Proton Inventory of Carboxypeptidase Y-Catalyzed Reactions

Shigeru KUNUGI,* Kazuo TANABE, and Mitsuhiro FUKUDA†
Department of Applied Chemistry, Fukui University, Fukui 910
†Department of Practical Life Studies, Hyogo University of Teacher Education, Yashiro-cho, Hyogo 673-14
(Received February 13, 1986)

Synopsis. The partial solvent isotope effect of D_2O on the carboxypeptidase Y-catalyzed hydrolysis of a peptide (3-(2-furyl)acryloyl-L-phenylalanyl-L-phenylalanine) and an ester (3-(2-furyl)acryloyl-L-phenylalanine ethyl ester) was investigated. The peptide hydrolysis showed a smaller kinetic isotope effect regarding the catalytic rate constant (k_{cat}) and the second-order rate constant (k_{cat}/K_m). k_{cat} Varied linearly on the heavy-water content, indicating that one proton is essentially transferred during the rate-determining step. On the other hand, an ester hydrolysis showed a larger, nonlinear effect, indicating the possibility of a transfer of two or more protons in the rate-determining step of the ester hydrolysis, possibly the deacylation step.

Serine carboxypeptidase [EC.3.4.16.1] is a group of *exo*-protease which have a crucial serine residue in the active site.[1] It is found in tissues from mold and yeast to plants and animals as an intercellular protease.[2] Some of the enzymes of this group are now commercially available, and are largely utilized in peptide sequencing. They are also useful for peptide syntheses through an enzymatic condensation reaction.[3]

One of the characteristics of enzymes of this class is that they often show esterase and/or amidase activities besides the intrinsic peptidase activity.[4-7] This is in contrast to carboxypeptidase A,[8] a well-known metallo-carboxypeptidase from mammalian. This endopeptidase-like activity of serine carboxypeptidase raises questions about the reaction mechanism, especially for the substrate binding to this enzyme.

We have investigated some of the details of reactions of these enzymes,[6,7,9,10] including the pressure dependence of the reaction rate, and found that the two different types of activities show contrasting characters: In a neutral pH region, the apparent activity of amidase or esterase increases with the pH while that of the peptidase increases with a decreasing pH. The latter is very sensitive to pressure.

For an enzyme isolated from yeast (carboxypeptidase Y), Douglas et al.[11] observed a burst process in a the reaction with a non-specific acylating reagent, trimethylacetyl *p*-nitrophenolate. Lobb and Auld[12] reported a presteady-state process in a reaction of this enzyme with a fluorescent depsipeptide substrate. Our preliminary study[13] on a chromophoric amino acid ester substrate using a stopped-flow method also indicated the existence of a presteady-state process. Coupled with the results of a trans-peptidation study catalyzed by this enzyme,[14] these results led us to postulate a so-called three-step mechanism including the acylated enzyme (Eq.1), just as in the case of the serine protease reactions, such as α-chymotrypsin or trypsin.

However, the rates of acylation (k_2) and the deacylation (k_3) are comparable in the case of carboxypeptidase Y-catalyzed hydrolysis of the peptide.[6] Under certain conditions a burst process has been observed,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (1)$$

(E, enzyme; S, substrate; E S, enzyme-substrate complex; ES′, acyl-enzyme; P_1, product; k_1, respective rate constant)

even for the peptide substrate.[13] These are fairly different from serine protease. To find more details regarding these constituent reaction steps, we studied the partial solvent isotope effect of deuterium oxide, known as a proton inventory technique that is useful for determining the behavior of protons during reaction pocesses.[15]

