Proton Magnetic Resonance Study of *Streptomyces* Subtilisin Inhibitor. pH Titration and Assignments of Individual Tyrosyl Resonances†

Shigeru Fujii, Kazuyuki Akasaka,* and Hiroyuki Hatano

ABSTRACT: This paper reports the pH titration at 25 °C and the assignment of aromatic proton resonances of three tyrosyl residues of each identical subunit of *Streptomyces* subtilisin inhibitor (M_r 23 000) by high-resolution ^{1}H NMR spectroscopy. The complete assignments of the specific tyrosyl resonances were made based on the results of the differential chemical modification of the tyrosyl residues with tetranitromethane followed by peptide analysis, independently of the knowledge of the crystal structure. pK_a values of Tyr-7, -75, and -93 were determined in a ^2H_2O solution to be 10.95, 11.8, and ≳12.6, respectively, at 25 °C, whereas pK_a values of nitrated Tyr-7 and -75 were determined to be 7.3 and 7.9, respectively. Tyr-93 was not nitratable under normal conditions. The strong resistance to nitration, together with the extremely high pK_a value and the high-field shifted positions of the ring proton resonances of Tyr-93 at neutral pH, strongly suggests that Tyr-93 takes part in a hydrogen bonding as a proton donor. Tyr-7 is more easily modified with tetranitromethane than Tyr-75, although in the crystal Tyr-75 is more exposed than Tyr-7. The result, together with the pK_a value of Tyr-75 significantly higher than that of a normal tyrosine, indicates that the microenvironment of Tyr-75 is more restricted in solution than in solid. These results imply that structural details of a protein may be quite similar in solution to those in the crystal in the rigid hydrophobic region of the protein but that in the surface region of the protein local structures may well differ between the solution and the crystal.

Streptomyces subtilisin inhibitor (SSI)[1] isolated from *Streptomyces albogriseolus* S-3253 (Murao & Sato, 1972; Sato & Murao, 1973) is unique in that it strongly inhibits microbial alkaline proteinases (Murao & Sato, 1972). The protein consists of two identical subunits, each having 113 amino acid residues including three tyrosyl residues and two disulfide bridges (Ikenaka et al., 1974). The reactive site toward subtilisin and chymotrypsin has been shown to be Met-73–Val 74 (Ikenaka et al., 1974).

The results of X-ray analysis of the protein and its complex with subtilisin (Mitsui et al., 1977; Mitsui et al., 1979a,b; Hirono et al., 1979) have shown the following concerning the microenvironment of the three tyrosyl residues in the crystal. Tyr-7 is located next to the six amino acid residues at the N terminal whose electron density was scarcely traceable, meaning a high degree of disorder. Tyr-7 is mostly exposed but one side of it is partially covered by Pro-9. There is no charged group within 10 Å of the phenol ring of Tyr-7. Tyr-75 lies adjacent to the reactive site and is located within the contact area on forming the complex with subtilisin, although its phenol ring is almost completely exposed in free SSI. There are two charged groups within 10 Å of Tyr 75, the ϵ-amino group of Lys-18 lying ~7 Å apart from the hydroxyl group of Tyr-75 and the carboxyl group of Asp-76 lying ~10 Å apart from the phenol ring. Neither Tyr-7 nor Tyr-75 would be able to take part in hydrogen bonding with neighboring residues. On the other hand, the phenol ring of Tyr-93 is located in a narrow cleft near the surface of the hydrophobic core and is mostly buried, closely surrounded by Leu-60, Asn-61 and Arg-95. The phenol OH of Tyr-93 is considered certain to be hydrogen bonded to the carboxylamide group of Asn-61. However, it is not clear from the crystal data only whether Tyr-93 or Asn-61 is a proton donor in this hydrogen bonding. The relative solvent (H_2O) accessibilities (Lee & Richards, 1971) for Tyr-7, -75, and -93 calculated on the basis of the X-ray data are 70, 84, and 5%, respectively, predicting that Tyr-93 is highly shielded from the solvent (Satow et al., 1980).

In contrast to the prediction from the X-ray data, the solvent perturbation UV spectroscopy has shown that all three tyrosyl residues are completely accessible to methanol and ethylene glycol, while they are, on the average, 89% accessible to poly(ethylene glycol) (Inouye et al., 1977). Experiments of the pH titration of the three tyrosyl residues were first conducted by Inouye et al. (1977) using a spectrophotometric method, and three widely different pK_a values (9.58, 11.10, and 12.42) for the three tyrosyl residues were reported at 25 °C.

On the other hand, utilizing ^{1}H NMR spectroscopy at 360 MHz, Akasaka (1978) was able to follow the pH titration of individual tyrosyl residues of SSI at 50 °C and determined pK_a values of respective tyrosyl residues to be 10.6, 11.2, and ≳11.5. On the basis of the known crystal structure (Mitsui et al., 1979a), the tyrosyl residue showing the unusually high pK_a value (≳11.5) was tentatively identified as Tyr-93. However, the complete assignment of all the tyrosyl residues to respective pK_a values has not been performed until present.

