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CATALYTIC ACTIVITY OF CARBOXYPEPTIDASE B AND OF CARBOXYPEPTIDASE Y WITH ANISYLAZOFORMYL SUBSTRATES

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Abstract: Anisylazoformyllysine ($CH_3OC_6H_4$–N=N–CO-Lys-OH) is rapidly hydrolyzed at the acyl-lysine linkage by the zinc-enzyme porcine carboxypeptidase B. The catalytic reaction is readily monitored spectrophotometrically by disappearance of the intense absorption (348.5 nm, ε 18400) of the azo chromophore, which chemically fragments after substrate cleavage. Carboxypeptidase Y has no activity toward this type of substrate.

A new type of assay substrate for proteolytic enzymes has recently been developed. As exemplified by carboxypeptidase A catalysis, substrate anisylazoformylphenylalanine (Aaf-Phe-OH, Figure 1) was shown to be cleaved efficiently by the enzyme with subsequent spontaneous fragmentation of the released acyl moiety.[1] The intense light absorption of the Aaf chromophore (portion $CH_3OC_6H_4$–N=N–CO-) is thereby abolished, allowing a convenient and sensitive spectrophotometric detection of catalysis. Similar substrates have been successfully employed with the bacterial zinc *endo*protease thermolysin.[2] The present communication examines the activity of this type of substrate towards two additional proteolytic enzymes, with both positive and negative results, indicating a potentially useful selectivity.

Because there are several metallo*exo*peptidases of interest that have substrate specificity for C-terminal lysine or arginine residues, we have now developed a preparation of anisylazoformyllysine (Aaf-Lys-OH, Figure 1). We demonstrate that it is efficiently hydrolysed by carboxypeptidase B. Consequently, the new substrate should be useful in assays for that enzyme and for other *exo*peptidases of similar specificity. In order to see whether this type of peptide analogue could be employed for categories of enzymes other than metalloproteases, we have also examined the activity of the serine protease carboxypeptidase Y toward the anisylazoformyl substrate for carboxypeptidase A, Aaf-Phe-OH, which it might be expected to hydrolyse. Substrate cleavage was not detected in that case.

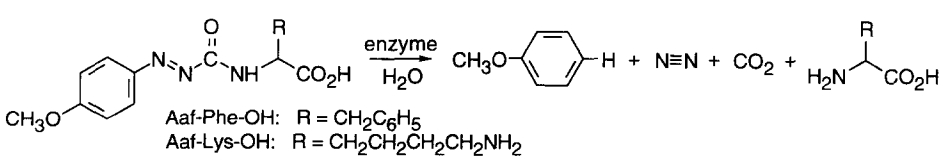

Figure 1. Structure of substrates and chemical transformation catalyzed by carboxypeptidases.

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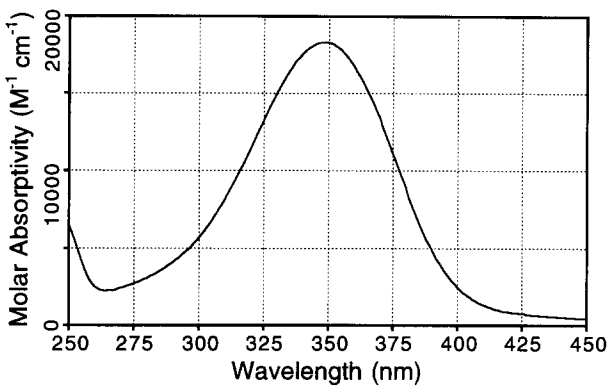


Figure 2. Spectrum in aqueous solution of Aaf-Lys-OH (λ_{max} 348.5 nm, ε 18400 M^{-1} cm^{-1}).

Results

In Figure 2 is depicted the ultraviolet-visible spectrum of Aaf-Lys-OH. The intense absorption centered on 348.5 nm completely disappears upon catalytic hydrolysis with porcine carboxypeptidase B, consonant with the chemical equation in Figure 1. By exploiting this feature in spectrophotometric measurements (using the method of initial rates), we have found for this substrate kinetic values of 76 (± 2) s^{-1} for k_{cat} and 0.81 (± 0.05) mM for K_m (measured at 390 nm with a pathlength of 1 mm, in 0.05 M Tris buffer at pH 7.9). Figure 3 displays the spectral change accompanying substrate cleavage, and demonstrates the expected linear dependence of catalysis rate upon concentration of carboxypeptidase B, which is requisite behavior for enzyme assay purposes.