Experimental

Three types of serine carboxypeptidases (carboxypeptidase Y from yeast, W from wheat and P from penicillium) were studied. They were obtained from Oriental Yeast Co., (Osaka; Lot. 21003001), Pentel Co., (Tokyo; Lot. 6930606) and the Protein Research Foundation, (Osaka; Lot. 099201), respectively. Chromophoric substrates, 3-(2-furyl)acryloyl-L-phenylalanyl-L-phenylalanine (Fua-Phe-Phe) and 3-(2-furyl)acryloyl-L-phenylalanine ethyl ester (Fua-Phe-OEt), were used as before.[6] Benzoyl-L-tyrosine-*p*-nitroanilide (Bz-Tyr-*p*NA) was from the Protein Research Foundation. 2-morpholinoethanesulfonic acid (MES) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were from the Dohjindo Laboratories (Kumamoto, Japan). Deuterium oxide (99.85%) and sodium deuterium oxide solution in D_2O were purchased from CEA (France). The absorbance spectrum was measured using a Uvidec type-650 spectrophotometer (Nippon Bunkou Co.). The pH was measured with a Hitachi-Horiba pH meter (F-7LC) and the pD value for a D_2O solution was calculated by adding 0.4 to the value read on the meter.[15] Most of the preparation of D_2O-containing solutions was carried out under a stream of dry nitrogen gas. The atom fraction of deuterium in an H_2O–D_2O mixture was calculated on the basis of weight. For a very low content, a NMR apparatus (JEOL GX-270) was also used.

Results

Figure 1 shows the pH or pD dependence of k_{cat} on the carboxypeptidase Y-catalyzed hydrolysis of Fua-Phe-Phe (**a**) and Fua-Phe-OEt (**b**). When the optimum values were compared, the kinetic deuterium-isotope effect ((k_{cat} in H_2O)/(k_{cat} in D_2O)=k_H/k_D) for the peptide substrate was found to be about 1.6, and that for the ester substrate was about 3.5. These values are somewhat larger than those for Z-Phe-Leu and Ac-Phe-OEt reported by Hayashi's group.[16] The K_m parameter was largely unaffected by the solvent D_2O, though not shown here. Thus a comparison of the second-order rate constant (k_{cat}/K_m) can also show the approximate effect on the catalytic process; for

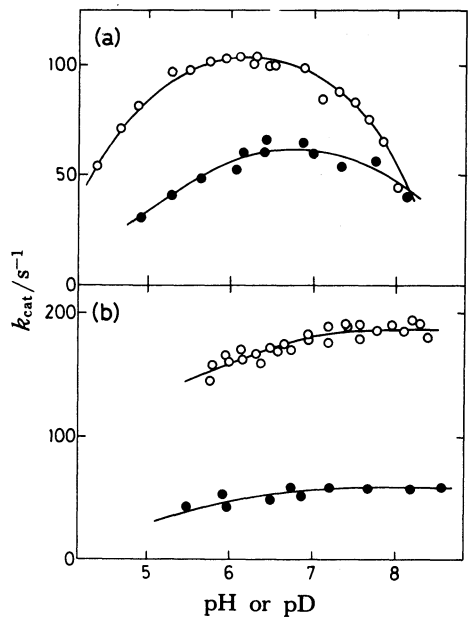

Fig. 1. The pH* dependence of k_{cat} for the carboxypeptidase Y-catalyzed hydrolysis of Fua-Phe-Phe (a) and of Fua-Phe-OEt (b). (○) in H_2O and (●) in D_2O, at 25 °C.
(a) [Fua-Phe-Phe]=0.46 mM, [E]=0.17 μM in H_2O and 0.21 μM in D_2O, containing 2.5% (v/v) CH_3CN.
(b) [Fua-Phe-OEt]=0.50 mM, [E]=0.10 μM in H_2O and 0.16 μM in D_2O, containing 2.0% (v/v) CH_3CN.

carboxypeptidase W, k_H/k_D of k_{cat}/K_m was 2.44 for Fua-Phe-OEt hydrolysis (at pH=7.60 and pD=8.01) and 1.13 for Fua-Phe-Phe hydrolysis (at pH=3.95 and pD=4.35). For carboxypeptidase P, only the peptide hydrolysis could be practically measurable and the k_H/k_D of k_{cat}/K_m was 1.11 (at pH=3.87 and pD=4.46). The effect was always small for a peptide hydrolysis.