In view of the discrepancies among the results obtained for the microenvironments of the three tyrosyl residues of SSI with different experimental techniques as described above, the complete and unambiguous assignment of the pH titration curves to individual tyrosyl residues and the characterization of their microenvironments in solution appear to be important.

In the present work, the complete assignment of the three tyrosyl residues to respective pH titration curves was under-

† From the Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606, Japan (S.F. and H.H.), and the Department of Molecular Physics, Max Planck Institute for Medical Research, 6900 Heidelberg, West Germany (K.A.). *Received April 16, 1980; revised manuscript received September 2, 1980.* This work was supported in part by a grant from the Ministry of Education, Science, and Culture of Japan, No. 134013 (1976–1977).

* Correspondence should be addressed to this author at the Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606, Japan.

[1] Abbreviations used: SSI, *Streptomyces* subtilisin inhibitor; ^{1}H NMR, proton nuclear magnetic resonance; TSP, sodium 3-trimethylsilylpropionate-2,2,3,3-d_4; Tris, tris(hydroxymethyl)aminomethane; δ, chemical shift downfield from TSP; ppm, parts per million.

0006-2960/81/0420-0518$01.00/0

taken, by the combined use of 360-MHz ^{1}H NMR and differential nitration followed by peptide analysis (Snyder et al., 1976). In spite of the high molecular weight (23 000) of the protein, pH titration of the individual tyrosyl residues at 25 °C was rendered possible by the combined use of (1) a high concentration of the protein solution (46 mg/mL), (2) the high-frequency ^{1}H NMR spectrometer (360 MHz), and (3) the convolution difference technique in the spectral analysis (Campbell et al., 1973), aided by the pH titration data initially carried out at 50 °C (Akasaka, 1978).

Experimental Procedures

Materials. Semipurified SSI was supplied by the courtesy of Dr. Murao of Osaka Prefectural University. It was further purified according to the previous method (Murao & Sato, 1972; Sato & Murao, 1973). Tetranitromethane was purchased from Maruwaka Chemical Co. (Osaka). 99.75% ^2H_2O from Merck was used for NMR measurements. TPCK-trypsin [trypsin treated with L-(tosylamido)-2-phenylethyl chloromethyl ketone to kill chymotryptic activity] was purchased from Millipore Co. (Freehold, NJ). Other reagents were obtained from Nakarai Chemical Co. (Kyoto).

Nitration of Tyrosyl Residues. SSI was dissolved in $^1/_{20}$ M Tris-HCl buffer at pH 8.0 at a concentration of 2.5×10^{-4} M. A 10% (v/v) ethanol solution of tetranitromethane was gradually added to the SSI solution at 10 or at 30 °C under stirring until a molar ratio of tetranitromethane to SSI became a 200-fold excess. The reaction was stopped by passing the reaction mixture on a Sephadex G-25 column (5 × 30 cm) equilibrated with the same Tris-HCl buffer. The nitrated SSI was cleaved into peptide fragments by reaction with BrCN and further with TPCK-trypsin (for the reaction at 30 °C) according to the method of Ikenaka et al. (1974) (See Results for detailed procedures). The nitration reaction followed directly in the NMR tube was performed under different conditions (See Results).

Amino Acid Analysis. The peptide fragments were dissolved in a 6 N HCl solution. The solutions (8 mL) were degassed and were protected from oxidation by adding ~1 mg of dithioerythritol. The solutions were kept for 22 h in a bath thermostated at 110 °C. The hydrolyzed amino acids were analyzed on a Hitachi Model KLA 3B amino acid analyzer.

NMR Measurements. ^{1}H NMR spectra were obtained at 360 MHz on a Bruker HX-360 spectrometer at the Max Planck Institute for Medical Research (Heidelberg) and partially at 200 MHz on a Varian XL-200 at the Medical School, Osaka University, both with standard 5-mm probes. Sample temperature cited is that of the probe temperature at the bottom of the sample tube, measured with the aid of a copper–constantan thermocouple. For the pH titration, dilute ^{2}HCl and NaO^{2}H solutions were used. All pH values given in this report are direct pH meter readings of the protein solution using a glass electrode standardized with ^1H_2O buffers. Chemical shifts were measured relative to an internal reference, TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d_4), and are believed to be accurate to ± 0.01 ppm.

Results

pH Titration at 25 °C. Figure 1 shows the aromatic region of the convolution difference 360-MHz ^{1}H NMR spectrum of SSI at 25 °C. In general, the ^{1}H NMR spectrum of a tyrosyl residue in a protein will consist of four doublets (A, B, C, and D), since four ring protons (2, 3, 5, and 6) should, in general, experience mutually different chemical shielding from the surrounding residues. When the internal rotation about the C_β–C_γ axis of a tyrosyl residue becomes higher, the

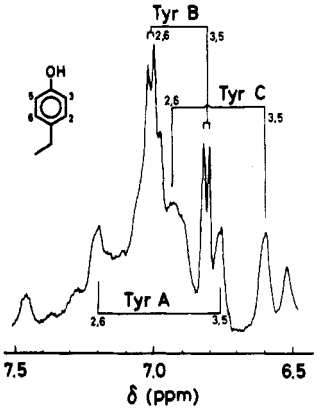

FIGURE 1: Convolution difference ^{1}H NMR spectrum of SSI in the aromatic proton region, taken at 360 MHz at 25 °C, pH 7.74. The protein concentration is 46 mg/mL in ^2H_2O. The chemical shifts are given relative to internal TSP.