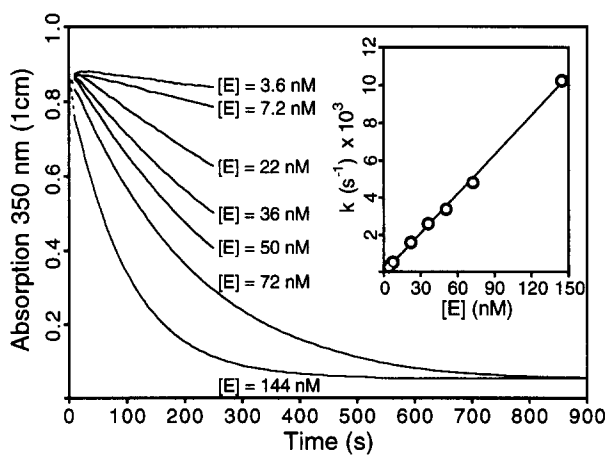


Figure 3. Reaction progress curves (spectral absorptivity versus time) for cleavage of Aaf-Lys-OH in buffered solution by porcine carboxypeptidase B, starting with 0.05 mM substrate and with varying concentrations of enzyme (pH 7.9, 350 nm, pathlength 1 cm). Inset: Derived rate constants as a function of enzyme concentration.

Although hippurylarginine[3] and salmine[4] have been recommended as assay substrates for carboxypeptidase B, the currently favored substrate for this class of enzyme appears to be *N*-[3-(2-furyl)acryloyl]-L-alanyl-L-lysine (Fua-Ala-Lys-OH), which is cleaved at the Ala–Lys linkage with an accompanying decrease of approximately 20% in spectral absorption at 324 nm.[5–9] We have undertaken a comparison of the latter substrate with Aaf-Lys-OH. A K_m value of 1.16 (± 0.20) mM was measured for Fua-Ala-Lys-OH with carboxypeptidase B, indicating enzymic affinity similar to that observed for Aaf-Lys-OH. In direct kinetic comparison we find specificity constants (k_{cat}/K_m) of 3.6×10^5 and 9.5×10^4 s^{-1} M^{-1} for Fua-Ala-Lys-OH and Aaf-Lys-OH, respectively. Although the furylacryloyl dipeptide appears to be 3.8-fold more active by that criterion, there are compensatory advantages for Aaf-Lys-OH. The change in spectral extinction at 350 nm accompanying complete substrate hydrolysis is ninefold greater for Aaf-Lys-OH (compared to that available maximally at 324 nm with Fua-Ala-Lys-OH); this provides slightly greater analytic sensitivity for the new substrate in practice. Moreover, use of the furylacryloyl dipeptide substrate entails complications from a high baseline, in that only a fractional decrease in a relatively strong shoulder absorption must be monitored within the range 310–335 nm;[6] this creates an upper limit upon useful substrate concentrations. In contrast, the stronger absorption of Aaf-Lys-OH is completely abolished upon hydrolysis. It is possible to follow catalysis kinetics at spectral wavelengths as long as 400 nm (Figure 2), so that baseline complications may be obviated (as was necessary to discern the K_m value). The greater range of wavelengths that are suitable for assay purposes with Aaf-Lys-OH could be advantageous in avoiding spectral interference from contaminants in clinical samples.

Carboxypeptidase Y is a glycoprotein serine *exo*peptidase produced by yeast. As an enzyme it exhibits a specificity for ordinary substrates which is quite similar to that of bovine carboxypeptidase A,[10,11] a metallopeptidase that readily cleaves Aaf-Phe-OH.[1] Consequently, the latter substrate was submitted to carboxypeptidase Y. Activity of the enzyme was first verified with substrate Fua-Phe-Phe-OH, for which a k_{cat} of 100 s^{-1} and a K_m of 0.025 mM at pH 6 has been reported,[12] kinetic parameters that we were able to confirm. However, no hydrolysis of Aaf-Phe-OH by carboxypeptidase Y was detectable. At a substrate concentration of 0.4 mM, we estimate that Aaf-Phe-OH is cleaved at best 10^5-fold more slowly than is Fua-Phe-Phe-OH. This contrasts with the behavior of carboxypeptidase A with these substrates, for which the furylacryloyl dipeptide proved only 16-fold more active by comparison of specificity constants k_{cat}/K_m.[1] Although this constitutes only one example, anisylazoformylpeptide derivatives appear *not* to be promising substrates for serine proteases.

