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The Role of a Tyrosyl Residue in the Mechanism of Action of Carboxypeptidase B: Luminescence Studies

(fluorescence/phosphorescence/enzyme mechanism)

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ABSTRACT **The luminescence spectra of carboxypeptidase B indicate specific differences between the zinc and apoenzyme due to the state of tyrosyl residues presumably at the active site. These differences disappear when enzyme–substrate or enzyme–inhibitor complexes are formed, suggesting that they may reflect the interaction of a tyrosyl residue in the native enzyme with the catalytically essential zinc atom. An interpretation of the role of that tyrosyl residue in the mechanism of action of carboxypeptidase B is presented.**

Chemical modifications have implicated a chemically hyperreactive tyrosyl residue in the function of carboxypeptidase B. Thus, nitration of the enzyme (1) markedly decreases the enzymic activity towards both basic and nonbasic substrates (2). These changes have been correlated with the nitration of active-site tyrosyl residues. Alkylation (3), iodination (5), acetylation (4), succinylation (6), and coupling with a diazonium salt (6) also involve tyrosyl residues with analogous catalytic consequences. The luminescence spectra of proteins provides an intrinsic sensitive signal of changes in stereochemical relationships directly related to the aromatic amino-acid side chains, especially tryptophan and tyrosine. Changes in the microenvironment of these residues may be thus observed especially in a protein like carboxypeptidase B possessing a reactive tyrosyl residue. We have, therefore, examined the luminescence properties of this enzyme. The results indicate differences in the tyrosyl state between the apo- and the zinc enzyme. These differences disappear on formation of enzyme–substrate or enzyme–inhibitor complexes.

METHODS

Apocarboxypeptidase B was prepared from porcine carboxypeptidase B [(Code COBC) Worthington Biochemical Corp.] by dialysis against multiple changes of 4 mM 1,10-phenanthroline in 0.01 M sodium acetate buffer (pH 5.5) followed by dialysis against the same buffer without phenanthroline, and finally against 0.05 M Tris·HCl–0.1 M NaCl at the desired pH. The apoenzyme was reconstituted by dialysis against 0.05 M Tris·HCl–0.1 M NaCl (pH 7.5) buffer containing 1 mM zinc sulphate (Merck Co.) followed by dialysis against metal-free buffer.

The metal content was determined by atomic absorption spectroscopy (Varian Techtron model AA5) and found to be 0.96–0.98 g-atom of Zn in the reconstituted enzyme and less than 0.03 g-atom of Zn in the apoenzyme. Enzymatic activities were estimated with basic and nonbasic peptide and ester substrates (2).

Fluorescence spectra were measured with a Hitachi–Perkin–Elmer spectrofluorometer model MPF-2. Phosphorescence measurements were performed with the same instrument, equipped with a phosphorescence attachment. Phosphorescence signals were obtained by use of a chopper of the Becquerell type rotating at 25 Hz to cut off short-lived signals (fluorescence and stray-light). Emission was observed at 90° to the incident beam. All spectra given are uncorrected for instrument response. Decay curves were obtained by use of a Tektronix storage oscilloscope. Lifetimes were calculated from the slopes of the curves obtained from the plots of logarithmic dependence of phosphorescence intensity with time. In measurements of tryptophan lifetime performed in the presence of the shorter lifetime tyrosyl signal, the slopes were taken from the linear part of the plots. Enzyme concentrations were 5.4 μM in the fluorescence measurements and 140 μM in the phosphorescence ones. All phosphorescence measurements were made on frozen buffer solutions.

