METAL–TYROSYL INTERACTION IN CARBOXYPEPTIDASES: PHOSPHORESCENCE STUDIES

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1. Introduction

Luminescence studies on the interaction of the active site Zn^{2+} ion with a tyrosyl residue in porcine carboxypeptidase B were recently reported [1]. It is pertinent to ask whether the tyrosine–metal system is specific for carboxypeptidase B or whether it is a feature involved in the mechanism of action common to all metallocarboxypeptidases. Moreover, is this interaction specific for zinc or can it be observed with another enzymatically active metallopeptidase. To elucidate these points we have now examined the phosphorescence emission spectra of the tyrosyl residues in carboxypeptidase A (CPA), carboxypeptidase B (CPB) and Cd-carboxypeptidase B.

2. Experimental

2.1. *Materials*

Porcine carboxypeptidase B (Worthington Biochemical Corp.) was purified on Sepharose-ε-aminocaproyl-D-Arg column [2]. Cadmium-carboxypeptidase B was prepared essentially as previously described [3]. The metal content determined by atomic absorption spectroscopy (Varian Techtron model AA5) was found to be 0.96–0.98 g atom metal per mol enzyme. Carboxypeptidase A_α (Sigma) was treated as described by Auld et al. [4] and then dissolved in 0.05 M Tris–1.0 M NaCl buffer to avoid precipitation. Samples were diluted prior to the measurement 5-fold by salt free buffer. Acetyl-L-arginine was obtained from Cyclo Co. and L-benzylsuccinic acid was prepared as previously described [5].

2.2. *Methods*

Carboxypeptidase A and B activities were determined spectrophotometrically at 225 nm with 10 mM tBoc–L–Ala–L–Ala–L–Phe (miles-Yda Co.) in 0.05 M Tris–0.1 M NaCl buffer (pH 7.5), 25°C.

Phosphorescence measurements were performed using a Hitachi-Perkin-Elmer spectrofluorometer model MPF-2A equipped with a phosphorescence attachment as described previously [11]. All phosphorescence measurements were made on frozen buffered solutions (liquid nitrogen, 77°K) using 100 μM CPB, 100 μM CPA and 70 μM Cd–CPB.

3. Results

The phosphorescence emission spectrum of CPB, when excited at 280 nm is characteristic of the highly structured emission spectrum of tryptophan at 400–600 nm with a signal of tyrosine at 350–400 nm (fig.1). The ratio of tyrosine/tryptophan emission reported as the ratio between the emission at 380 and 440 nm, (I_{380}/I_{440}) was found to depend on the protein concentration. This is illustrated in fig.1 for two concentrations of CPB. Similar variations in the ratio I_{380}/I_{440} were observed in 1:1 mixtures of tyrosine and tryptophan at concentrations 4×10^{-3} M–4×10^{-5} M. Therefore, the phosphorescence data presented and compared in this work were measured in all cases at the same protein concentration.

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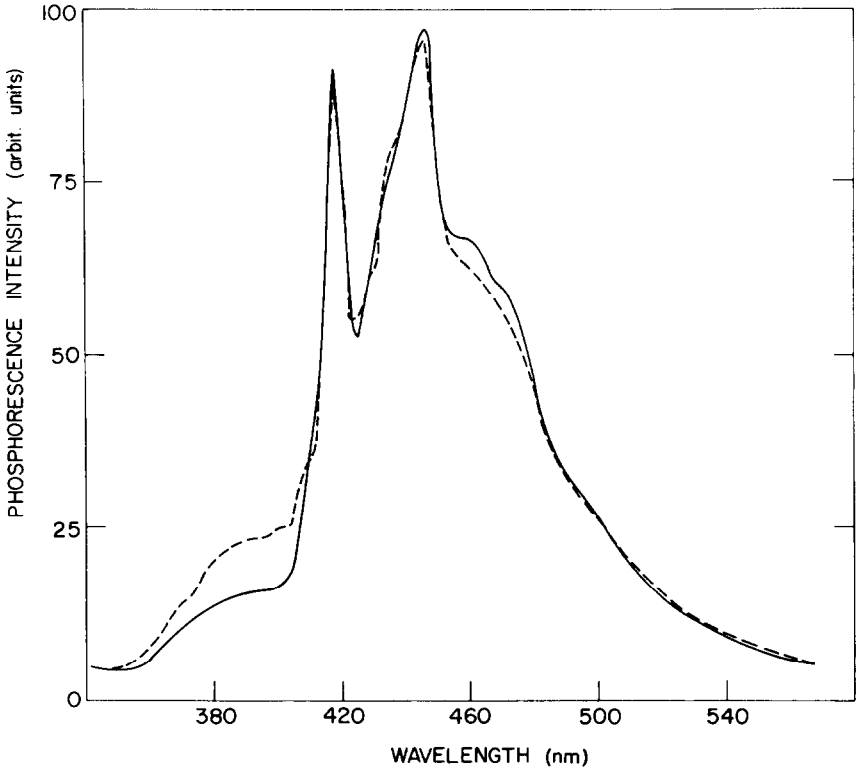

