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Second Derivative Spectral Properties of Tryptophan and Tyrosine Residues in Proteins. Effects of Guanidine Hydrochloride and Dodecyl Sulfate on the Residues in Lysozyme, Ribonuclease and Serum Albumin

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The 2nd derivative spectra of tryptophan and tyrosine, and of ribonuclease (as a tyrosine-rich protein) and lysozyme (as a tryptophan-rich protein) were recorded under various conditions. On the basis of these spectra, the spectral bands of tryptophan and tyrosine residues in proteins were analyzed. Furthermore, the effects of the anionic surfactant sodium dodecyl sulfate on the environment around tryptophan and tyrosine residues in bovine serum albumin were studied.

Keywords——derivative spectrophotometry; tyrosine residue; tryptophan residue; ribonuclease; lysozyme; serum albumin; sodium dodecyl sulfate; guanidine hydrochloride

Spectrophotometry is very useful for analysis of chemical compounds, because it eliminates the effect of contaminants, which sometimes disturb specific reactions between the test compounds and chemical reagents added for color reactions in chemical analysis. However, in spectrophotometry, the spectral bands of test compounds should be clearly distinguished from those of contaminants. We have shown that derivative spectrophotometry is useful for the analysis of aromatic amino acids, such as phenylalanine, tryptophan and tyrosine; in the alkaline region, derivative spectral bands of these three amino acids can be separated.[1a)] However, the analysis of these amino acid residues in proteins is more difficult, because the spectral bands are modified by the environments around the chromophores.

Recently we showed that derivative spectrophotometry can be used for analysis of the state and amount of phenylalanine residues in proteins.[1)] The second derivative spectral bands of phenylalanine are observed between 240 and 270 nm, where there is no absorption due to tryptophan or tyrosine at neutral pH. Furthermore, above 270 nm phenylalanine has no influence on the spectra of the latter two aromatic amino acids. Thus, the spectral bands due to phenylalanine residues are completely separated from those of other aromatic amino acids, though the absorption of phenylalanine residues in the normal absorption spectrum is very weak and is masked by the strong absorptions of tryptophan and tyrosine residues.

In contrast, analysis of spectral bands due to tryptophan and tyrosine residues in proteins is complex, because of the overlapping of absorptions. There has been no fundamental study on this problem, although the characterization of these bands under certain conditions has been attempted.[1a,2,3)]

In this study we measured the second drivative spectra of the amino acids tryptophan and tyrosine above 270 nm under various conditions, and compared them with the spectra of proteins containing large amounts of either tryptophan or tyrosine. We used lysozyme and ribonuclease as tryptophan-rich and tyrosine-rich proteins, respectively. On the basis of the observed spectral properties, we analyzed the derivative spectra of serum albumin under

various conditions.

Experimental

N-Acetyl ethyl esters of L-tryptophan and L-tyrosine were from Sigma Chemical Co., St. Louis (U.S.A.), and Tokyo Kasei Kogyo, Tokyo (Japan), respectively. Lysozyme (egg white, crystallized) and ribonuclease (bovine pancreas, crystalline) were obtained from P-L Biochemicals Inc., Milwaukee (U.S.A.), and serum albumin (bovine, crystallized and lyophilized) was from Sigma Chemical Co. Sodium dodecyl sulfate (SDS) and guanidine hydrochloride were specially prepared reagents for protein research from Wako Chemical Industries, Osaka (Japan). These compounds were dissolved in 0.1 M sodium phosphate buffer, pH 7.0, unless otherwise indicated. Concentrations of amino acids and proteins were determined spectrophotometrically as reported previously.[1a,b]

Absorption spectra were recorded with a Shimadzu spectrophotometer, model UV-300. Derivative absorption spectra were recorded with a derivative attachment, model DES-1, connected to the spectrophotometer. The derivative wavelength difference, $\Delta\lambda$, was 1 nm.