RESULTS

Fluorescence. The fluorescence spectra of zinc carboxypeptidase B at pH 7.5 excited at 275 and 295 nm is shown in Fig. 1*A*. These emission spectra normalized at 350 nm are virtually identical at longer wavelengths, while they differ below 350 nm, indicating the existence of a tyrosyl emission

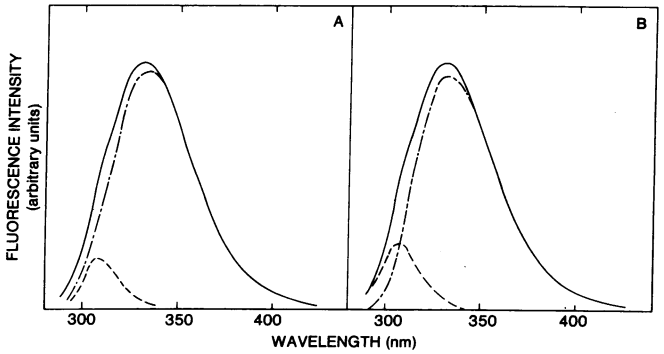

FIG. 1. Fluorescence spectrum of (*A*) zinc-carboxypeptidase and (*B*) apocarboxypeptidase. Conditions: 0.05 M Tris·HCl–0.1 M NaCl at pH 7.5 and 298 K°. (——) excited at 275 nm; (—·—) excited at 295 nm; (– – –) difference spectrum (275 nm compared to 295 nm).

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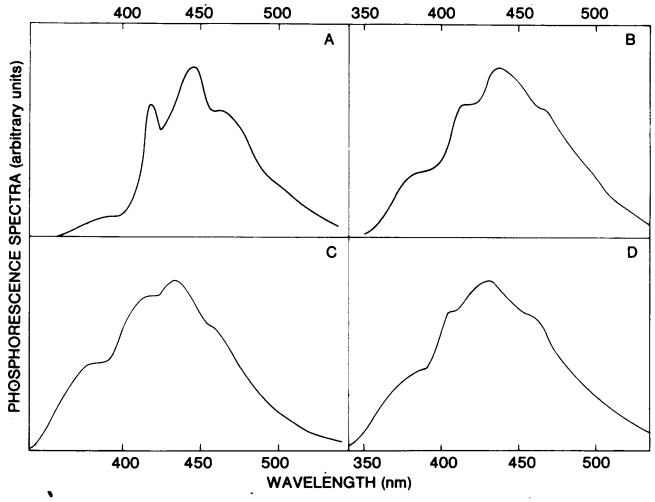

FIG. 2. Phosphorescence spectrum of (*A*) Zn-enzyme, (*B*) apoenzyme, (*C*) Zn-enzyme in the presence of 10 mM L-arginine, (*D*) Zn-enzyme in the presence of 10 mM *N*-acetyl-L-arginine. Conditions: 0.05 M Tris·HCl–0.1 M NaCl at pH 7.0 and 77°K. Excitation at 280 nm. All spectra were normalized at the wavelength of maximum tryptophanyl emission.

(7). The difference spectrum, moreover, (*lowest curve*, Fig. 1*A*) is characteristic of tyrosine. Similar spectral properties are observed in the emission of the apocarboxypeptidase (Fig. 1*B*). However, the emission intensity of the tyrosine (relative to that of tryptophan) is lower in the zinc enzyme than in the apoenzyme. The quantum yield was almost the same for both the native enzyme and the apoenzyme, and it was independent of pH in the range 6.2–9.0 in both derivatives. The substrate *N*-acetyl-L-arginine and the specific inhibitor L-arginine (8), which neither absorb nor emit in the range investigated, did not affect the tryptophanyl fluorescence properties of the enzyme.

