^{1}H-Nuclear Magnetic Resonance Studies on Interaction between 4-[4-(*N*,*N*-Dimethylcarbamoylmethoxycarbonylmethyl)phenoxycarbonylphenyl]guanidinium Methanesulfonate and Trypsin

Osamu MATSUMOTO, Hideki MASUDA, Tooru TAGA, Yoshihiro KURODA and Katsunosuke MACHIDA*

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan. *Received December 12, 1989*

The 600 MHz ^{1}H-nuclear magnetic resonance spectra of a mixed aqueous solution of trypsin and a synthesized trypsin inhibitor, 4-[4-(*N*,*N*-dimethylcarbamoylmethoxycarbonylmethyl)phenoxycarbonylphenyl]-guanidinium methanesulfonate (FOY-305) were measured. Signals assigned to the inhibitor protons indicated that the hydrated fragment of the inhibitor formed a complex with trypsin. The signal change during 4 h of incubation at 37 °C was interpreted as reflecting a successive process of the partial release of the fragment and its weak recombination with the enzyme around the active site. The result is consistent with the disorder of the fragment in the crystal structure of the inhibitor: trypsin complex.

Keywords trypsin; inhibitor; complex; ^{1}H-NMR

The binding and release process between trypsin and its inhibitors is very important in understanding the inhibition mechanism at the molecular level. 4-[4-(*N*,*N*-Dimethylcarbamoylmethoxycarbonylmethyl)phenoxycarbonylphenyl]guanidinium methanesulfonate (FOY-305) (Fig. 1) is a potent competitive inhibitor of trypsin.[1] The inhibitor is known to bind to trypsin at the P1 site, and the stereo structure of the inhibitor: trypsin complex has been studied by X-ray crystal analysis[2] on the basis of the crystal structures of trypsin[3] and the inhibitor molecule itself.[4] In the crystal of the complex, several inhibitor fragments were located in disorder around the active site of trypsin. Such an inhibitor binding complex in the solid state is also assumed to exist in the solution under the same condition as that used in crystal soaking. To confirm the complex formation in solutions, 600 MHz ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra of the mixed solutions of inhibitor and trypsin were studied in this work.

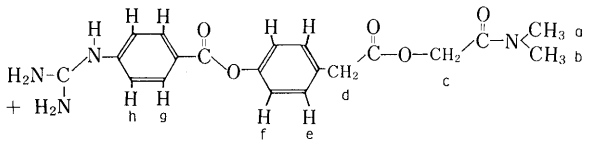

Fig. 1. Structure of the Inhibitor

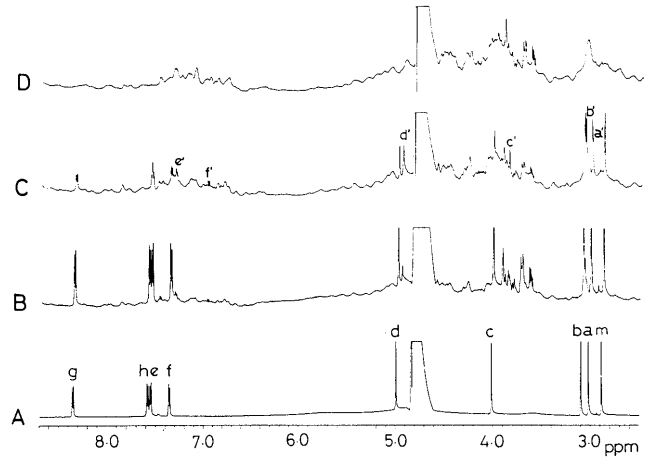

Fig. 2. The 600 MHz ^{1}H-NMR Spectra of the D_2O Solutions of (A) FOY-305, (B) 1:10 Trypsin:FOY-305. (C) 1:2 Trypsin:FOY-305 and (D) Trypsin

For all solutions the concentration of FOY-305 was 2.0 mM and the pH was 5.0 (uncorrected) at 27 °C. Signals a to h were assigned to H_a to H_h in Fig. 1. Signal m was assigned to the methyl protons of methanesulfonate.