Results and Discussion

Spectral Properties of Tryptophan and Tyrosine

Figure 1 shows the 2nd derivative spectra of the *N*-acetyl ethyl ester of tryptophan in phosphate buffer, pH 7.0, and in *n*-propanol between 270 and 320 nm. In phosphate buffer, there are 3 peaks and 3 troughs, which are referred to as P-1 to P-3, and T-1 to T-3, respectively, in order from longer to shorter wavelengths: the peaks are at 293.0 (P-1), 285.5 (P-2) and 274.0 nm (P-3), and the troughs at 288.5 (T-1), 281.5 (T-2) and 271.5 nm (T-3). The spectrum in *n*-propanol is characterized by shifts of the positions of peaks and troughs by 1.0 to 2.0 nm to higher wavelengths and increases in the absorbance relative to the spectrum in phosphate buffer.

Figure 2 shows the 2nd derivative spectra of the *N*-acetyl ethyl ester of tyrosine in phosphate buffer, pH 7.0, and in *n*-propanol. The spectrum in phosphate buffer shows 2 main

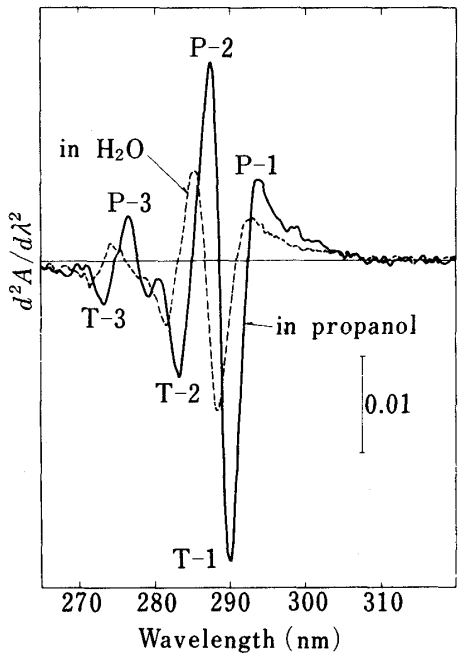

Fig. 1. 2nd Derivative Spectra of the *N*-Acetyl Ethyl Ester of Tryptophan in Phosphate Buffer, pH 7.0 (Dotted Line) and in *n*-Propanol (Solid Line)

Concentration of tryptophan: 9.01×10^{-5} M.

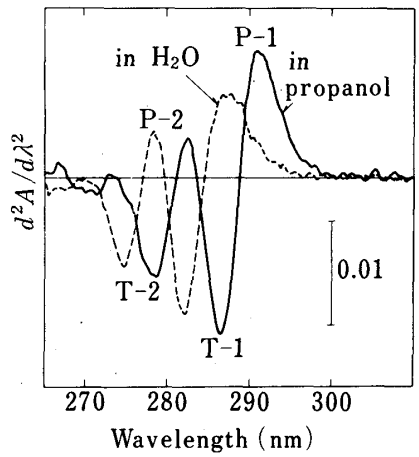

Fig. 2. 2nd Derivative Spectra of the *N*-Acetyl Ethyl Ester of Tyrosine in Phosphate Buffer, pH 7.0 (Dotted Line), and in *n*-Propanol (Solid Line)

Concentration of tyrosine: 3.79×10^{-4} M.

peaks at 287.5 (P-1) and 278.5 nm (P-2), and 2 troughs at 282.0 (T-1) and 274.5 nm (T-2). However, some of these positive and negative peaks consist of doublet or triplet bands, which are not observed in the spectrum of tryptophan. The positions described above are those of the main bands. As shown in Fig. 2, the spectrum of tyrosine in *n*-propanol is shifted by 3.5—4.5 nm to higher wavelengths than that in water. P-1 and P-2 are at 291.0 and 282.5 nm, and T-1 and T-2, at 286.5 and 279.0 nm, respectively. It is noteworthy that the change in the optical intensities with change from a polar to an apolar environment is not great, in contrast to the case of tryptophan.