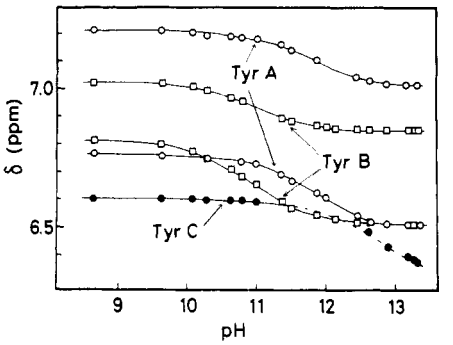

FIGURE 2: pH titration curves of aromatic protons of the three tyrosyl residues of SSI, measured at 360 MHz at 25 °C. Protein concentration, 46 mg/mL. The chemical shifts are given relative to internal TSP.

signals merge into a pair of doublets, the lower field doublet normally corresponding to the 2 and 6 protons and the higher field one to the 3 and 5 protons of the phenol ring (Snyder et al., 1976). In Figure 1, Tyr A, B and C represent a pair of ring proton signals of the three tyrosyl residues, respectively. Only Tyr B showed a clear doublet pair at 25 °C whereas signals of Tyr A and C showed only limited resolution. However, the scalar coupling between the 2 and 6 protons and the 3 and 5 protons for each of the three tyrosyl pairs was confirmed by spin decoupling. At 50 °C, the signals of Tyr A become clearly resolved into a pair of doublets, while those of Tyr C become at least partially resolvable (Akasaka, 1978).

pH titration curves of the ring proton signals of the three tyrosyl residues are shown in Figure 2. Both Tyr A and B exhibit normal pH titration curves, except that their pK_a values are high. The pair of signals of Tyr A gave identical pK_a values within the experimental error and so does the pair of Tyr B. The signal from the 3 and 5 protons of Tyr C was not traceable in the whole pH range because of its broadness and the overlap with other aromatic signals and was omitted in the titration curve of Figure 2. At 50 °C, however, the same signal is traceable in a wide pH range (Akasaka, 1978). The dotted line for the 2 and 6 protons of Tyr C is tentatively drawn, because the signal could not be traced between pH 11 and 12.5. It is to be noted that even at neutral pH the signal of the 2 and 6 protons of Tyr C appears at a significantly high field and remains at the same position at least below pH 11.

Nitration and Assignment of Individual Tyrosyl Resonances. Figure 3 shows the aromatic region of ^{1}H NMR spectra of SSI before the nitration reaction (a) and after the nitration reaction (b), taken at 50 °C. After the reaction, the

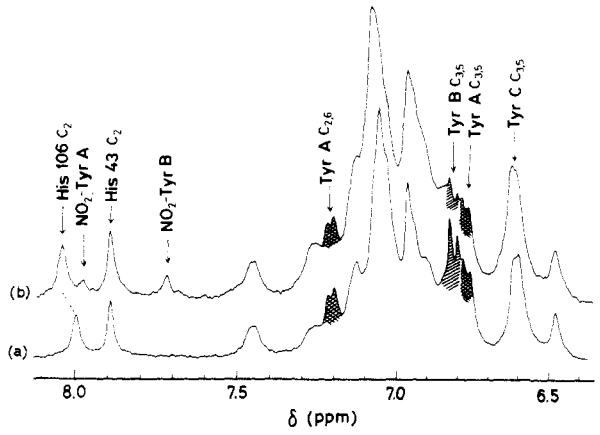

FIGURE 3: Aromatic region of ^1H NMR spectra of SSI and nitrated SSI taken at 360 MHz at 50 °C, pH 8.03 in ^2H_2O–borate buffer. The reaction with tetranitromethane was carried out for 7 h at 25 °C, directly in the NMR sample tube. 7 μL of an ethanol-d_6 solution of tetranitromethane (10% v/v) was added to the protein solution of 23 mg/0.5 mL. (a) Before nitration reaction. (b) After nitration reaction. Note that the resonance of Tyr B (hatched) has decreased faster than those of Tyr A (crosshatched), whereas the resonance of Tyr C is virtually unchanged. The newly appearing peaks at $\delta = 7.72$ and $\delta = 7.97$ can be assigned to the C_2 protons of nitrated Tyr B and nitrated Tyr A, respectively. The lower field shift of the C_2 proton resonance of His-106 in (b) is due to a slight change of pH with the nitration reaction.

ring proton signal of Tyr B decreased quite significantly, whereas that of Tyr A also decreased but to a much smaller extent. The signal of Tyr C was found almost unchanged. Concomitant with the decrease of the signals of Tyr A and B, two new signals (NO_2-Tyr A and NO_2-Tyr B) appeared in the lower field of Figure 3, where the increase of signal intensity of NO_2-Tyr A corresponded to the decrease of signal intensity of Tyr A and that of NO_2-Tyr B to the decrease of Tyr B. These signals, showing singlet patterns, are assignable to the C_2 protons of NO_2-Tyr A and NO_2-Tyr B (Snyder et al., 1976). Signals of the C_5 and C_6 protons of NO_2-Tyr A and B are hidden under other resonances. From the pH dependence of the chemical shift of the C_2 proton resonances, the pK_a value of NO_2-Tyr A was determined to be 7.9 and that of NO_2-Tyr B to be 7.3 at 25 °C. The differential pK_a value (0.6 pH unit) between NO_2-Tyr A and NO_2-Tyr B corresponds rather well with the differential pK_a value (0.85 pH unit) between Tyr A and Tyr B of intact SSI.

