Notes

[Chem. Pharm. Bull. 31(3)1067—1072(1983)]

Apparent Autolysis of the N-Terminal Tetrapeptide of Vasoactive Intestinal Polypeptide (VIP)[1)]

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(Received August 19, 1982)

The hydrolysis of a tetrapeptide, H–His–Ser–Asp–Ala–OH, corresponding to the N-terminal portion of vasoactive intestinal polypeptide (VIP) was examined. At pH 5.0, Ala was released at a much faster rate than His, while the release of His was predominant at pH 7.78. When examined by nuclear magnetic resonance spectroscopy, the pD dependence of chemical shift was especially pronounced at the His residue, but in a different manner from that observed in a His monomer. On the basis of calculation of rotamer population ratios, a relationship between the predominant G′ conformation of the His residue and faster hydrolysis of the tetrapeptide at lower pH is postulated.

Keywords——hydrolysis of His–Ser–Asp–Ala; N-terminal tetrapeptide of vasoactive intestinal polypeptide; hydrolysis of *p*-nitrophenyl acetate; NMR spectra of His–Ser–Asp–Ala; rotamer population

In the course of our synthetic studies on vasoactive intestinal polypeptide (VIP), we observed the unusual phenomenon that the N-terminal His residue was released from the deprotected heptacosapeptide during incubation with mercaptoethanol in aqueous solution for reduction of the Met(O) residue.[2)] The N-terminal part of VIP is composed of three functional amino acids, His–Ser–Asp, which are known to be the active catalytic triad of serine proteases.[3)] The hydrolysis of the peptide bond, which looks like autolysis, might result from interaction of the three functional groups mentioned above. We therefore synthesized the N-terminal tetrapeptide of VIP, H–His–Ser–Asp–Ala–OH, and carried out conformational analysis by nuclear magnetic resonance (NMR), as well as examination of its catalytic activity for the hydrolysis of *p*-nitrophenyl acetate (*p*NPA), a substrate of serine proteases. Though we have not yet reached a definitive conclusion regarding this apparent autolysis, we observed that at pH 5.0, Ala was released at a much faster rate than that of His from this model peptide. These experimental results are reported in this paper.

The model peptide, H–His–Ser–Asp–Ala–OH, was prepared by the azide condensation of two dipeptide units, Z(OMe)–His–Ser(Bzl)–$NHNH_2$[4)] and H–Asp(OBzl)–Ala–OBzl,[5)] followed by removal of all the protecting groups.

First, the catalytic activity of the peptide for the hydrolysis of *p*NPA was measured as a rough criterion of its nucleophilicity, according to Katchalski *et al.*[6)] Relationships between reaction rate (v) and *p*NPA concentration, peptide concentration and pH in this hydrolytic reaction are shown in Figs. 1, 2 and 3 respectively.

The v-S (substrate, *p*NPA) concentration relationship, as well as the v-peptide concentration relationship, gave a straight line without showing any hyperbolic section due to catalyst-substrate complex,[7)] as can be seen in Figs. 1 and 2. The results imply that this reaction proceeds simply as a bimolecular-type reaction. However, the rapid increase of the reaction rate above pH 7.0, as shown in Fig. 3, suggested the predominant participation of the imidazole function of the His residue and the N^{α}-amino group in this hydrolytic reaction.

In terms of the catalytic activity of the tetrapeptide for the hydrolysis of *p*NPA, the

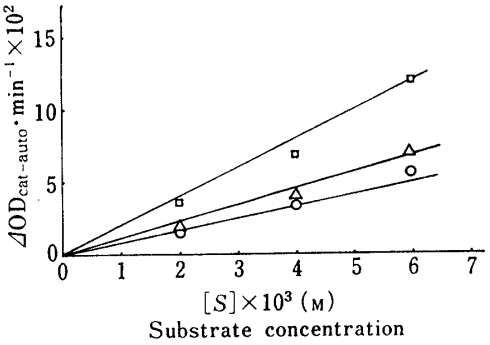

Fig. 1. Reaction Rate-Substrate Concentration Relationship for the Hydrolysis of *p*NPA by H–His–Ser–Asp–Ala–OH (pH 7.75)

Peptide concentration: —□—, 0.246×10^{-3} M; —△—, 0.164×10^{-3} M; —○—, 0.082×10^{-3} M.