When measured in an H_2O-D_2O mixture, the k_{cat} value changed as shown in Fig. 2. In the peptide hydrolysis (**a**) the rate constant varied almost linearly with a change in the D_2O content, while the rate of ester hydrolysis (**b**) varied considerably in a concave manner. According to a simplified theoretical treatment of the proton inventory, the rate constant in an H_2O-D_2O mixture can be expressed, provided that all the protons contribute to the total effect in equal amounts,[15] as

$$k = k_{H_2O}(1-n+n\phi)^{\nu}, \quad (2)$$

where n and ν indicate the D_2O content and the number of protons concerning the rate-determining step, respectively. ϕ is a deuterium isotope effect in an elementary step. A nonlinear regression of the data showed us that the ester hydrolysis can be represented by a ϕ value of 0.54 for ν=2 (two-proton assumption) and 0.66 for ν=3 (three-proton assumption). We can mention that at least two protons take part in the rate-determining process of ester hydrolysis while only one proton is apparently concerned in the rate-determining step of the peptide hydrolysis.

Discussion

A proton inventory technique has been successfully

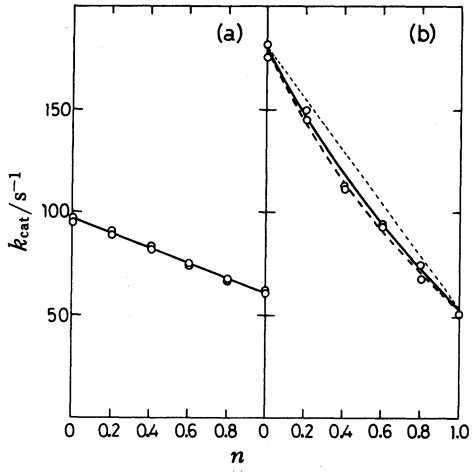

Fig. 2. The partial solvent isotope effect as a function of atom fraction of deuterium (n) for the k_{cat} of the carboxypeptidase Y-catalyzed hydrolysis of Fua-Phe-Phe (a) and of Fua-Phe-OEt (b), at 25 °C. [E]=0.11 μM, containing 2.5% (v/v) CH_3CN.
(a) [Fua-Phe-Phe]=0.40 mM. (——) ν=1, ϕ=0.63.
(b) [Fua-Phe-OEt]=0.52 mM.
(——) ν=2, ϕ=0.54, (----) ν=3, ϕ=0.66 for Eq. 2.
(----) ν=1, ϕ=0.29.

applied to the analysis of an enzyme reaction, especially for the reaction involving serine proteases. In the cases of a serine proteases such as α-chymotrypsin or elastase, a coupled movement of protons was reported for the hydrolysis of specific peptide anilide substrates,[15,17] largely related to the character of the acylation step, while most of the deacylation processes gave a very simple partial isotope effect.[15,18] The coupled movement of the protons during the acylation step of these enzymes was explained based on the so-called charge-relay mechanism involving a concerted proton movement within the Ser-His-Asp residues. This was originally postulated by Blows's group[19] on the basis of their X-ray crystallographical studies, though this mechanism (especially the existence of a very week carbonic acid side chain of Asp 102) has been recently questioned.[20]

Compared to these serine proteases, we know far less about the active site of serine carboxypeptidase. Hayashi et al.[21] showed that a His residue was crucial for the catalytic activity of carboxypeptidase Y. This gives us a picture of an active site similar to that of serine protease; a cooperative function of the Ser and His residues. We have reported after a systematic study of the steady-state kinetics of this enzyme that the peptide hydrolysis by serine carboxypeptidase is partly rate-controlled by the acylation step and that the ester hydrolysis is mostly deacylation-controlled.[6] The present results involving proton inventory indicates that the acylation reaction for these enzymes involves a transfer of one proton, probably between Ser and His residues. On the other hand, the deacylation process is more or less cooperative and a coupled movement of proton(s), in addition to the one between the hydration water and the His residue, is to be considered. Based on a new understanding of the proton movement of the serine protease reaction,[20] a mechanism including the

relaying of ptotons (not a charge) is still considered to be meaningful, especially when the substrate is bound to the active site. Therefore, the present result for carboxypeptidase Y might be explained by a coupled proton transfer occurring when the acyl part of the substrate is co-valently attached to the active site of the enzyme. However, the possibility of other mechanisms, such as a structural change of the protein or a change in the hydration state, might also be considered.

We gratefully acknowledge the advice and encouragement of Professors Norio Ise (Kyoto University) and Yoshiharu Nakamura (Fukui University) throughout the study.

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