Discussion

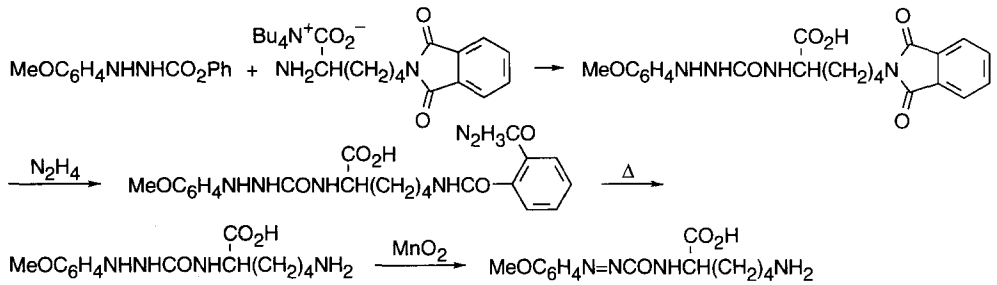
Our results indicate that anisylazoformyllysine may be a useful substrate for metallopeptidases that require a cationic amino acid residue in the P_1' position within substrates. It is noteworthy that an internal-quench fluorescent-type of substrate for an enzyme having this specificity may be difficult to secure (due to absence of excitation acceptor or donor in P′), so that a sensitive spectral absorption assay is likely to retain practical significance. The preparation described in the Experimental Section for Aaf-Lys-OH involves several steps, but is operationally simple, merely involving mixing of reagents and collecting of precipitates. As for other, physiologically significant enzymes potentially capable of cleaving Aaf-Lys-OH, carboxypeptidase N has been shown to be similar to carboxypeptidase B in terms of substrates accepted.[13] Carboxypeptidase M and carboxypeptidase T are other candidate enzymes of related specificity, for which the new substrate might prove useful.[14,15]

The serine protease carboxypeptidase Y was unable to cleave Aaf-Phe-OH, which is an excellent substrate for the zinc-enzyme carboxypeptidase A. No explanation for such a negative result can presently be advanced. However, should this reactivity represent a general pattern, it might provide an additional technique for categorizing newly-discovered proteolytic activity.

Experimental Section

Materials. Affinity-purified porcine pancreatic carboxypeptidase B (EC 3.4.17.2) and bakers yeast carboxypeptidase Y (EC 3.4.16.1) obtained from Sigma Chemical Co. (Nos. C 6518 and C 3888, respectively) were used as received. Enzyme concentrations were estimated from UV absorption: carboxypeptidase B, $E^{1\%}_{278nm}$ = 21.4 with M_r 34,500;[3] carboxypeptidase Y, $E^{1\%}_{280nm}$ = 15.0 with M_r 61,000.[16] Substrates *N*-[3-(2-furyl)acryloyl]-L-alanyl-L-lysine (Fua-Ala-Lys-OH, No. F 5882) and *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-L-phenylalanine (Fua-Phe-Phe-OH, No. F 7133) were also from Sigma. Buffer employed in solutions for kinetics was tris-*N*-(hydroxymethyl)aminomethane (Tris).

Substrate N^2-(4-methoxyphenylazoformyl)-L-lysine (anisylazoformyllysine, Aaf-Lys-OH) was assembled according to the following scheme.



N^6-Phthaloyl-L-lysine[17] was converted from its hydrochloride (Sigma No. P 3058) by neutralization with potassium hydroxide in ethanolic solution followed by collection of the precipitate. A solution of 0.989 g (2.82 mmol) of the N^6-phthaloyl-L-lysine containing 1 equiv of remnant potassium chloride in 3 mL (2.82 mmol) of methanolic tetrabutylammonium hydroxide (Aldrich No. 23,018-9) was rotary evaporated. The residual oil was taken up in 6 mL of dry *N,N*-dimethylformamide. To this solution 0.646 g (2.5 mmol) of phenyl 4-methoxyphenylhydrazoformate[1,2] was added with magnetic stirring under an inert atmosphere. After 2 h at room temperature the reaction mixture was acidified with 1 N hydrochloric acid to a pH of 2–3 according to pH paper, yielding a yellow precipitate that was collected. An additional small amount of product was recovered by ethyl acetate extraction. After drying, 0.826 g (75%) of coupling product N^2-(4-methoxyphenylhydrazoformyl)-N^6-phthaloyl-L-lysine was obtained, mp 170 °C (dec), IR (KBr) 1710 cm^{-1}.

To 0.5 g (15 mmol) of 95% hydrazine in 8 mL of ethanol, 1.138 g (2.58 mmol) of N^2-(4-methoxyphenylhydrazoformyl)-N^6-phthaloyl-L-lysine was added, and the solution was heated to 45 °C for 2 h. A white precipitate formed and after cooling it was collected. After washing thoroughly with ethanol, 1.1 g (90%) of a ring-opened adduct was obtained, N^2-(4-methoxyphenylhydrazoformyl)-N^6-(2-carbazoylbenzoyl)-L-lysine, mp 175 °C (dec), IR (KBr) 1400, 1550–1580, 1625–1660 cm^{-1}.