It was found that the difference in the rate of nitration between Tyr A and Tyr B decreased with increasing temperature, e.g., when the modification was carried out at 30 °C, pH 8.0, for 90 min, Tyr A and Tyr B were modified to a comparable extent. We therefore modified SSI by nitration at 10 °C. The comparison of peak area of C_2 protons of the nitrated tyrosyl residues (NO_2-Tyr A and NO_2-Tyr B) with the area of the C_2 proton of His-43 on the 200-MHz ^1H NMR spectrum (Figure 4a) gave an estimate of 25% and 80% of nitration for Tyr A and Tyr B, respectively. The modified SSI was then selectively cleaved at the carboxyl ends of the three methionyl residues by reaction with BrCN (Ikenaka et al., 1974). The resultant mixture of peptides was separated into three fractions through a column of Sephadex G-75 (5 × 100 cm) (note that one of the disulfide bridges (Cys-71–Cys-101) keeps two of the fragments inseparable). The first fraction of these contains the peptide Asn-1–Met-70, and the second and the third contain the peptide Cys-71–Met-103 and the peptide Asn-104–Phe-113, respectively. Figure 4b,c shows 200-MHz ^1H NMR spectra of the two fractions, Asp-1–Met-70 and Cys-71–Met-103, respectively. The NMR

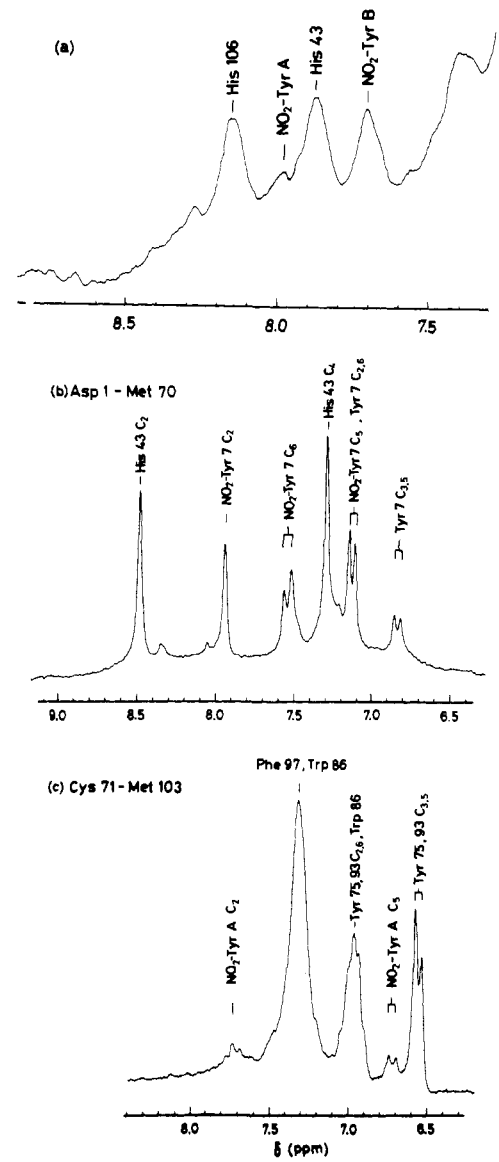

FIGURE 4: 200-MHz ^1H NMR spectra of SSI modified with tetranitromethane. The modification was carried out for 90 min at 10 °C and pH 8.0. (a) The C_2 proton region of nitrotyrosyl residues of nitrated SSI. (b) The aromatic region of the peptide Asp-1–Met-70 of the nitrated SSI. (c) The aromatic region of the peptide Cys-71–Met-103 of the nitrated SSI.

spectrum of the first fraction (Figure 4b) indicates that ~75% of Tyr-7 was nitrated, in agreement with the nitration (~80%) of Tyr B. On the basis of this result, Tyr B was assigned to Tyr-7. The spectrum of the second fraction (Figure 4c), containing two tyrosyl residues (Tyr-75 and Tyr-93), shows that ~15% of the two tyrosyl residues was nitrated. If the nitrated tyrosyl residue arises solely from Tyr A as Figure 3 suggests, the above result leads to a conclusion that ~30% of Tyr A was nitrated, in agreement with the nitration (~25%) of Tyr A in Figure 4a.