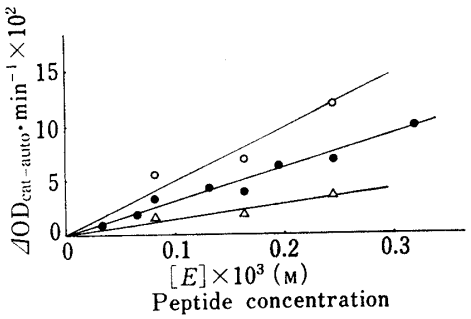

Fig. 2. Reaction Rate-Peptide Concentration Relationship for the Hydrolysis of *p*NPA by H–His–Ser–Asp–Ala–OH (pH 7.75)

*p*NPA concentration: —○—, 6.00×10^{-3} M; —●—, 4.00×10^{-3} M; —△—, 2.00×10^{-3} M.

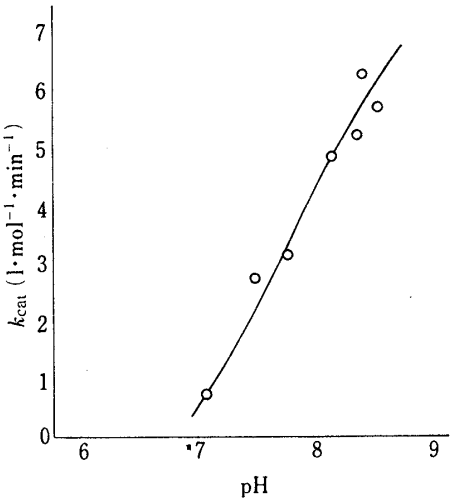

Fig. 3. Reaction Rate-pH Relationship for the Hydrolysis of *p*NPA byH–His–Ser–Asp–Ala–OH

*p*NPA concentration; 0.50×10^{-3} M. H–His–Ser–Asp–Ala–OH concentration; 0.15×10^{-3} M.

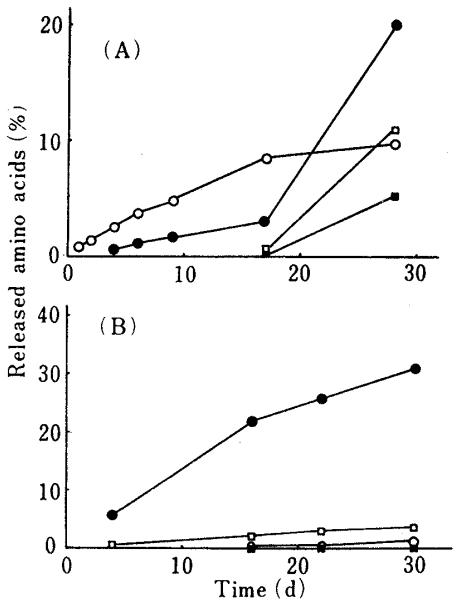

Fig. 4. Time Course for the Hydrolysis of H–His–Ser–Asp–Ala–OH at 37°C

(A) in water (pH 5.0)
(B) in 0.2 M Tris–HCl buffer (pH 7.78)
—●—, His; —□—, Ser; —■—, Asp; —○—, Ala.

second-order rate constants (k_{cat}) calculated according to Imanishi *et al.*[8] were 4.0—5.0 $l \cdot mol^{-1} \cdot min^{-1}$ at around pH 8.0 as shown in Fig. 3. These values are *ca.* 1/2000—1/2500 of that of chymotrypsin ($k_{cat}=10^4$ $1 \cdot mol^{-1} \cdot min^{-1}$).[6] From these experimental data, it can be concluded that this small peptide has no ability to act as an enzyme-model peptide.

Next, the behavior of the tetrapeptide in water at pH 5.0 and in Tris–HCl buffer at pH 7.78 was examined and the amounts of amino acids released were plotted against time as shown in Fig. 4-A and B, respectively.

As shown in Fig. 4-A, at pH 5.0, the release of C-terminal Ala was marked in an early stage of incubation at 37°C and reached 8 to 10% after 28 d. The release of the N-terminal His was slower than that of Ala in this early stage, but increased sharply after 18 d, then reached *ca.* 20% after 28 d. A certain amount of released Ser and Asp was also detected after incubation for about 17 d. As shown in Fig. 4-B, release of His is noteworthy at pH 7.78,

but release of Ala, as well as other amino acids, was judged to be somewhat slower than that observed at pH 5.0. Since hydrolysis of the ester or amide bond is supposed to proceed much faster at higher pH, the above phenomena imply the presence of some subtle relationship between the rate of hydrolysis and the conformation of this tetrapeptide molecule.