Dissolution of 1.42 g (3 mmol) of N^2-(4-methoxyphenylhydrazoformyl)-N^6-(2-carbazoylbenzoyl)-L-lysine in 30 mL of dimethyl sulfoxide yielded a clear yellow solution. Upon stirring at 45 °C for 4 h, a white precipitate

formed. The suspension was diluted with 100 mL of ethyl acetate and the solid was collected, giving 0.93 g (100%) of deprotected material, N^2-(4-methoxyphenylhydrazoformyl)-L-lysine, mp 175 °C (dec).

To a solution of 50 mL of methanol containing 1.066 g (3.4 mmol) of N^2-(4-methoxyphenylhydrazoformyl)-L-lysine was added 1.49 g (17 mmol) of manganese dioxide. The resulting suspension was stirred magnetically for 0.5 h and then was filtered. A filter agent was added to the resulting solution, and then a second filtration was performed. The solid obtained by rotary evaporation of the filtrate was precipitated from methanol with ethyl ether to give 0.60 g (57%) of a yellow powder of N^2-(4-methoxyphenylazoformyl)-L-lysine (Aaf-Lys-OH), dried over P_2O_5 under vacuum for 72 h at 5 °C, mp 210–215 °C (dec); IR (KBr) 1400, 1560–1600, 1695 cm^{-1}; UV (H_2O) 348.5 nm (ε 18400 M^{-1} cm^{-1}); ^1H NMR (D_2O) δ 1.49 (m, 2H), 1.72 (m, 2H), 1.88 (m, 1H), 1.96 (m, 1H), 3.00 (t, 2H, J = 7.5 Hz), 3.93 (s, 3H), 4.31 (dd, 1H, J = 5.0, 8.2 Hz), 7.14 (d, 2H, J = 8.8 Hz), 7.95 (d, 2H, J = 8.8 Hz). Anal. Calcd for $C_{14}H_{20}N_4O_4 \cdot 0.75H_2O$: C 52.33, H 6.59, N 17.43. Found: C 52.19, 52.32, H 6.71, 6.78, N 17.16, 17.21. The solid appears to be stable for several months in a freezer. It is recommended that stock solutions of the anisylazoformylamide substrate be shielded from light to avoid *cis–trans* isomerization of the azo linkage.

Substrate anisylazoformylphenylalanine (Aaf-Phe-OH) was secured as previously described.[1] It is non-crystalline in the carboxylic acid form, and consequently it formerly was converted to the solid potassium salt. The latter material appears to be unstable toward storage, and we now recommend employment of the substrate in the form of a malonamamidine salt. Malonamamidine hydrochloride [$H_2NCOCH_2C(NH_2)_2^+Cl^-$, Aldrich No. 17,651–6] was converted to the free base by neutralization of 63.2 mg (0.37 mmol) of the salt with 1 equiv of potassium hydroxide in ethanolic solution. After filtration of potassium chloride the resulting solution was added dropwise to a solution of 118.8 mg (0.36 mmol) of anisylazoformylphenylalanine in 2 mL of methanol. The semi-solid obtained upon evaporation of the reaction mixture was precipitated from methanol with ethyl ether. Well-formed crystals suitable for storage (81% recovery) were obtained after an additional recrystallization (MeOH-Et_2O), mp 170 °C (dec). The malonamamidine cation was independently shown to cause no perturbation of catalysis for either carboxypeptidase A or carboxypeptidase Y in the mM concentration range.

Methods. Velocity measurement for catalytic hydrolysis of the substrates by carboxypeptidase B was carried out at 25 °C in 0.05 M Tris buffer (pH 7.9), with spectrophotometric (320–400 nm, 0.05–1 cm pathlength) analysis and the method of initial rates. As an illustrative example, an initial rate of change in spectral extinction for Aaf-Lys-OH of –0.11 a.u. min^{-1} (dA/dt) was obtained at 350 nm, 1 cm path, [S] = 0.05 mM, [E] = 36 nM. Kinetic parameters k_{cat} and K_m were secured by a direct fit of velocity data to the Michaelis-Menten equation by the method of least squares. Alternatively, values for k_{cat}/K_m were obtained by fitting complete reaction progress curves to an exponential decay function ($[S]_0 < K_m$). Attempted kinetic assay with carboxypeptidase Y was similar (pH 6). All pH values in this article are calibrated pH meter readings uncorrected for ionic strength effects. Tolerances listed are standard errors from least-squares analysis.

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