Fig.1. Phosphorescence emission spectra of 5×10^{-4} M (– – –) and 1×10^{-3} M (———) carboxypeptidase B, in 0.05 M Tris–0.1 M NaCl buffer pH 7.0 and 77° K; excitation at 280 nm.

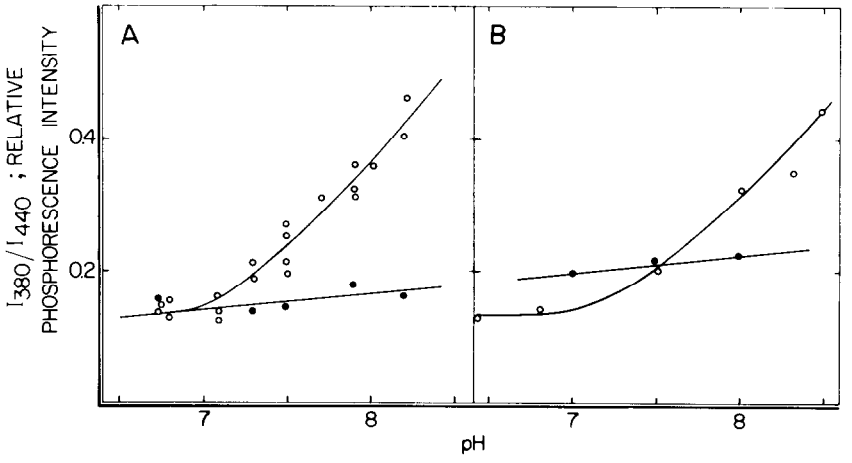

Fig.2. Ratio of phosphorescence emission intensity at 380 nm and 440 nm as a function of pH [A] (○) CPA; (●) CPA in the presence of 0.1 mM L-benzylsuccinic acid in 0.05 M Tris–0.2 M NaCl buffer. [B] (○) CPB; (●) CPB in the presence of 0.1 mM L-benzylsuccinic acid in 0.05 M Tris–0.1M NaCl buffer. Excitation at 280 nm.

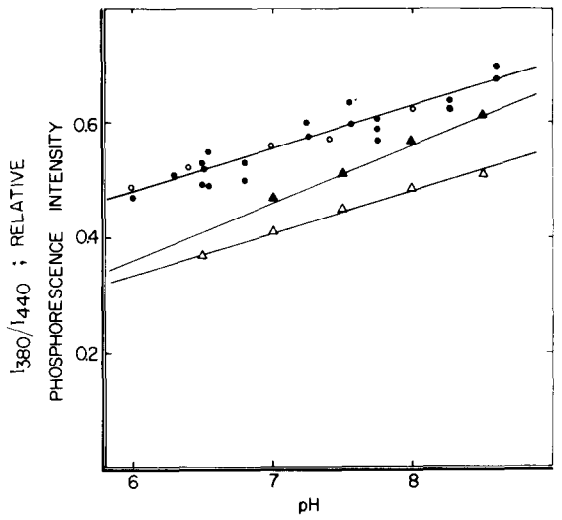

Fig.3. Ratio of phosphorescence emission intensity at 380 nm and 440 nm as a function of pH. (•) Cd–CPB in 0.05 M Tris–0.1 M NaCl buffer containing 1 mM Cd^{+2}; (○) Cd–CPB in the presence of 10 mM *N*-acetyl-L-arginine; (▲) Zn–CPB in the presence of 10 mM *N*-acetyl-L-arginine; (△) bovine serum albumin. Excitation at 280 nm.