Phosphorescence. The results obtained from low-temperature phosphorescence measurements are shown in Fig. 2. It should be noted that the enzyme exposed to 77°K retains its activity when returned to room temperature (298°K). The luminescence properties of the native enzyme (Fig. 2*A*) are characteristic of the highly structured spectrum of tryptophan at 400–600 nm with a signal of tyrosine at 350–400 nm (9). On the other hand, the phosphorescence spectrum

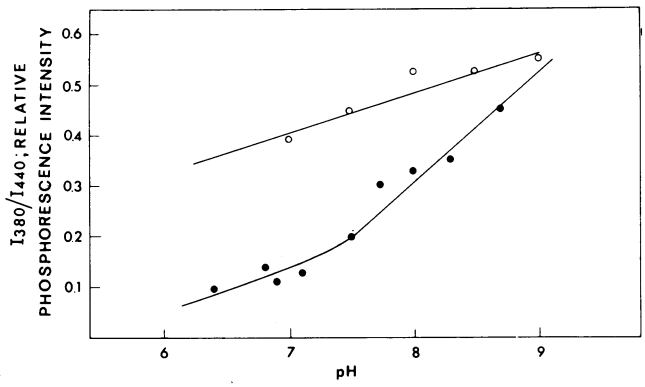

FIG. 3. Ratio of phosphorescence emission intensity at 380 nm and 440 nm as a function of pH; (●) Zn-enzyme; (○) apoenzyme.

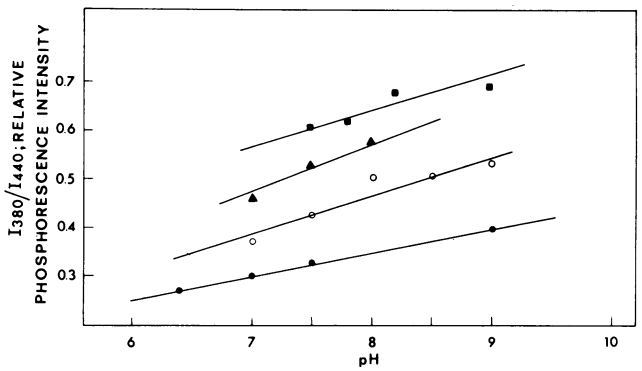

FIG. 4. Ratio of phosphorescence emission intensity at 380 nm and 440 nm as a function of pH. (●) bovine-serum albumin; (○) apoenzyme; (▲) Zn-enzyme in the presence of 10 mM *N*-acetyl-L-arginine; (■) Zn-enzyme in the presence of 10 mM L-arginine.

characteristic of the apoenzyme (Fig. 2*B*) is perturbed and exhibits a 4-fold increase in the tyrosine/tryptophan ratio of emission intensity. The metalloenzyme reconstituted from the apoenzyme initially exposed to low temperature regained full enzymic activity, and its phosphorescence spectrum was identical to that of the native enzyme.

Substances that bind to the active site of carboxypeptidase B alter its phosphorescence spectrum. Addition of 10 mM of the competitive inhibitor L-arginine, conditions under which nonspecific binding is minimized (2), causes perturbation of the phosphorescence spectrum accompanied by an increase in the ratio of the tyrosine/tryptophan emission intensity (Fig. 2*C*). Similar results were obtained with the substrate *N*-acetyl-L-arginine (Fig. 2*D*). The changes observed are attributed to the protein since both the inhibitor and substrate are devoid of phosphorescence signal in the region under inspection. The effect of pH on the ratio of the tyrosine/tryptophan emission reported as the ratio between the emission at 380 nm (typical only of tyrosine) and at 440 nm (typical of tryptophan) is shown in Fig. 3. The ratio of emission of the apoenzyme gradually increases with pH while that of the zinc enzyme changes more dramatically around pH 7.5. Similarly, the pH-dependence of that ratio is shown in Fig. 4 for bovine-serum albumin and for carboxypeptidase in the presence of substrate and inhibitor. Both proteins

TABLE 1. *Lifetime of tryptophan and tyrosine emission in carboxypeptidase B (CPB) in the presence of Zn, substrate (N-acetyl-L-arginine), and inhibitor (L-arginine).*