The NMR spectra of H–His–Ser–Asp–Ala–OH in D_2O at pD 5.2 and 7.0 were measured using DSS [3-(trimethylsilyl)-propanesulfonic acid Na salt] as a reference, as shown in Fig. 5. The pD dependence of the chemical shift is especially pronounced at the His residue in that the signal splitting of $C_\beta H_2$ of His is quite different at the two pDs, 5.2 and 7.0. The signal is composed of 8 lines at pD 5.2, which can be assigned as an AB part of an ABX pattern. A and B correspond to the two C_β-protons, and X to the C_α–proton. At pD 7.0, the signal becomes only 2 lines. This indicates that there is no difference in chemical shift at this pD for the A and B protons, or in vicinal coupling, *i.e.*, $\delta_{AB}=0$ and $J_{AX}=J_{BX}$. It seems worthwhile to note that pH dependence of the spectra observed here is opposite to that recorded in the His monomer.[9]

The vicinal couplings, J_{AX} and J_{BX}, are listed in Table I, in which the constants for Asp and Ser residues are also included. With these constants, we calculated the population ratios of three possible rotamers (Fig. 6; G, G′ and T) for His, Asp and Ser at two pDs according to the Kopple modification of the Karplus relation.[10] First, theoretical coupling constants (J_t and J_g) were calculated at two angles, $\chi=180°$ and 60°, according to equation (1) and then rotamer populations (P_T, $P_{G'}$, and P_G) were calculated according to equation (2),

$$(1)\quad J_{HH}=11.0\cos^2\chi-1.4\cot\chi+1.6\sin^2\chi$$

$$J_t=12.4\ \text{Hz}(\chi=180°)\quad\text{and}\quad J_g=3.25\ \text{Hz}(\chi\pm60°)$$

$$(2)\quad P_T=\frac{J_{AX}-Jg}{J_t-Jg}\quad P_{G'}=\frac{J_{BX}-J_g}{J_t-J_g}\quad P_G=1-P_T-P_{G'}$$

where J_{AX} and J_{BX} are the vicinal couplings for H_α–$H_{\beta1}$ and $H_{\beta2}$, respectively. Table II shows the rotamer populations for the three residues thus calculated.

In terms of His, the ratios of rotamer populations are nearly equal at higher pD, then G′