Records of the spectra of these two amino acids at various molar ratios of water to *n*-propanol showed that all the peaks moved to higher wavelengths with increase in the proportion of *n*-propanol. Similar tendencies were observed when the spectra were recorded in chloroform and *n*-hexane (data not shown). Thus, changes in the environment of the amino acids from an apolar to a polar aqueous region can be monitored by measuring the changes in the positions and absorbances of the spectral bands in the 2nd derivative spectra: a blue-shift with decrease in the optical intensity for tryptophan, and a large blue-shift with only a slight change in the absorbance for tyrosine.

In the normal spectrum, the molar absorption coefficient at the λ_{max} of tryptophan is about 4 times greater than that of tyrosine[4]: ε of tryptophan at 279.9 nm is 5550 and that of tyrosine at 274.5 nm is 1420. As shown in Figs. 1 and 2, this relation also holds for the 2nd derivative spectra of these amino acids in water, since the curvatures are similar to those of their normal spectra.

Derivative Spectra of Ribonuclease and Lysozyme

It was expected that the derivative spectra of tryptophan- and tyrosine-rich proteins would provide useful information about the spectral properties of tryptophan and tyrosine residues in proteins. Figure 3 shows the 2nd derivative spectra of ribonuclease in phosphate buffer, pH 7.0 in 6 M guanidine hydrochloride, and in a solution of 1.80×10^{-2} M SDS. Ribonuclease contains 6 tyrosine residues, but no tryptophan residues.[5] The spectrum in phosphate buffer, pH 7.0, shows peaks at about 290.5 (P-1) and about 281.5 nm (P-2), and troughs at 285.5 (T-1) and 277.0 nm (T-2). Some peaks and troughs consist of triplet bands, as

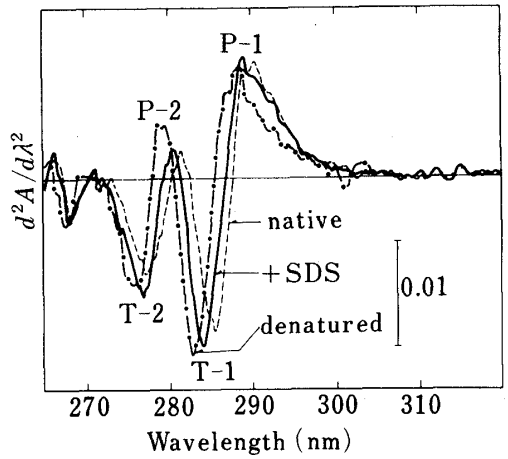

Fig. 3. 2nd Derivative Spectra of Ribonuclease under Various Conditions

Dotted line: in phosphate buffer, pH 7.0.
Chained line: in 6 M guanidine hydrochloride.
Solid line: in 1.80×10^{-2} M SDS ($D/P = 165$).
Concentration of ribonuclease: 1.09×10^{-4} M.

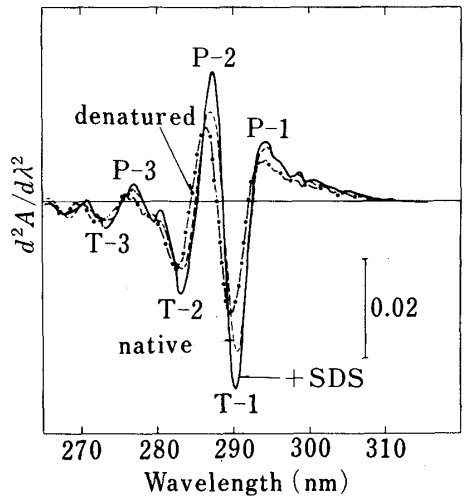

Fig. 4. 2nd Derivative Spectra of Lysozyme under Various Conditions

Dotted line: in phosphate buffer, pH 7.0.
Chained line: in 6 M guanidine hydrochloride.
Solid line: in 4.5×10^{-3} M SDS ($D/P = 165$).
Concentration of lysozyme: 2.73×10^{-5} M.