System	pH	τ tyrosine (sec)	τ tryptophan (sec)
Zn-CPB	6.8	1.5	4.1
	7.8	0.9	4.2
	9.0	0.9	4.7
apo-CPB	7.5	1.5	4.3
	8.0	1.6	4.2
	9.0	1.8	4.7
Zn-CPB + inhibitor	7.3	1.5	4.1
	7.8	1.8	4.2
	9.0	1.3	4.1
Zn-CPB + substrate	7.8	1.6	4.2

show the same gradual increase in the I_{380}/I_{440} with pH, although the magnitude of that ratio in each case is different. The phosphorescence lifetime of tyrosine (at 380 nm) and tryptophan (at 440 nm) at various pH values for the derivatives of carboxypeptidase are summarized in Table 1. The table shows that the lifetime of tryptophan and tyrosine emissions in apoenzyme, and the enzyme–substrate and enzyme–inhibitor complexes, are independent of the pH, range investigated. In contrast, the tyrosine emission lifetime of the zinc enzyme is pH-dependent, i.e., 1.5 sec and 0.9 sec at pH 6.8 and 7.8, respectively. The corresponding lifetimes of the tryptophan emission are essentially unchanged.

DISCUSSION

Carboxypeptidase B contains both tyrosyl and tryptophanyl residues, and like proteins of this class, shows mainly tryptophanyl fluorescence (Fig. 1). As shown, there is a tyrosyl contribution to the fluorescence of carboxypeptidase between 300 and 350 nm, but its magnitude is small relative to the total emission. Therefore, changes in the intensity of the emission at 337 nm are mainly related to changes of the emission intensity of tryptophan. Addition of an inhibitor, removal of the metal from the enzyme, or change of the pH in the range measured do not affect this fluorescence signal significantly. The population of singlet level is, therefore, constant. As a consequence, the population of the triplet level must in turn be constant, as was also suggested for another system (10). Furthermore, as shown in Table 1, the tryptophanyl phosphorescence lifetime is constant, and hence the phosphorescence intensity should not change under these conditions. Thus, the ratio Tyr/Try in the phosphorescence possibly reflects change in the state of the tyrosyl residues.

The fluorescence measurements at pH 7.5 indicate the presence of tyrosyl contribution as manifested in the difference spectra. Such signals have been observed in several proteins containing both tyrosyl and tryptophanyl residues (11). The fact that the tyrosyl signal in the zinc enzyme is lower than in the apoenzyme, might result from quenching due to the presence of the metal ion, or to other features of tyrosine environment. Phosphorescence studies, discussed below, are more helpful in this regard since the tyrosyl and tryptophanyl emissions are well separated from each other.

The phosphorescence spectrum of zinc carboxypeptidase at pH 7.0 (Fig. 2) clearly shows a small but detectable tyrosyl signal. The removal of the zinc atom and the addition of a substrate or an inhibitor lead to various degrees of increase in the tyrosyl emission intensity. That this increase in intensity is not accompanied by the corresponding changes in lifetime suggests a change in the rate of intersystem crossing, possibly due to microenvironmental changes. It seems likely that the major tyrosyl-phosphorescence changes are due to a change in the state of a tyrosyl residue. Having most, or all, of the observed signals arise from a single residue is not surprising since it is known that in many proteins the tyrosyl contribution to the phosphorescence emission is not always observed (12–14). The phosphorescence changes in the presence of substrate or inhibitor serve as a strong indication for the participation of the tyrosyl residue in the active center of the enzyme.

Further identification of the tyrosyl residue responsible for the observed luminescence properties was attempted by nitration of the enzyme (1) in order to cause quenching of the phosphorescence by the formation of nitrotyrosyl residues. However, the wide absorption band of the nitrotyrosine in the range of 350–400 nm caused quenching of the tyrosyl as well as the tryptophanyl residues. It was not possible, therefore, to follow the quenching of tyrosine relative to tryptophan in that system. Examination of aminotyrosyl carboxypeptidase (1) was also inconclusive because (*a*) the aminotyrosyl residue was phosphorescent, both in model compounds and in the enzyme, and (*b*) the phosphorescence showed a red shift, which impaired the resolution of the spectrum. Acylation of the tyrosyl residue was without success for the same reasons.