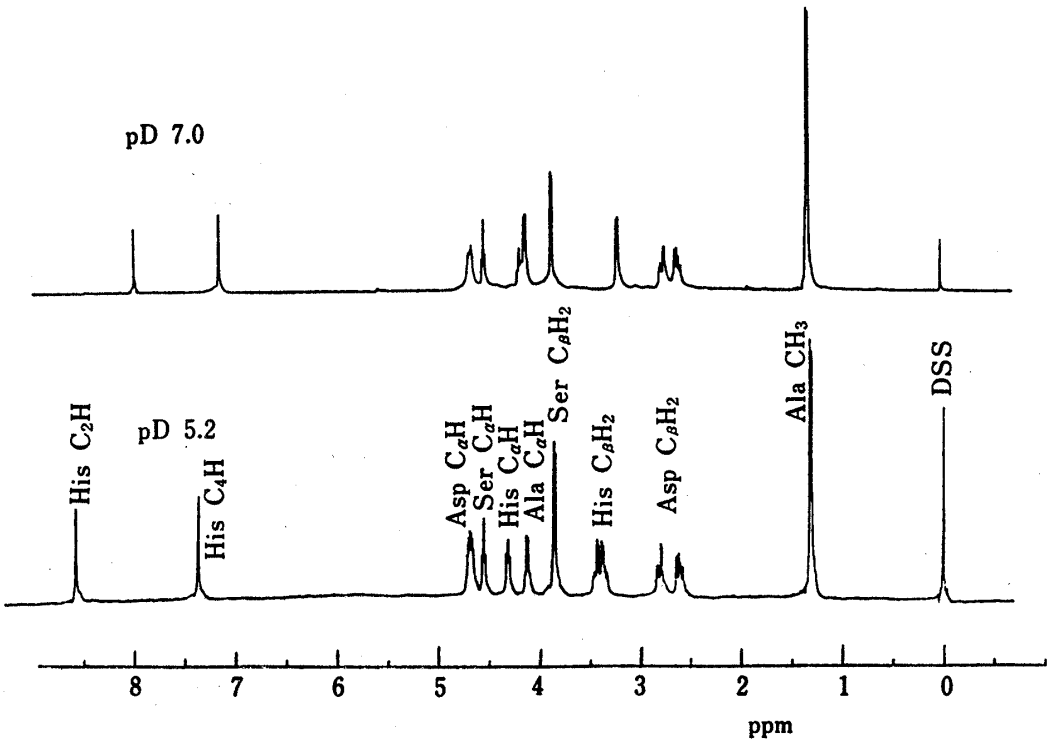

Fig. 5. The 400 MHz ^{1}H-NMR Spectra of H–His–Ser–Asp–Ala–OH at pDs 5.2 and 7.0

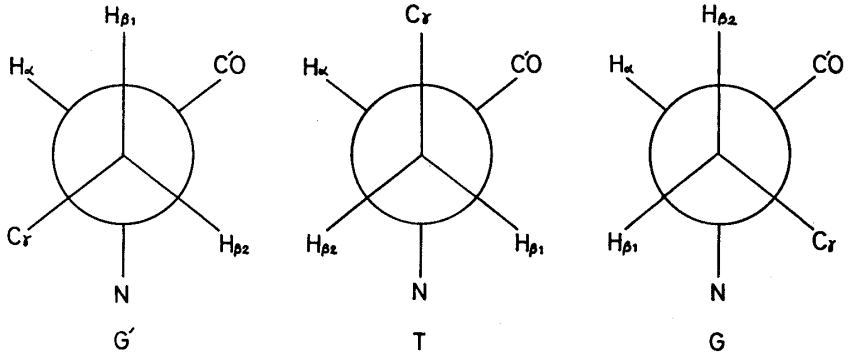

Fig. 6. Definition of the Different Rotamers around the C_α–C_β bond

TABLE I. Vicinal Couplings for $C_\beta H_2$ (Hz)

	pD	J_{AX}	J_{BX}
His	5.2	5.79	7.78
	7.0	6.35	6.35
Ser	5.2	6.28	6.28
	7.0	6.15	6.15
Asp	5.2	3.37	9.20
	7.0	4.13	9.06

TABLE II. Rotamer Populations

	pD	P_T	P_G	$P_{G'}$
His	5.2	0.278	0.227	0.495
	7.0	0.339	0.322	0.339
Ser	5.2	0.331	0.338	0.331
	7.0	0.317	0.366	0.317
Asp	5.2	0.011	0.339	0.650
	7.0	0.096	0.269	0.635

becomes predominant at lower pD, where T and G are nearly equal. For Asp, the rotamer populations are in the order G′>G>T at both pDs. For Ser, the three rotamers are equivalent and there is no pD dependence. In conclusion, pD dependence of the side chain conformation is noticeable in His, which takes the G′ conformation as the most preferable one at low pD. These results imply the presence of a subtle relationship between the predominant G′ conformation of the His residue at low pD and the faster hydrolysis of the tetrapeptide in lower pH, described above. In order to obtain more precise information about this relationship, it seems necessary to carry out conformational analysis of the entire molecule, including the peptide backbone in future.

As mentioned above, our present conformational studies are not conclusive. However, it can be predicted that not only the release of His,[2] but also the hydrolysis of the Asp–Ala bond may take place when VIP is stored in water, even at pH around 7. Such phenomena may account in part for the instability of secretin in solution,[11,12] since the N-terminal tripeptide unit, H–His–Ser–Asp, of secretin is identical with that of VIP.

Experimental

Thin layer chromatography (TLC) was performed on silica gel (Kieselgel G, Merck) and *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8: 3: 1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4: 1:

1: 2). Leucine-aminopeptidase (LAP) was purchased from Sigma (Lot. No. 79C-8110). NMR spectra were taken with a JEOL FX-400 machine (400 MHz for H) in D_2O at 27°C. Ultraviolet spectrum (UV) absorption was measured with Hitachi-Perkin Elmer 139 spectrophotometer.