To make sure that in the second fraction nitration occurs selectively on Tyr A but not on Tyr C, we modified SSI with tetranitromethane at 30 °C. The ^1H NMR spectrum of this modified SSI showed that Tyr A as well as Tyr-7 was almost fully nitrated. The modified SSI was cleaved with BrCN, and then the peptide Cys-71–Met-103 was separated through the column of Sephadex G-75. The peptide fraction was further cleaved with TPCK-trypsin (Ikenaka et al., 1974), and then the resultant mixture of smaller peptides was separated into three fractions through a column of Sephadex G-25 (5 × 110

Table I: Amino Acid Composition of Peptide Fragments of Nitrated SSI[a]

amino acid	Asn-1–Met-70[b]	Cys-71–Met-103[b]	Val-74–Lys-89[c]	Val-91–Arg-95[c]
Asp	5.68 (5)	2.85 (3)	2.01 (2)	
Thr	6.83 (7)	0.95 (1)	0.98 (1)	
Ser	4.17 (5)	1.77 (2)		1.08 (1)
Glu	2.69 (2)	4.48 (4)	0.98 (1)	0.97 (1)
Pro	6.07 (6)	2.04 (2)	1.10 (1)	
Gly	8.10 (8)	2.17 (2)	2.01 (2)	
Ala	16.17 (16)			
Val	6.07 (6)	6.11 (6)	3.70 (4)	1.01 (1)
Leu	6.45 (7)	2.04 (2)	2.01 (2)	
Tyr	0.26	1.50	0.08	0.84
NO_2-Tyr	0.61 (1)	0.34 (2)	0.85 (1)	0.07 (1)
Phe		1.19 (1)		
Lys	0.95 (1)	0.52 (1)	0.85 (1)	
His	1.06 (1)			
Arg	2.08 (2)	2.04 (2)		0.97 (1)

[a] Numbers in parentheses indicate the theoretical values based on the amino acid sequence (Ikenaka et al., 1974). Cystine was not detected because of dithioerythritol which was applied upon the hydrolysis of the peptides. [b] Peptide fragments of SSI nitrated at 10 °C (cf. Figure 4). [c] Peptide fragments of SSI nitrated at 30 °C (cf. Figure 5).

cm). The first fraction (Val-74–Lys-89) and the third fraction (Val-91–Arg-95) contained Tyr-75 and Tyr-93, respectively, as the result of amino acid analysis shows (Table I). The NMR spectra (Figure 5a,b) of the two fractions clearly indicate that Tyr-75 was almost fully nitrated, whereas Tyr-93 was scarcely nitrated. On the basis of this result, Tyr A and Tyr C can be assigned to Tyr-75 and Tyr-93, respectively. The high resistance of Tyr C against nitration reaction indicates that Tyr C exists in an environment well shielded from the attack of the reagent, tetranitromethane. On the basis of the above results, we also conclude that at 10 °C ~30% of Tyr-75 was nitrated, whereas the nitration of Tyr-93 was close to none.

The amino acid analysis of the fragments of the modified SSI at 10 and at 30 °C was carried out, and the results are summarized in Table I. Table I shows that the cleaved and separated peptides are those expected from the amino acid sequence of SSI (Ikenaka et al., 1974). Table I also indicates that in the degree of nitration the results of amino acid analysis agree with those from ^{1}H NMR within experimental error. The chemical shift parameters and the pK_a values of individual tyrosyl residues determined above are summarized in Table II.

Discussion

The microenvironments of the three tyrosyl residues of SSI in solution will be discussed below in contrast with the corresponding microenvironments in the crystal (Mitsui et al., 1979a). In the first place, we consider Tyr-93 which shows a peculiar pH titration curve far apart from that of a free tyrosine. The pK_a value ($\gtrsim$12.6) is abnormally high by at least 2 pH units from that of a free tyrosine (10.0; Snyder et al., 1976). A similar pK_a value is reported for Tyr-53 of lysozyme (Tojo et al., 1966; Hayashi et al., 1968; Imoto et al., 1972), in which case the tyrosyl residue is buried in the hydrophobic core and is deprotonated only upon unfolding of the protein by alkaline denaturation. Similarly, protonation of His-43 of SSI which lies in the middle of the hydrophobic core was found to take place only upon acid denaturation at pH 3.25 (Fujii et al., 1980). However, in the case of Tyr-93 of SSI, the pH titration proceeds within the native conformation of the protein before alkaline denaturation takes place well above pH 13 at 25 °C (Figure 2). This observation shows that, despite its high pK_a value, Tyr-93 is not totally buried but lies in a position to be able to be in contact with the solvent (water). Therefore,