observed in the spectrum of tyrosine, and their positions are very similar (at about 1 nm lower wavelengths) to those of the respective bands of tyrosine in *n*-propanol. On denaturation of ribonuclease with 6 M guanidine hydrochloride, all the spectral bands shifted by 1.5—2.5 nm to lower wavelengths without appreciable change in their absorbances: P-1 to 288.5 nm, P-2 to 279.5 nm, T-1 to 283.0 nm and T-2 to 275.5 nm. These positions are very similar to those of tyrosine in water. Thus, conversion of the tyrosine residue to a polar environment causes a blue-shift with only a slight change in the absorbance of the spectrum. Similar spectral properties are observed with a solution of ribonuclease in the presence of 1.80×10^{-2} M SDS, corresponding to a molar ratio of surfactant (D) to protein (P), D/P, of about 165. However, in this case the blue-shift was smaller (about 1 nm) than that on denaturation of ribonuclease.

Since ribonuclease contains 6 tyrosine residues but no tryptophan residues, the average state of tyrosine residues can be determined by comparison of its spectra with those of the amino acid tyrosine under various conditions. From the fact that the positions of peaks and troughs in native ribonuclease are consistently at about 1 nm lower wavelength than those of tyrosine in *n*-propanol, it is concluded that the tyrosine residues in native ribonuclease are located, on average, in regions where the hydrophobicity is a little less than that of *n*-propanol. This must be related to the fact that 3 of the 6 tyrosine residues are located inside the protein molecule.[6] Since the positions of the peaks and troughs of denatured ribonuclease are very similar to those of tyrosine in water, all the tyrosine residues are concluded to be exposed almost completely by denaturation. In the presence of a high concentration of SDS ($D/P=165$), the tyrosine residues in ribonuclease are only partly exposed, since the degree of the blue-shift is less than that on denaturation of ribonuclease with guanidine hydrochloride.

Figure 4 shows the spectra of lysozyme in the native state, after denaturation by guanidine hydrochloride, and in the presence of SDS at $D/P=165$. The spectrum of native lysozyme is very similar to that of tryptophan in *n*-propanol, *i.e.*, 294.5 (P-1), 290.5 (T-1), 287.5 (P-2), 283.5 (T-2), 277.0 (P-3) and 273.5 nm (T-3). Though lysozyme contains 3 tyrosine residues as well as 6 tryptophan residues,[7] its spectral properties are very similar to those of tryptophan (*cf.* Fig. 1), and the spectral bands of tyrosine residues are masked completely by the strong absorbances of tryptophan residues. Thus, the spectrum of lysozyme above 270 nm is regarded as reflecting that of tryptophan residues.

All the peaks and troughs in the spectrum of lysozyme in 6 M guanidine hydrochloride are smaller than those observed in the spectrum of native lysozyme, and show a slight blue-shift (0.5—1.0 nm). These spectral changes are characteristics of those of tryptophan residues transferred to a polar environment from an apolar environment, as observed with the spectra of tryptophan shown in Fig. 1. On the other hand, addition of SDS to a solution of native lysozyme caused increases in all peaks and troughs but with little change in the positions. Thus, on addition of a high concentration of SDS ($D/P=165$), tryptophan residues in lysozyme are concluded to be transferred to a more hydrophobic environment than that in native lysozyme. Probably they become involved in micelle-like clusters of SDS-lysozyme complexes, unlike tyrosine residues in ribonuclease. The tryptophan residues in lysozyme may constitute part of the binding sites for SDS.

Spectral Properties of Bovine Serum Albumin

Figure 5 shows the 2nd derivative spectra of native and denatured bovine serum albumin. This protein contains 2 tryptophan and 19 tyrosine residues.[8] The spectrum of native albumin has peaks at 293.0 (P-1), 289.0 (P-2), 281.5 (P-3) and 271.0 nm (P-4), and troughs at 289.5 (T-1), 285.0 (T-2) and 277.5 nm (T-3). From the spectra of lysozyme and ribonuclease, the residues responsible for the spectral bands of native serum albumin are deduced to be as follows: P-1, P-2 and P-4 are due to a composite of tryptophan and tyrosine; T-1 may correspond to T-1 of tryptophan, but tyrosine has a considerable positive absorption at this

wavelength; T-2, P-3 and T-3 are mainly due to tyrosine. On denaturation with guanidine hydrochloride, all the spectral bands except P-1 and T-1 showed a blue-shift of about 2 nm. The increases in height of P-2 and depth of T-2 on denaturation were particularly marked. Changes in the optical properties around P-2 and T-2 are concluded to be due mainly to the blue-shifts of P-1 and T-1 in the spectrum of tyrosine residues (*cf.* Figs. 2 and 3) caused by their exposure. However, these spectral bands of tyrosine residues overlap those of tryptophan (P-2 and T-2 of tryptophan, *cf.* Figs. 1 and 4), since the blue-shift associated with denaturation is large with tyrosine residues, but small with tryptophan residues.