Synthesis of the Tetrapeptide

a) **Z(OMe)–His–Ser(Bzl)–Asp(OBzl)–Ala–OBzl**——Z(OMe)–Asp(OBzl)–Ala–OBzl[5] (1.23 g, 2.24 mmol) was treated with TFA–anisole (3 ml–2 ml) in an ice-bath for 60 min, then excess TFA was removed by evaporation. The oily residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (10 ml) containing Et_3N (0.31 ml, 2.24 mmol). To this ice-chilled solution, the azide [prepared from 1.02 g, 2.0 mmol of Z(OMe)–His–Ser(Bzl)–$NHNH_2$[4]] in DMF (10 ml) and Et_3N (0.28 ml, 2.0 mmol) were added, and the mixture was stirred at 4°C for 40 h. The solvent was evaporated off and the residue was treated with H_2O. The resulting powder was washed with H_2O and AcOEt and recrystallized from MeOH and AcOEt; yield 1.23 g (71%), mp 121—124°C, $[\alpha]_D^{18}$ −16.0° (c=0.5, DMF), Rf_1 0.65. Amino acid ratios in 6 N HCl hydrolysate: His 1.04, Ser 0.81, Asp 1.00, Ala 0.95 (recovery of Asp 85%). *Anal.* Calcd for $C_{46}H_{50}N_6O_{11}\cdot H_2O$: C, 62.71; H, 5.95; N, 9.54. Found: C, 62.43; H, 5.77; N, 9.23.

b) **H–His–Ser–Asp–Ala–OH**——Z(OMe)–His–Ser(Bzl)–Asp(OBzl)–Ala–OBzl (431 mg, 0.50 mmol) was treated with TFA–anisole (2 ml–0.4 ml) in an icebath for 60 min to remove the Z(OMe) group, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and dissolved in MeOH (15 ml) containing 5% Pd/C and a few drops of AcOH. Next, to remove the Bzl groups, H_2 gas was bubbled through the solution for 8 h. The solution was filtered, the filtrate was concentrated and the residue was dissolved in H_2O (5 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 2 g) for 30 min, then filtered and the filtrate was lyophilized. The resulting powder was dissolved in a small amount of H_2O and the solution was applied to a column of Sephadex G-10 (1.5×95 cm). Individual fractions (6 ml each) were collected and ninhydrin color was detected on TLC. Tube Nos. 31—34, which were ninhydrin-positive, were collected and the solvent was removed by lypohilization to give a white powder; yield 80.9 mg (33%), $[\alpha]_D^{18}$ −24.7° (c=0.4, 0.1 N AcOH), Rf_2 0.09. Amino acid ratios in 6 N HCl hydrolysate and LAP digest (numbers in parentheses): His 1.05 (1.05), Ser 0.80 (1.00), Asp 1.00 (1.00), Ala 0.97 (1.00), recovery of Asp 84% (86%). *Anal.* Calcd for $C_{16}H_{25}N_6O_8\cdot 1/2\ H_2O$: C, 43.93; H, 5.76; N, 19.21. Found: C, 43.97; H, 5.61; N, 18.80.

Determination of pNPA Hydrolytic Activity——The rate of hydrolysis of *p*NPA was measured in 1/15 M phosphate buffer containing 11.8% dioxane at 25°C at 400 nm, according to Katchalski *et al.*,[6] and the results are shown in Figs. 1, 2 and 3. The second-order rate constant, k_{cat} was determined according to Imanishi *et al.*[8] by means of the following formula.

$$k_1 t = \ln\frac{OD_\infty - OD_0}{OD_\infty - OD_t} \qquad k_{cat} = \frac{k_1 - k_W}{[E]}$$

k_1: pseudo-first-order rate constant for hydrolysis of *p*NPA by the peptide.
k_W: rate constant for spontaneous hydrolysis of *p*NPA.
$[E]$: concentration of the peptide solution.
OD: optical density.

Incubation of H–His–Ser–Asp–Ala–OH——A solution of H–His–Ser–Asp–Ala–OH (4.15 mg, 9.7 μmol) in H_2O (2.0 ml, pH 5.0 adjusted with AcOH) and a solution of the above tetrapeptide (4.15 mg, 9.7 μmol) in Tris–HCl buffer (2.0 ml, pH 7.78) were each incubated at 37°C . An aliquot of each solution, after dilution with 0.12 M sodium citrate buffer (pH 2.2), was tested on an amino acid analyzer periodically. The results are shown in Fig. 4-A and B.

Acknowledgement The authors wish to thank Dr. Michio Yoneyama, Faculty of Science, Hokkaido University, for his valuable advice.

References and Notes

1) Amino acids and the peptide and its derivative mentioned here are of the L-configuration. Abbreviations used: *p*NPA=*p*-nitrophenyl acetate; Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, DMF=dimethylformamide, TFA=trifluoroacetic acid.
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