Experimental

Trypsin (type I powder from bovine pancreas) was obtained from Sigma Chemical Company. The inhibitor, FOY-305, was provided by Ono Pharmaceutical Company. Each was dissolved in D_2O at concentrations of *ca.* 2.0 mM. The mixtures with 10:1 and 2:1 mole ratios were prepared by direct dissolution of the trypsin powder into the 2.0 mM solution of the inhibitor. The pH of solution was adjusted to 5.0±0.5 (uncorrected). The ^{1}H-NMR spectra of these solutions at 27 °C were recorded on a Bruker AM-600 spectrometer, using internal reference HDO (Fig. 2).

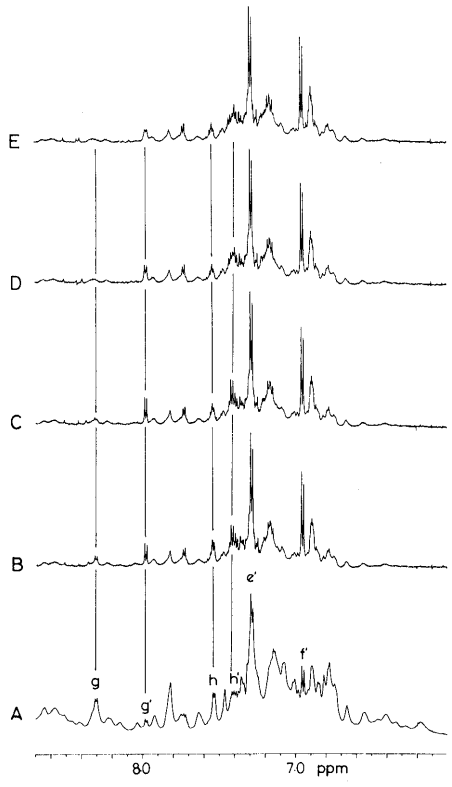

Fig. 3. The Time Course of 600 MHz ^{1}H-NMR Spectra of the D_2O Solutions of 1:1 Mole Ratio Trypsin and FOY-305 Mixture (A) 1 h, (B) 1.5 h, (C) 2 h, (D) 3 h and (E) 4 h after the Mixing

The solutions contained concentrations of 2.0 mM of each of FOY-305 and trypsin and the pH was 8.0 (uncorrected) at 37 °C. Signals e to h were assigned to H_e to H_h in Fig. 1. In Fig. 3-B—E the doublet peaks were stressed by irradiating residual HDO resonance at about 4.7 ppm.