Furthermore, both the optical intensity and the position of T-1, where there is a trough in the spectrum of tryptophan residues but considerable positive absorption of tyrosine residues, are little changed on denaturation. This is because the negative absorbance due to tryptophan and positive absorbance of tyrosine tend to change toward the base line, with little change in the positions of their peaks, as observed with the spectra of ribonuclease and lysozyme. Decrease in absorbance at about 293 nm (P-1) is due mainly to the exposure of tyrosine residues, since the absorbance of tyrosine residues in ribonuclease at about this wavelength decreases greatly on denaturation (*cf.* Fig. 3), but the change in absorbance of tryptophan residues in lysozyme on denaturation is not great (*cf.* Fig. 4).

The spectral bands of tyrosine and tryptophan residues are not separated clearly when both residues are exposed, as is the case upon denaturation. However, the total exposure of tryptophan and tyrosine residues can be monitored when i) there is little change in the optical properties of T-1, ii) blue-shift and increase in the intensities of P-2 and T-2 occur, and iii) decrease in the absorbance at P-1 occurs. The blue-shifts and associated increases in P-2 and T-2 are a sensitive index of exposure of these two aromatic amino acid residues, especially tyrosine residues.

Figure 6 shows the 2nd derivative spectrum of bovine serum albumin in the presence of SDS at the molar ratio of the detergent to albumin of 49 ($D/P=49$). This spectrum differs greatly from those of native albumin and guanidine hydrochloride-denatured albumin. In the presence of SDS, the spectrum is characterized by a red-shift and increase in the absorbance of P-1, and intensification of T-1, P-2, T-2 and T-3 in comparison with the spectrum of native

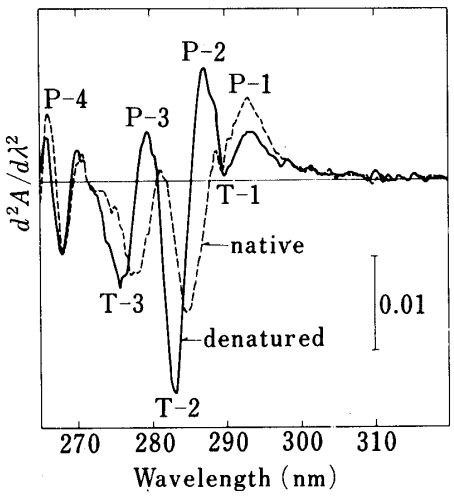

Fig. 5. 2nd Derivative Spectra of Native and Denatured Bovine Serum Albumin

Dotted line: in phosphate buffer, pH 7.0.
Solid line: in 6 M guanidine hydrochloride.
Concentration of bovine serum albumin: 2.34×10^{-5} M.

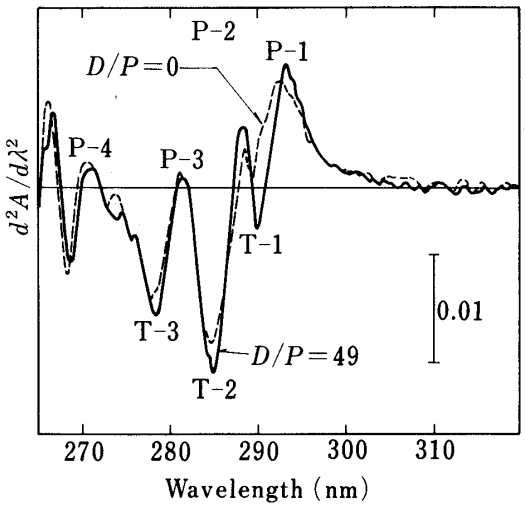

Fig. 6. Effect of SDS on the 2nd Derivative Spectrum of Bovine Serum Albumin at Various Molar Ratios of SDS to Albumin (D/P)

