Study of ionization of tyrosine residues in proteins by second-derivative UV spectroscopy

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Four spectrally different forms of tyrosine residues were shown to be present in proteins, namely, nonionized residues, either buried or exposed to solvent, and ionized residues buried or exposed to solvent. A method for determining the pK_a values of the tyrosine residues in proteins was proposed. It is based on the decrease in the absorption intensity in the second derivative of the UV spectrum at 284.2 nm, which is the wavelength of the isobestic point corresponding to the transition of the nonionized tyrosine residues from the buried to the exposed state. Several proteins were studied by this method; the results obtained were found to be close to the corresponding published data. This method is simpler than the conventional UV titration.

Key words: ionization of tyrosine in proteins, UV absorption spectra, second derivative.

Information on the dissociation constants of tyrosine residues in proteins is important for the investigation of the microenvironment of these groups and the structure of the protein as a whole in solution. These constants are normally found from the variation of the intensity of the absorption of ionized tyrosine residues in proteins as a function of the pH at a wavelength of 293—295 nm, *i.e.*, near the absorption maximum of these groups, which is shifted 8—10 nm to longer wavelengths with respect to the absorption maximum of the nonionized residues.[1] In some cases, NMR spectroscopy is used; this makes it possible to determine pK_a of the tyrosine groups in a protein from the dependence of the chemical shift of the carbon atoms in the aromatic ring on the pH of the medium.[2]

Recently, even derivatives of the absorption intensity with respect to wavelength have been widely used to interpret spectral data. Their advantage over the ordinary spectrum is that the half-widths of peaks are much smaller. This makes it possible to distinguish peaks that overlap in the ordinary spectrum. For example, the use of the second and fourth derivatives in the studies of the UV spectra of proteins makes it possible to distinguish the absorption peaks of individual aromatic amino acids. The minimum in the second derivative of the spectrum and the maximum in the fourth derivative coincide in wavelength with a maximum or a shoulder in the initial spectrum, respectively.[3—6]

We assumed that, owing to the high resolution of peaks, the even derivatives of the spectrum would also be convenient for the investigation of the ionization of tyrosine residues in proteins. In this study, we used the second derivative of the UV spectrum (SDUS), since in higher-order derivatives, peaks of ionized tyrosine residues cannot be distinguished from the background.[5] In the fourth derivative of the spectrum and in higher-order derivatives, the intensity of the peaks of ionized tyrosine residues is negligibly small due to the large half-widths of these peaks in the initial spectrum ($d^nA/d\lambda^n = K \cdot A/\Gamma^n$, where Γ is the half-width of a peak in the spectrum and K is a constant).

In the present study, we are proposing and using a method for investigating the ionization of tyrosine residues in proteins. The method is based on monitoring the decrease in the intensity of the second derivative of the spectrum of a protein at the wavelength of the isobestic point corresponding to the transition between buried and exposed nonionized tyrosine residues (284.2 nm).

Experimental

The following proteins and reagents were used: porcine insulin from Eli Lilly (USA), α-chymotrypsin, Carlsberg subtilisin, and bis-tris-propane from Sigma (USA), subtilisin 72 from the Moscow Plant of Enzymic Preparations, α-chymotrypsinogen from BDH (Great Britain), bovine serum albumin and the ethyl ester of *N*-acetyl-L-tyrosine from Reanal (Hungary). Ribonuclease A, homogeneous according to SDS-electrophoresis, was kindly provided by G. N. Rudenskaya.

The spectra of the proteins and their second derivatives were obtained on a Shimadzu 265 FW spectrophotometer (Japan). The measurements were carried out in the 275—300 nm range of wavelengths with a slit width of 1 nm and at the "slow" rate of scanning. The spectrum was differentiated by a built-in microprocessor with a step of 1 nm. The concentration of the protein in the cell with an optical length of 10 mm varied between 0.4 and 2 mg mL^{-1}.

Alkaline denaturation of albumin and Carlsberg subtilisin was carried out at pH 12 and at a concentration of the protein of 1 mg mL^{-1}.