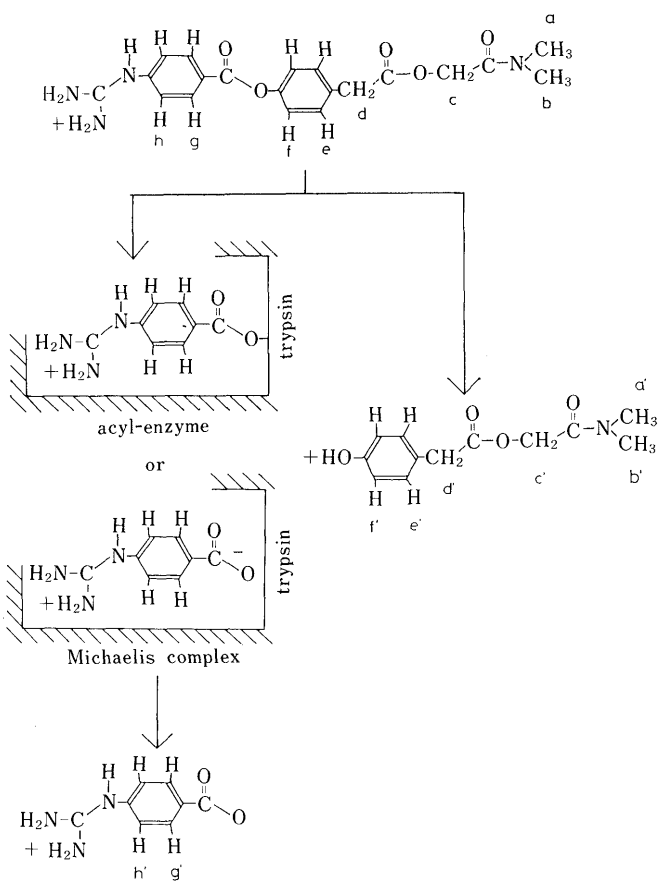

Fig. 4. Hypothesized Hydrolysis Process

The 1 : 1 mole ratio solution with 1 mg/ml $CaCl_2$ and DCl–Tris buffer was prepared at a concentration of *ca.* 2 mM. The pH was 8.0 (uncorrected). The solution was incubated at 37 °C, and the ^{1}H-NMR spectra were recorded after 1, 1.5, 2, 3 and 4 h after the time of the mixing (Fig. 3). The chemical shifts were referenced to the methyl proton peak of the methanesulfonate ion.

Results and Discussion

In analogy with the ^{1}H-NMR spectra of *N*,*N*′-dimethylformamide and *N*,*N*′-dimethylacetamide,[5] peaks a (2.99 ppm) and b (3.07 ppm) in Fig. 2 were assigned to the *cis* and *trans* N-methyl protons of the inhibitor, respectively, whereas peaks c (3.99 ppm) and d (4.97 ppm) were assigned to the protons of the two methylene groups. The doublet peaks e ($\delta=7.525$ ppm, $J=6$ Hz) and f ($\delta=7.330$ ppm, $J=12$ Hz) were assigned to the aromatic protons of the phenylacetate group based on the results of spin decoupling experiments and the Diehl rule.[6] The doublet peaks g ($\delta=8.325$ ppm, $J=6$ Hz) and h ($\delta=7.555$ ppm, $J=6$ Hz) were assigned to the aromatic protons of *p*-guanidinobenzoyloxy group by the spin decoupling experiments.

In the mixed state, new peaks (a′—d′ in Fig. 2-C) corresponding to a—d appeared at slightly higher field ($\delta H_a - \delta H_{a'} = 0.007$, $\delta H_b - \delta H_{b'} = 0.013$, $\delta H_c - \delta H_{c'} = 0.157$ and $\delta H_d - \delta H_{d'} = 0.050$ ppm). The intensities of the peaks a′—d′ increased in proportion to the mole ratio of trypsin. New peaks (e′ and f′) corresponding to e and f were also observed at higher field. The shifts of peaks e and f with the addition of trypsin were much larger than those for a—d: $\delta H_e - \delta H_{e'} = 0.244$ and $\delta H_f - \delta H_{f'} = 0.388$ ppm. To confirm assignment of the new peaks, we measured the ^{1}H-NMR spectrum of the 2 mM inhibitor solution hydrolyzed by 40% (w/v) sodium deuterioxide, and observed four doublets at ($\delta=7.875$ ppm, $J=6$ Hz), ($\delta=7.051$ ppm, $J=4.2$ Hz), ($\delta=7.042$ ppm, $J=6$ Hz) and ($\delta=6.610$ ppm, $J=8.4$ Hz). The spin decoupling experiments revealed that the first two doublet peaks formed a pair. The last two doublets correspond clearly to peaks e′ and f′ of the mixed solution, indicating that the phenylacetate group is hydrolyzed in the mixed solution of trypsin. The sharpness of the new peaks e′ and f′ can be attributed to the free motion of the hydrolyzed phenylacetate fragment removed from the specific pocket of trypsin. The intensities of peaks g and h decreased in parallel with those of a—f on the increase of the mole ratio of trypsin, but no alternative peaks to g and h could be detected in the spectrum of the mixture. Probably, new signals corresponding to H_g and H_h are so broadened that they are hidden in the trypsin background of the spectrum. This interpretation is reasonable since the molecular motion of the *p*-guanidinophenyl group is thought to be strongly restricted by a tight binding to the active site of trypsin. The form of the binding may be an acyl-enzyme form which is an intermediate state in the hydrolysis process, or a Michaelis complex in which a hydrolyzed product, *p*-guanidinobenzoate, binds to the specific pocket of trypsin. The formation of these intermediates is shown in Fig. 4 schematically. It is known that a similar inhibitor, ethyl *p*-guanidinobenzoate, forms an acyl-enzyme complex with trypsin.[7] According to Kayama *et al.*, the protease activity of trypsin inhibited by FOY-305 is almost completely recovered after incubation at 37 °C for 2 h.[8] In order to detect any spectral evidence of such a binding and release process, we measured the time dependent ^{1}H-NMR spectra of the inhibitor : trypsin complex incubated at 37 °C.