Dotted lines: $D/P=0$ (native albumin).
Solid line: $D/P=49$.
Concentration of bovine serum albumin: 2.68×10^{-5} M.

albumin. The change in absorbance at T-1 is especially marked. This may be due mainly to the involvement of tryptophan residues in the apolar region. This view is supported by the increase in optical intensity with red-shift of P-1 and T-1, since the absorption intensities of the amino acid tryptophan at P-1 and T-1 become large upon its transfer from water to *n*-propanol, as shown in Fig. 1. The increase in depth of T-2 with a slight red-shift is a reflection of an increase in hydrophobicity in the environment of tryptophan residues.

However, SDS does not seem to affect the state of tyrosine residues greatly. SDS is suggested to cause slight exposure of tyrosine residues, since there is only a small increase in absorbance and a slight blue-shift of P-2, and only a small change in the spectral properties of P-3. Such a slight exposure was also found by Aoki *et al.* by laser Raman spectrometry.[9] According to our preliminary study, the effect of SDS on the state of these two residues is complex and dependent on the concentration of SDS. Quantitative analysis of the effect of SDS will be the subject of further study.

In this study we showed that the spectral properties of the tyrosine-rich protein ribonuclease and the tryptophan-rich protein lysozyme are essentially the same as those of tyrosine and tryptophan in a hydrophobic environment, respectively. Thus, the state of these amino acid residues under various conditions can be assigned from the spectra of tyrosine and tryptophan.

With proteins which contain spectrophotometrically corresponding amounts of these two amino acid residues, such as serum albumin, the analysis was not always successful, especially with denatured protein at pH 7. On denaturation, the spectral bands characteristic of tyrosine and tryptophan residues changed and overlapped. When the anionic detergent SDS was added to serum albumin, the changes of environment around tyrosine and tryptophan residues could be monitored, because SDS has been suggested to cause different effects on these amino acid residues.

The results presented here suggest that derivative spectrophotometry can be used for the determination of aromatic amino acid residues in proteins. For this purpose, all the aromatic amino acid residues should be in the same environment. Thus the study on the relationship between the spectral properties and the environments around aromatic amino acid residues is important. The results in this study provide a starting point to understanding this relationship. Further study on the quantitative analysis of amino acid residues in proteins by derivative spectrophotometry is in progress.

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References

1) *a*) T. Ichikawa and H. Terada, *Chem. Pharm. Bull.*, **29**, 438 (1981); *b*) *Idem, Biochim. Biophys. Acta*, **494**, 267 (1977); *c*) *Idem, ibid.*, **580**, 120 (1979); *d*) *Idem, ibid.*, **671**, 33 (1981).
2) C. Balestrierie, G. Colonna, A. Giovane, G. Irace, and L. Servillo, *Eur. J. Biochem.*, **90**, 433 (1978).
3) C. Balestrierie, G. Colonna, A. Giovane, G. Irace, and L. Servillo, *Anal. Biochem.*, **54**, 370 (1980).
4) J. N. Solli and T. T. Herskovitz, *Anal. Biochem.*, **54**, 370 (1973).
5) D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 227 (1963).
6) C. Tanford, J. D. Hauenstein, and D. Rands, *J. Am. Chem. Soc.*, **77**, 6409 (1956).
7) R. E. Canfield, *J. Biol. Chem.*, **238**, 2698 (1963).
8) J. R. Brown, "Albumin Structure, Function and Uses," ed. by V. M. Rosenoer, M. Oratz, and M. A. Rothschild, Pergamon Press, Oxford, 1977, p. 27.
9) K. Aoki, H. Okabayashi, S. Maezawa, T. Mizuno, M. Murata, and K. Hiramatsu, *Biochim. Biophys. Acta*, **703**, 11 (1982).