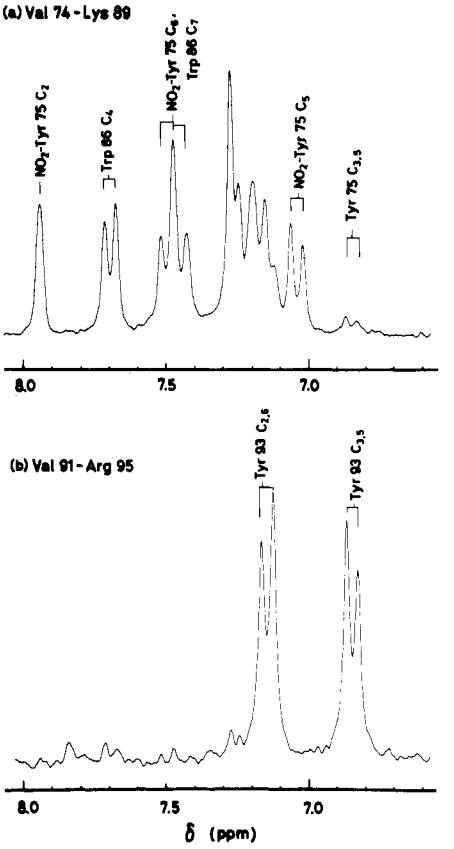

FIGURE 5: 200-MHz ^{1}H NMR spectra of peptide fragments of SSI modified with tetranitromethane at 30 °C. (a) The aromatic region of the peptide Val-74–Lys-89 of the nitrated SSI. (b) The aromatic region of the peptide Val-91–Arg-95 of the nitrated SSI.

Table II: Chemical Shift Parameters of Tyrosyl and Nitrotyrosyl Residues of SSI

		SSI						nitrated SSI			
	proton type	25 °C[a]			50 °C[b]			25 °C[c]			degree of nitration[d] (%)
		δ_{HA}	δ_{A^-}	pK_a	δ_{HA}	δ_{A^-}	pK_a	δ_{HA}	δ_{A^-}	pK_a	
Tyr-7	2,6	7.02	6.84	10.95	7.03	6.84	10.6				75
	3,5	6.81	6.53	10.95	6.81	6.52	10.6				
NO_2-Tyr-7	2							7.88	7.61	7.3	
Tyr-75	2,6	7.21	7.03	11.8	7.21	7.02	11.2				30
	3,5	6.77	6.54	11.8	6.77	6.54	11.2				
NO_2-Tyr-75	2							8.08	7.82	7.9	
Tyr-93	2,6				6.95						~0
	3,5	6.61	$\lesssim$6.40	$\gtrsim$12.6	6.61	~6.5	$\gtrsim$11.5				

[a] Taken at 360 MHz. [b] Taken at 360 MHz (Akasaka, 1978). [c] Taken at 200 MHz. [d] The nitration reaction was carried out for 90 min at 10 °C and pH 8.0 (see Experimental Procedures for details).

the reason for its high pK_a value is not because the residue is buried deep inside the hydrophobic core but rather because the residue is involved in a specific interresidue interaction. An interresidue interaction that causes a large positive shift of the pK_a value would be hydrogen bonding in which the tyrosyl OH acts as the hydrogen donor. This hydrogen bonding hypothesis is also consistent with the high-field shift of the 3 and 5 proton resonances of Tyr-93 (δ = 6.61) below the pH titration range.

The X-ray crystallography carried out by Mitsui et al. (1979a) shows that the OH group of Tyr-93 indeed forms hydrogen bond with Asn-61. However, the X-ray result does not tell which one of the two residues is acting as the proton donor. The positive shift of the pK_a value and the high-field shift of the resonance positions observed in Figure 2 are only consistent with the case in which Tyr-93 acts as the proton donor. A similar high pK_a value (11.7) and high-field shift of ring proton resonances have also been observed for Tyr-23 of basic pancreatic trypsin inhibitor (Snyder et al., 1976), which also is considered to form a hydrogen bond with a main chain carbonyl as a proton donor (Huber et al., 1971).

While Tyr-93 is allowed to come in contact with water as discussed above, it is tightly protected from the attack of tetranitromethane. This is not surprising, since the tetranitromethane molecule is considerably larger than the water molecule. On the other hand, on the basis of the crystal data, the solvent (H_2O) accessibility for Tyr-93 has been calculated to be only 5% (Satow et al., 1980), whereas the solvent perturbation experiments show that it is 100% accessible to methanol and ethylene glycol (Inouye et al., 1977). It is to be noted that the continuity of the NMR titration curve of the 3 and 5 protons of Tyr-93 (Figure 2) will be realized, if the exchange between the protonated (HA) and the deprotonated (A^-) forms of Tyr-93 is rapid enough to average out the two resonance positions (ν_{AH} and ν_{A^-}) into a single resonance position. Specifically, in the reaction

$$AH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A^- + H^+ \qquad (1)$$

where k_1 and k_{-1} represent monomolecular rate constants with respect to AH and A^- species, the inequality must hold at the midpoint of pH titration (Carrington & McLachlan, 1967)

$$k_1 = k_{-1} > \frac{2\pi|\nu_{AH} - \nu_{A^-}|}{2\sqrt{2}} \simeq 170\ s^{-1} \qquad (2)$$

in which we have used $|\nu_{AH} - \nu_{A^-}| \approx 76$ Hz from Figure 2. The above inequality requires that there should, at least, be a water channel to the OH group of Tyr-93. However, it does not necessarily require that the whole ring of Tyr 93 is well exposed to the solvent.

The result of X-ray analysis has shown that Tyr-93 lies in a narrow cleft surrounded by the side chains of Leu-60, Asn-61, and Arg-95 (Satow et al., 1980). It appears quite likely that this narrow cleft becomes a water channel to Tyr-93, particularly in view of the possibility that the water channel can be a dynamic one in solution, opening and closing as the side chains of Arg-95 and Leu-60 fluctuate. The discrepancy in the solvent accessibility between the crystal data and the solvent perturbation data may also originate partly from this effect.