By comparing the spectra of the incubated mixture with those of the inhibitor itself under the same conditions, pH 8.0 at 37 °C, the peaks e′ ($\delta=7.28$ ppm, $J=12$ Hz), f′ ($\delta=6.945$ ppm, $J=6$ Hz), g′ ($\delta=7.975$ ppm, $J=6$ Hz) and h′ ($\delta=7.405$ ppm, $J=6$ Hz) were assigned to the hydrated inhibitor fragments. Peaks e and f attributed to the phenylacetate ring in Fig. 2 are not clearly observed in Fig. 3, probably because they overlap with trypsin peaks. Peaks g and h in Fig. 3-A gradually diminished with elapsed time and almost disappeared after 3 h (Fig. 3-B—D). Instead, new sharp doublet peaks g′ and h′ appeared as a result of incubation in the region where no inhibitor peaks were observed at 27 °C, pH 5.0. These doublets attained the maximum peak intensities after 2 h (Fig. 3-A—C). It was confirmed from the spin decoupling experiment that peaks g′ and h′ were due to the guanidinobenzoyl group corresponding to g and h of the undissociated inhibitor. The sharp feature of the peaks g′ and h′ during the first 2 h is attributable to the free molecular motion of the acyl fragment released from trypsin within this time interval. During the next 2 h, peaks g′ and h′ broadened gradually and peak g′ shifted slightly to the lower field ($\delta g' = 0.03$ ppm), while peaks e′ and f′ retained their position and sharpness throughout the incubation (Fig. 3-B—E). This result suggests that the *p*-guanidinobenzoyl fragment partially recombines with trypsin while most of the phenylacetyl fragment remains in the solution. The intensities of peaks g′ and h′ are much smaller than those

of peaks e′ and f′ because the recombined fragment did not contribute to the former or the fragment was still trapped in the active pocket. In the X-ray analysis, the difference Fourier synthesis revealed some extra contours in the density map, probably due to the *p*-guanidinobenzoyl fragments attached to less specific interaction sites around the specific pocket in disorder. This crystallographic observation is reasonably explained as a consequence of the recombination between the *p*-guanidinobenzoyl fragment and trypsin suggested from the ^{1}H-NMR spectra.

References

1) Y. Tamura, M. Hirado, K. Okamura, Y. Minato and S. Fujii, *Biochim. Biophys. Acta*, **484**, 417 (1977).
2) O. Matsumoto, T. Taga, T. Higashi, M. Matsushima and K. Machida, in preparation.
3) W. Bode and P. Schwager, *J. Mol. Biol.*, **98**, 683 (1975).
4) O. Matsumoto, T. Taga and K. Machida, *Acta Crystallogr., Sect., C*, **45**, 913 (1989).
5) J. V. Hatton and R. E. Richards, *Mol. Phys.*, **3**, 253 (1961); *idem, ibid.*, **5**, 139 (1962).
6) P. Diehl, *Helv. Chim. Acta*, **44**, 829 (1961).
7) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **242**, 5782 (1967).
8) N. Kayama, K. Sakaguchi and S. Fujii, *Gendai Iryou*, **12**, 1 (1989).