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DETECTION OF AN ANHYDRIDE INTERMEDIATE IN THE CARBOXYPEPTIDASE A CATALYZED HYDROLYSIS OF A PEPTIDE SUBSTRATE BY SOLID STATE NMR SPECTROSCOPY AND ITS MECHANISTIC IMPLICATION

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Abstract: We have detected an anhydride intermediate in the CPA catalyzed proteolytic reaction of Gly-Tyr. It appears that since the zinc-bound water molecule which is believed to attack the scissile amide carbonyl carbon in the hydrolysis reaction is excluded by the *N*-terminal amino group of Gly-Tyr, the carboxylate of Glu-270 becomes to attack the amide bond to generate the anhydride intermediate.

Carboxypeptidase A (CPA) is a much studied prototypical zinc containing proteolytic enzyme which selectively cleaves the C-terminal amino acid residue having a hydrophobic side chain.[1] This enzyme has received increasing attention as a model for a large family of zinc proteases which serve as targets for the design of inhibitors of therapeutic potential.[2] Although its catalytic mechanism has been the subject of intensive scrutiny over last several decades, no general consensus has yet been reached and detailed mechanism still remains the subject of heated debate.[3] The debate centers on the identity of the intermediate involved in the enzymic reaction. We wish to report herein the detection of an anhydride intermediate that has been proposed for the CPA-catalyzed hydrolysis of peptide substrates using the low-temperature time-resolved solid-state NMR technique.[4]

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Glycyl-L-tryrosine (Gly-Tyr) is a slowly hydrolyzed substrate for CPA.[5] For the present study, the Gly-Tyr was doubly enriched at the scissile carbonyl carbon and amide nitrogen (99% in both ^{13}C and ^{15}N) to enhance the sensitivity of ^{13}C NMR and to remove the ^{13}C–^{14}N quadrupolar coupling.[6] Figure 1 shows ^{13}C CP-MAS solid state NMR spectra taken at 223 K of the freeze-quenched mixture of CPA (2 mM) and the substrate (3 mM) after the indicated time of mixing (the time of enzymic reaction).[7,8] The strong resonance

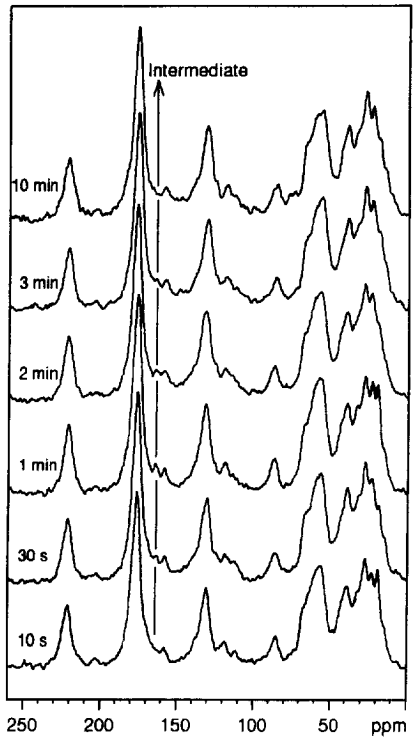

Figure 1. ^{13}C CP-MAS solid-state NMR spectra at 223 K of the mixture of CPA (2 mM) and amido-[^{13}C, ^{15}N] Gly-Tyr (3 mM), which was freeze-quenched by spraying into liquid propane after mixing for the indicated time in Tris buffer (50 mM) of pH 7.0 containing 1 M NaCl. Spectra were taken at a frequency of 75.47 MHz on a Bruker DPX 300 spectrometer with a double resonance CP-MAS probe using a 7 mm zirconia rotor with spinning speed of 3.4 kHz (±5 Hz). The raw data were acquired with 2.5 s recycle time, 1 ms contact time, and 8000 scans, and processed with 100 Hz line broadening.