The effect of pH on the ratio of the emission intensity at 380 nm and 440 nm of both CPA and CPB is similar and changes around pH 7.5 (fig.2). In both cases after addition of the competitive inhibitor L-benzylsuccinic acid the dependence of I_{380}/I_{440} on the pH is changed into a moderate increase of this ratio with the pH. It should be noted that CPA, CPB and Cd–CPB exposed to 77°K regained full enzymic activity when returned to room temperature (298°K).

As shown in fig.3 the pH dependence of tyrosine-tryptophan intensity of Cd–CPB differ from that of the Zn–CPB. The changes around pH 7.5 typical for the zinc enzyme (fig.2) disappears in the Cd-CPB. Fig.3 compares also the pH dependence of the intensity ratio I_{380}/I_{440} for Cd–CPB and Zn–CPB in the presence of acetyl-L-arginine, and that of bovine serum albumin which is considered as a 'normal' model [1].

The phosphorescence lifetime of tryptophan (at 440 nm) was about 3.5 sec for CPA and about 4 sec for Zn– and Cd–CPB, and were independent of the pH. The tyrosyl emission lifetime (at 380 nm) for CPA was 0.8 ± 0.2 sec at all pH values measured and thus the variability of the lifetimes was close to the experimental error (which is about 10%). In the case of Cd–CPB emission lifetimes of tyrosine (0.9 sec) and tryptophan (4 sec) in the presence or absence of the inhibitor, or changes in pH, remained constant within experimental error.

4. Discussion

In both zinc carboxypeptidases A and B the emission intensity is pH-dependent (fig.2) and around pH 7.5 there is a dramatic increase in the tyrosyl emission. In a previous study [1] we have shown that when the tryptophan lifetime is constant, as was observed here for the carboxypeptidases, changes in the I_{380}/I_{440} ratio reflect variations in the state of the tyrosyl residues. In Zn–CPB the pH-dependent increase in the tyrosyl emission is accompanied by a decrease in the lifetime from 1.6 sec at pH 6.8 to 0.9 sec at pH above 7.5. This phosphorescent behaviour was attributed to the ionization of a tyrosyl residue. In Zn–CPA, the tyrosyl lifetime is short even at pHs below 7.5, and the variations of the lifetime throughout the pH range investigated are small and therefore it is difficult to interpret them in terms of the ionization state of the tyrosyl residue.

It has been shown by Mason and his co-workers [6] that the tryptophan triplet yield depends on the amino acid concentration. As this dependency is not necessarily equal for the two amino acid residues investigated here, i.e. tyrosyl and tryptophanyl residues, this may affect the ratio between the phosphorescence emission of tyrosine and tryptophan (I_{380}/I_{440}), as indeed was observed (fig.1). We preferred to work in aqueous media in order to allow comparison of enzymic activity and structure in aqueous solution. Thus, due to the opaque glass obtained by freezing the buffered enzyme solutions there is a contribution of the scattering of the fluorescent light which is dependent on the optical density of the medium. Since scattering is dependent on λ^{-4}, this effect will contribute to the changes observed in the I_{380}/I_{440} ratio. In order to overcome the problems arising from these contributions, all measurements were carried out at constant enzyme concentration.

In contrast to the phosphorescent behaviour of the Zn–CPA and Zn–CPB, a more gradual increase of

I_{380}/I_{440} with pH is observed for the enzyme–inhibitor complex (fig.2). The inhibitor used in this study, L-benzylsuccinic acid, is a very potent competitive inhibitor for both carboxypeptidases [5,7] and hence its concentrations in the phosphorescent measurements were low (0.1 mM) so that absorption of the inhibitor at 280 nm and its phosphorescence emission can be neglected. The phosphorescent behaviour of CPA and CPB described here and in a previous report [1], strongly suggests the presence of zinc interaction with a tyrosyl residue (presumably tyrosine-248) in the active site of CPB and CPA. The addition of substrate [1] or inhibitor to the enzyme, disrupts the zinc–tyrosine interaction. The proposal that the tyrosyl residue is in close proximity to the zinc is in agreement with the previous data of Vallee's group demonstrating that, in solution, arsanilazotyrosine-248 of arsanilazocarboxypeptidase A form an intramolecular co-ordination complex with the zinc atom of this enzyme [8,9].