Tyr-7 is most easily nitrated, and its pK_a value is most normal among the three tyrosyl residues (pK_a = 10.95 at 25 °C in ^2H_2O). Moreover, the sharp doublet signals of the 3 and 5 protons and of the 2 and 6 protons of Tyr-7 (see Figure 1) indicate that at 25 °C, Tyr-7 is allowed to rotate rapidly about its C_β–C_γ bond (Wüthrich & Wagner, 1975). All the above evidence points to the notion that Tyr-7 is little involved in interresidue interaction. In the crystal, the phenol ring of Tyr-7 is found mostly exposed, but one side of it is partially covered by Pro-9. Its solvent accessibility is predicted to be 70%. On the other hand, the X-ray analysis also shows that the electron clouds of six amino acid residues from the N terminal are scarcely traceable (Mitsui et al., 1979a), i.e., the part involving at least these six residues is disordered in the crystal. Therefore, it appears reasonable that, in solution, Tyr-7 next to these six residues is allowed internal rotation and exists in the most unrestricted microenvironment among the three tyrosyl residues.

Finally, we discuss Tyr-75 which lies near the reactive site (Met-73–Val-74). Tyr-75 has a markedly high pK_a value (11.8 at 25 °C) and is more slowly modified with tetranitromethane than Tyr-7, at least below room temperature. These results strongly suggest that Tyr-75 is involved in some kind of interresidue interaction in solution. The difference between its pK_a value at 25 °C and that at 50 °C (0.6 pH unit) is also too large to be explained only by the ΔH of ionization of the phenol group of a free tyrosine (~6200 cal/mol, corresponding to only half of the ΔH observed) (Greenstein & Winitz, 1961). This result also shows that Tyr-75 is involved in an interresidue interaction at 25 °C, which is slightly weakened at 50 °C. Unlike Tyr-7, the ring proton resonances of Tyr-75 are broad at 25 °C, suggesting that the rotation about the C_β–C_γ is hindered in Tyr-75 due to interresidue interaction. On the other hand, the X-ray analysis shows that in the crystal Tyr-75 is almost completely exposed (solvent accessibility 84%), having no apparent interaction with other residues. From these results, we conclude that the microenvironment of Tyr-75 in solution is considerably more restricted than that predicted from the crystallographic study.

The above discrepancy between the microenvironments of tyrosyl residues of SSI in solid and in solution raises a question as to the validity of the naive and straightforward extension of crystallographic data to the solution conformation of a protein. NMR spectroscopy has been used to supplement and complete the conformations of biopolymers presented by X-ray crystallography. A number of unpublished results of ours on the conformation of SSI and many other works on other proteins (basic pancreatic trypsin inhibitor, lysozyme) (Wüthrich et al., 1977; Perkins & Dwek, 1980) have, in most cases, shown that the conformations of proteins in solution and in crystal are nearly identical, as far as the core region of the protein is concerned. Inspired by the present work, we are led to the conclusion that, although the conformation of the rigid core of the protein may remain nearly identical both in solution and in the crystal owing to a cooperative interplay of hydrogen bonding, ionic interaction, and hydrophobic bonding, the microenvironments of the residues located on the surface region of the protein may well differ between the solution and the crystal, as is the case for Tyr-75 of SSI.

Acknowledgments

We are greatly indebted to Professor S. Murao of the University of Osaka Prefecture and Professor K. Hiromi of Kyoto University for the promotion of the research project on SSI. K.A. thanks Professor K. H. Hausser and the members of the Department of Molecular Physics, the Max Planck Institute for Medical Research (Heidelberg), for their kind cooperation during his visit under the sponsorship of an Alexander-von-Humboldt fellowship (1977–1978). Thanks are also due to Professor T. Yamano of Osaka University for allowing us to use the Varian XL-200 spectrometer and to

Professor W. Hengstenberg and Dr. U. Schmidt-Aderjan of M.P.I. for their cooperation with the nitration of tyrosine.

References

Akasaka, K. (1978) in *Proceedings of the European Conference on NMR of Macromolecules* (Conti, F., Ed.) pp 475–483, Lerici, Rome.

Campbell, I. D., Dobson, C. M., Williams, R. J. P., & Xavier, A. V. (1973) *J. Magn. Reson. 11*, 172–181.

Carrington, A., & McLachlan, A. D. (1967) in *Introduction to Magnetic Resonance*, Chapter 12, Harper and Row, New York.

Fujii, S., Akasaka, K., & Hatano, H. (1980) *J. Biochem. (Tokyo) 88*, 789–796.

Greenstein, J. P., & Winitz, M. (1961) in *Chemistry of the Amino Acid*, Vol. 1, pp 492–494, Wiley, New York.

Hayashi, K., Shimoda, T., Imoto, T., & Funatsu, M. (1968) *J. Biochem. (Tokyo) 64*, 365–370.