signal appeared at 175 ppm is ascribed to the peptide carbonyl carbons of CPA and those at 131 and 221 ppm correspond to its rotational sidebands. There appeared a small but distinctive new resonance signal at 164 ppm in about 30 s and the intensity of the new peak increased progressively with its maximum shown in around 1 min. The time course of the signal build-up was in line with the rate of product formation estimated from the ^{1}H NMR of the free Gly generated by the enzyme. The peak at 164 ppm is thought to be arisen from the ^{13}C of a mixed anhydride that is formed as a result of attacking the carboxylate of Glu-270 on the amide carbonyl of Gly-Tyr. This assignment is consistent with the general trend observed for the ^{13}C NMR

signals of free carboxylates, which experience an upfield shift by about 10 ppm upon forming anhydrides.[9,10] When the mixture of CPA and the substrate was incubated in the presence of 2-benzylsuccinate (2 mM),[11] a potent competitive inhibitor which is known to bind to the active site of CPA, the signal at 164 ppm did not appear. Similarly, the new signal did not appear when CPA was mixed with unlabeled Gly-Tyr. Above results taken together suggest strongly that the new ^{13}C NMR signal shown at 164 ppm is due to the anhydride intermediate that is formed in the enzymic proteolytic reaction.

Two schools of thoughts are currently prevailing in explaining the catalytic action of CPA. One school believes that there occurs a nucleophilic attack on the scissile carbonyl carbon of the substrate by the carboxylate of Glu-270 to form an anhydride intermediate which is subsequently hydrolyzed (anhydride pathway) (Figure 2A).[12] The other stipulates a tetrahedral transient intermediate that is generated by the nucleophilic attack on the peptide carbonyl by the zinc bound water molecule (general base pathway) (Figure 2B).[13] A most conclusive way to settle the dispute on the catalytic mechanism of CPA would thus be to

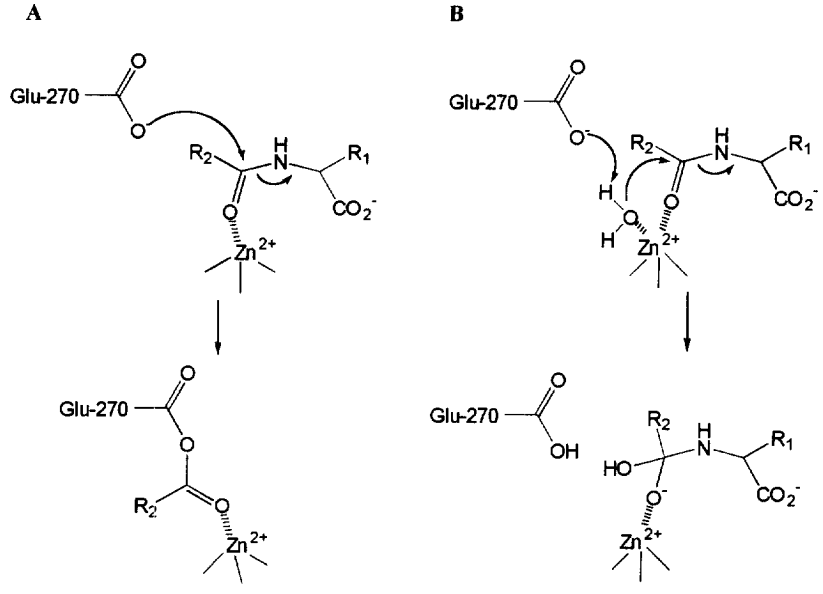

Figure 2. Schematic representations for the formations of anhydride intermediate (anhydride pathway, **A**) and tetrahedral transition state (general base mechanism, **B**) proposed for the CPA-catalyzed hydrolysis of substrates