The question arises as to whether the existance of the tyrosine–zinc interaction in the active site of carboxypeptidases is due to the presence of a specific metal, i.e. zinc, or can it be observed with another enzymatically active metallo-carboxypeptidase. To elucidate this point we have substituted the zinc ion in the active site of CPB by cadmium, a substitution which yields an enzymatically active peptidase [3]. The comparison between the phosphorescent emission of Zn–CPB (fig.2B) and Cd–CPB (fig.3) clearly demonstrates different behaviour. The changes of I_{380}/I_{440} with pH typical to the Zn–enzyme disappears in the Cd–enzyme, where a moderate increase in I_{380}/I_{440} is observed. This phosphorescent behaviour is similar to that seen for bovine serum albumin, and to that of the enzyme–inhibitor complexes of Zn–CPA and Zn–CPB. The formation of Cd–enzyme complex with *N*-acetyl-L-arginine, did not change the pH-dependency nor the tyrosyl lifetime values. Therefore, it seems most likely that the complex of tyrosine–metal characterized by phosphorescence, is not expressed in the Cd–CPB. The diminished interaction between the Cd^{2+} ion and the tyrosyl residue may result from changes in the distance between the two so that they are further apart. In addition, conformational changes due to different geometries of the cadmium and zinc ions [10] might lead to 'wrong' orientation of the metal and the tyrosyl residue. Thus, the microenvironment of the tyrosyl residue which is thought to facilitate hydrolysis of substrates [1,11,12] is different in the zinc and cadmium enzymes. The interaction between the metal ion and the tyrosyl residue in these carboxypeptidases may therefore be indicative of the ability of the enzyme to hydrolyze peptide bonds. Indeed it has been shown recently [3] that Cd–CPB does not catalyze the hydrolysis of dipeptides while it is fully active in the hydrolysis of larger substrates (tri- and tetrapeptides). The lack of hydrolysis of dipeptides can thus be explained by the unavailability of the tyrosyl residue in the proper orientation. On the other hand, in larger substrates, additional interaction between the latter and the enzyme subsites will enforce the proper catalytically active orientation of the reactants and hence these substrates will be hydrolyzed by Cd–CPB. It should be noted that kinetic studies also indicated a possible role for the side chain of the larger substrates for the proper alignment of substrate along the active site of Cd–CPB [3]. Experiments currently in progress comparing the chemical hyperreactivity of the tyrosyl residue in Cd– and Zn–CPB will help in further evaluation of the suggestions made in this report.

References

[1] Shaklai, N., Zisapel, N. and Sokolovsky, M. (1973) Proc. Natl. Acad. Sci. USA 70, 2025–2028.
[2] Sokolovsky, M. (1974) in: Methods in Enzymol. (Jacoby, W. B. and Wilchek, M. eds.) Academic Press N.Y.
[3] Zisapel, N. and Sokolovsky, M. (1973) Biochem. Biophys. Res. Commun. 53, 722–729.
[4] Auld, D. S., Latt, S. A. and Vallee, B. L. (1972) Biochemistry 11, 4994–4999.
[5] Byers, L. D. and Wolfenden, R. (1973) Biochemistry 12, 2070–2078.
[6] Shiga, T., Mason, H. S. and Simo, C. (1966) Biochemistry 5, 1877–1885.
[7] Zisapel, N. and Sokolovsky, M. (1974) Biochem. Biophys. Res. Commun. 58, 951–959.
[8] Johansen, J. T. and Vallee, B. L. (1971) Proc. Natl. Acad. Sci. USA 68, 2532–2535.
[9] Johansen, J. T., Livingston, D. M. and Vallee, B. L. (1972) Biochemistry 11, 2584–2588.
[10] Vallee, B. L. and Williams, R. J. P. (1968) Proc. Natl. Acad. Sci. USA 59, 498–502.
[11] Lipscomb, W. N. (1970) Accounts. Chem. Res. 4, 81–89.
[12] Sokolovsky, M. and Zisapel, N. (1974) in: Arieh Berger Memorial issue, Israel J. Chem. 12, 631–641.