Hirono, S., Nakamura, K. T., Iitaka, Y., & Mitsui, Y. (1979) *J. Mol. Biol. 131*, 855–869.

Huber, R., Kukla, D., Rühlmann, A., & Steigemann, W. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 141–151.

Ikenaka, T., Odani, S., Sakai, M., Nabeshima, Y., Sato, S., & Murao, S. (1974) *J. Biochem. (Tokyo) 76*, 1191–1210.

Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972) *Enzymes, 3rd Ed. 7*, 665–686.

Inouye, K., Tonomura, B., Hiromi, K., Sato, S., & Murao, S. (1977) *J. Biochem. (Tokyo) 82*, 1207–1215.

Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379–400.

Mitsui, Y., Satow, Y., Sakamaki, T., & Iitaka, Y. (1977) *J. Biochem. (Tokyo) 82*, 295–298.

Mitsui, Y., Satow, Y., Watanabe, Y., & Iitaka, Y. (1979a) *J. Mol. Biol. 131*, 697–724.

Mitsui, Y., Satow, Y., Watanabe, Y., Hirono, S., & Iitaka, Y. (1979b) *Nature (London) 277*, 447–452.

Murao, S., & Sato, S. (1972) *Agric. Biol. Chem. 36*, 160–163.

Perkins, S. J., & Dwek, R. A. (1980) *Biochemistry 19*, 245–258.

Sato, S., & Murao, S. (1973) *Agric. Biol. Chem. 37*, 1067–1074.

Satow, Y., Watanabe, Y., & Mitsui, Y. (1980) *J. Biochem. (Tokyo) 88*, 1739–1755.

Snyder, G. H., Rowan, R. R., III, & Sykes, B. D. (1976) *Biochemistry 15*, 2275–2283.

Tojo, T., Hamaguchi, K., Imanishi, M., & Amano, T. (1966) *J. Biochem. (Tokyo) 60*, 538–542.

Wüthrich, K., & Wagner, G. (1975) *FEBS Lett. 50*, 265–268.

Wüthrich, K., Wagner, G., Richarz, R., & De Marco, A. (1977) in *NMR in Biology* (Dwek, R. A., Campbell, I. D., Richards, R. E., & Williams, R. J. P., Eds.) pp 51–62, Academic Press, New York.

Persistence of Segregated Phospholipid Domains in Phospholipid–Lipopolysaccharide Mixed Bilayers: Studies with Spin-Labeled Phospholipids†

Yutaka Takeuchi‡ and Hiroshi Nikaido*

ABSTRACT: When lipopolysaccharides from *Escherichia coli* B were sonicated together with pure spin-labeled phospholipids without the addition of unlabeled phospholipids, extensive line broadening was observed due to the close proximity of spin-labeled molecules to each other, a result suggesting that spin-labeled phospholipids existed in segregated domains containing few lipopolysaccharide molecules. Such mixed bilayers were incubated under various conditions, including the addition of NaCl and $MgCl_2$ to the medium and the incorporation of the major outer membrane protein, porin, into the bilayer, and the intermixing of the domains was followed by the decrease in line width. The diffusion of the labeled phospholipids into lipopolysaccharide domains was hardly detectable when the mixed bilayer contained spin-labeled phospholipids and lipopolysaccharide in approximately equimolar ratios. Although progressive diffusion was observed when a 17-fold molar excess of lipopolysaccharide was present, it was very slow even under the optimal conditions, usually requiring several days for a nearly complete mixing. In another series of experiments, spin-labeled phospholipids were diluted with a 100-fold excess of unlabeled phospholipids and then mixed with lipopolysaccharides. In these experiments, the fluidity of the domains containing spin-labeled phospholipids was shown to be identical, even after 3 days of incubation, with the fluidity of bilayers containing only phospholipids, in contrast to the expectation of the diminished fluidity if phospholipid molecules became finely interspersed with lipopolysaccharide molecules. These two different lines of approach therefore supported the idea that phospholipid (and most probably lipopolysaccharide) domains in mixed bilayers tend to be rather stable and persist for long periods of time.

The outer membrane of Gram-negative bacteria contains two classes of amphiphilic lipids, phospholipids and lipopolysaccharides [for a review, see Nikaido & Nakae (1979)]. In *Escherichia coli* and *Salmonella typhimurium*, about 80% of the phospholipids are phosphatidylethanolamine (PE),[1] and

† From the Department of Microbiology and Immunology, University of California, Berkeley, California 94720. *Received July 14, 1980.* This work was supported by grants from the National Institutes of Health (AI-09644) and American Cancer Society (BC-20).

‡ Fellow in Cancer Research supported by Grant DRG 321-FT of the Damon Runyon-Walter Winchell Cancer Fund. Present address: Department of Medical Chemistry, Kansai Medical School, Moriguchi-shi, Osaka, Japan.

[1] Abbreviations used: LPS, lipopolysaccharide; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PE*, spin-labeled PE; PG*, spin-labeled PG; 5- (or 12-) nitroxide stearate, 4,4-dimethyloxazolidinyl-*N*-oxy derivative of 5- (or 12-) ketostearic acid; ESR, electron spin resonance; Hepes, *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

0006-2960/81/0420-0523$01.00/0