anion affinity constants is mainly determined by the dissociation of the coordinated water molecule, the hydroxide ion itself being competitive with anions.

The spectroscopic data allow us to estimate the structure of the cobalt enzyme–anion systems. The high molar absorbance of the electronic spectra of the anion derivatives are indicative of tetracoordination [20, 21]; also the large T_1^{-1} values of the histidine 4-H protons are consistent with the large nuclear relaxing capability displayed by pseudo-tetrahedrally-coordinated cobalt(II) ions on nearby nuclei, as proposed by cobalt(II) carbonic anhydrase systems [22] and for simple inorganic cobalt(II) containing compounds [23]. Since there is no evidence for the presence of coordinated water in the anion adducts of CoCPA, whereas there is evidence from NMR that the two histidines are bound to the metal, the donor set can be proposed to be formed by two histidine nitrogens, one oxygen from Glu-72 and the anion itself. The variation of proton histidines T_1^{-1} and electronic spectra upon anion binding are indicative of a substantial change

in the chromophore, the values of the unligated CoCPA being consistent with five coordination [15, 21]. X-ray data on CoCPA crystals [5] have shown that Glu-72 is bidentate in the solid state. The variation in electronic parameters upon anion binding could be accounted for by a change in the binding behavior of Glu-72, *i.e.* bidentate in the unligated system and monodentate (or at least one Co–O distance much larger that the other) in the presence of N_3^- and NCO^-. This possibility holds only if the solid state structure is maintained in solution.

The present research has shown that the enzyme carboxypeptidase A can interact with anions in a fashion which can be understood in terms analogous to those which account for the interaction of anions with carbonic anhydrase, and that analogously to the latter system, such interactions cause large variations in the electronic properties of the metal ion which may even reflect a change in coordination number.

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