characterize the intermediate in the enzymic reaction. In fact, there have been numerous attempts to detect spectroscopically the intermediates postulated for the proposed catalytic reaction paths, but they were successful only with ester substrates.[12a, 14-16] The present detection of the anhydride intermediate appears to support the anhydride mechanism for the CPA catalyzed peptide hydrolysis, but a note of caution is appropriately due. Lipscomb and colleagues were able to determine the crystal structure of CPA complexed with Gly-Tyr to show that the peptide chelates the active site Zn^{+2} with exclusion of the zinc bound water.[5] The amide oxygen and the free *N*-terminal amine of Gly-Tyr are involved in the chelation.[5] In the absence of the catalytic water at the active site, there is expected to operate an alternative catalytic path in which the carboxylate of Glu-270 attacks the scissile peptide bond. This may be account for the fact that Gly-Tyr is an unusually poor substrate, the rate of the hydrolysis being reduced by 5000 fold.[17] In this regard, it is worthy of noting that molecular dynamic calculations carried out by Banci et al. for the enzymic reaction of CPA revealed that both mechanisms are structurally feasible.[18] Lastly, we have recently reported that *O*-(hydroxyacetyl)-L-β-phenyllactic acid is a mechanism-based inactivator for CPA, which is designed on the basis of the postulation that such a ligand binds the enzyme in a bidentate fashion with exclusion of the zinc bound water molecule, causing thus the carboxylate of Glu-270 to attack at the ester carbonyl carbon.[19] The present observation supports the rationale used for the design of the CPA inactivator.

In summary, we have successfully detected by the solid state NMR spectroscopic method an anhydride intermediate in the CPA-catalyzed proteolysis using a slowly hydrolyzed dipeptide (Gly-Tyr) as substrate. The results may appear to suggest that an anhydride intermediate is involved in the proteolytic reaction of CPA, but in light of the X-ray structural data reported for the CPA·Gly-Tyr complex, Gly-Tyr is an exceptional peptide substrate which is forced to take the anhydride pathway in the absence of the catalytic water molecule at the active site.

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6. Amido-[^{13}C,^{15}N]Gly-Tyr was synthesized starting with Glycine-1-^{13}C and L-Tyrosine-^{15}N following a standard method.
7. CPA was purchased from Sigma Chemical Co. and used without further purification. The buffer solution containing CPA (2 mM) and Gly-Tyr (3 mM) was loaded into a Hamilton gas-tight syringe. After mixing for discrete time intervals, the mixture (300 μl) was sprayed through 0.1 mm nozzle into an aluminum receptacle containing liquid propane using a syringe pump. The finely powdered frozen sample was packed at the bottom of the NMR rotor that was immersed in the liquid propane. The receptacle was transferred to an aluminum block equilibrated at 223 K under nitrogen gas, and the propane was removed under vacuum at 223 K. The rotor was transferred swiftly to precooled (223 K) NMR probe, and CP-MAS spectra were obtained immediately. The enzymic activity of the CPA sample was unchanged when assayed after the freeze-quenching.
8. The free substrate and the reaction product of ^{13}C-Gly were not detected because the spin locking fields that provide excellent cross-polarization for rapidly frozen solutions of CPA and enzyme-bound species also provide very poor cross-polarization for the small molecules due to their relative isotropic motion in the frozen solution. Resonance signal assignable to bound Gly-Tyr was not appeared, which is presumably due to its extremely low concentration. The K_m value of Gly-Tyr for CPA was reported to be 0.7 mM (Ludwig, M. L.; Lipscomb, W. N. *Inorg. Biochem.* **1973**, *1*, 438 – 487). The resonance appeared at about 20 ppm was identified as signal due to residual propane in the sample.
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10. It may be arguable that the new signal is due to an active site zinc-coordinated Gly-Tyr. However, the ^{13}C solution NMR experiment in which $ZnCl_2$ is added to the solution of Gly-Tyr showed that the carbonyl carbon signal shifts towards downfield. Furthermore, the pH dependent ^{13}C NMR study of

Gly-Tyr indicated that the carbon signal shifts towards the further downfield region at higher pH, revealing that the new signal being a zinc-bound Gly-Tyr is highly improbable.

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