The difference between the phosphorescent behavior of the tyrosyl residue in the zinc enzyme on the one hand and the apoenzyme on the other is clearly seen in Fig. 3. Around pH 7.5 there is a dramatic increase in the tyrosyl emission of the zinc enzyme accompanied by a decrease in the lifetime to 0.9 sec, but not in the apoenzyme. The effect of pH on phosphorescence of tryptophan, tyrosine, and proteins has been recently reported (15). A great increase of intensity of the phosphorescence of tyrosinate ion over tyrosine, accompanied by a decrease in lifetime, was observed. We can therefore attribute the phosphorescent behavior of the zinc enzyme to the ionization of the tyrosyl residue. Ionization around this low pH is unusual for tyrosine. In principle, the pH dependence of the phosphorescence intensity reflects the ground state pK, since reequilibrium in the excited state would be precluded in a rigid medium. However, any attempt to interpret a pK value would be meaningless, since the rapid decrease in temperature to 77°K leaves the system in a frozen state where equilibrium is not achieved even in the ground state (16).

In contrast to the behavior of the zinc enzyme, a more gradual increase of the I_{380}/I_{440} with pH is observed for the apoenzyme and also with the enzyme–substrate and enzyme–inhibitor complexes (Fig. 4). Similar behavior is observed with bovine-serum albumin, which does not contain any unusual tyrosyl residues and hence might be considered as a "normal" model. Similarly, as shown in Table 1, there are no changes in tyrosyl lifetime in the enzyme–substrate or enzyme–inhibitor complexes within the pH range investigated. Thus, the unusual behavior of the tyrosyl residue, seen in the native enzyme is not seen in the apoenzyme, the enzyme–substrate, and the enzyme–inhibitor complexes.

That the ionization of tyrosine is responsible for the unusual pH dependence of the I_{380}/I_{440} in the native enzyme is in agreement with the earlier assumption that we are following a tyrosyl residue, since it is difficult to assume that many tyrosines will be ionized at such low pH. In addition, the hyperreactivity of a single tyrosine towards chemical modification (1, 3–6) also supports this assumption, and tempts us to suggest that it is the same tyrosyl residue involved, i.e., tyrosine-"248" (1, 3–6).

It can be concluded, therefore, that in the presence of metal the tyrosyl residue is present in a microenvironment that results in a perturbed phosphorescent signal and a lowered pK. One of the possibilities for such an effect is zinc interaction with the tyrosyl residue. Removal of the metal, or addition of substrate or inhibitor to the enzyme, disrupts the tyrosine–zinc interaction. The tyrosyl residue is thought to facilitate hydrolysis of the substrate by interacting with the susceptible peptide bond, which is thought to

be polarized through the cooperative action of the zinc atom, as proposed for carboxypeptidase A (17). In our terms, the zinc atom would interact directly with the tyrosyl residue in the absence of substrate but would be displaced by it when present. The chemical hyperreactivity of the tyrosyl residue could then be ascribed to its interaction with zinc, displacing a proton to result in an ionized species. Further experiments are clearly needed to test this hypothesis. However, the proposal that the tyrosyl residue of carboxypeptidase B is in close proximity to zinc is in agreement with other recent data demonstrating that, in solution, arsanilazotyrosine-248 of arsanilazocarboxypeptidase A (18) and the corresponding tyrosine of carboxypeptidase B (6) form an intramolecular coordination complex with the zinc atom of these enzymes.

It should be noted that both a zinc atom and tyrosyl residue are components of the active center of several metallopeptidases, e.g., carboxypeptidases, thermolysin (19), and aminopeptidases (20). It is pertinent to ask whether the tyrosine–metal system is a general and common pathway in the mechanism of action of all of these enzymes. There is no answer to this question thus far, but studies along these lines are currently in progress in this laboratory.

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