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Titration was carried out in a 0.05 *M* borate (pH 9.0—11.0) or borate-alkaline (pH 11.5—13.0) buffer solution. To determine abnormally low pK_a (pH 7.0—9.5), the 0.05 *M* bis-tris-propane buffer was used. The pH of the reaction mixture was changed by adding 1 *N* NaOH. To be sure that the nature of the buffer solution had no effect on the determination of pK_a values, tyrosine residues in several proteins were titrated in the pH range of 9—9.5 both in borate and in bis-tris-propane buffers.

A typical experiment was carried out in the following way: 4 mg of a protein was dissolved in 4 mL of a 0.05 *M* buffer solution (bis-tris propane, pH 6.3, or borate, pH 9.1). After measuring the pH value of the resulting solution, a 1 mL aliquot was withdrawn, and the UV spectrum of the protein in the 275—300 nm range was recorded; the second derivative of the spectrum was obtained, and its intensity at a wavelength of 284.2 nm was determined. After the measurements, the aliquot was returned to the initial solution. Then, to increase the pH of the solution, 10—20 mL of 1 *N* NaOH was added to it with stirring; after that, a 1 mL aliquot was withdrawn again to determine the spectral changes, and then the next step of titration was carried out. The titration experiment was repeated 4 or 5 times.

The change in the pH of a buffer solution depends linearly on the volume of alkali added. Therefore, we considered that the dilution during titration and the change in the pH are related to each other by the following formula: $A(\%) = A_0 - a(\mathrm{pH} - \mathrm{pH}_0)$, where $a = (0.5—1)\%$ is the dilution coefficient determined experimentally, A_0 is the percentage of ionized tyrosine residues in the protein without allowance for dilution, A is the same value with allowance for dilution, and pH_0 is the pH value at which the titration was started. All the titration curves presented in this paper were constructed with allowance for dilution.

When working with solutions with pH > 11, the initial solution of the protein was replaced by another one every 10—15 min or more frequently, to prevent denaturation from affecting the results of measurements. In each particular case, the time interval in which denaturation could be neglected was determined. Spectral changes corresponding to the transition of ionized tyrosine residues from the buried to the exposed form were regarded as the criterion for the occurrence of denaturation processes. The experiments (see below) showed that at pH < 11.8, substantial spectral changes (more than 3% of the overall intensity of the absorption of tyrosine in the protein) in the proteins under consideration start after 30 minutes of incubation.

The Sigma Plot program package (Jandel Scientific) was used to construct titration curves and to take dilution into account.

Results and Discussion

The absorption maxima of aromatic amino acids are known[3,5] to shift to longer wavelengths by 2—5 nm as the hydrophobicity of the environment increases. Previously, we found[3] that the transition between the buried and exposed forms of nonionized tyrosine residues is characterized by an isobestic point in the SDUS (the wavelength at the minimum of the SDUS for exposed tyrosine residues is 282.3 nm, that for buried tyrosine residues is 286.1 nm, and the wavelength of the isobestic point is 284.2 nm).

The definition of the notions "buried" and "exposed" groups is somewhat ambiguous. Therefore, it is necessary to explain what we mean by buried and exposed groups. A tyrosine residue whose aromatic system participates in hydrophobic interactions in the protein molecule and is thus eliminated from contacts with the solvent is referred to as buried. Conversely, the aromatic system of an exposed group participates in van der Waals interactions with solvent molecules. The case in point is the accessibility of the benzene ring of tyrosine to the solvent, because it is this ring that determines the position of the absorption band corresponding to this group in the SDUS. From X-ray diffraction data, it is known[7] that the accessibilities of the polar and nonpolar parts of an amino-acid residue in a protein to water molecules can be different.

It should be noted that the presence of the isobestic point in the SDUS of nonionized tyrosine residues in proteins, shown experimentally,[3] indicates that the buried and exposed states of tyrosine residues in a protein are discrete. The wavelength of the isobestic point for this transition was found[3] to be constant for all proteins.

When we studied the SDUS of proteins after a sharp increase in the pH of the medium, we noticed that the absorption maximum of the tyrosine residues not only shifts to longer wavelengths by 5—7 nm following their ionization but also undergoes subsequent changes. For example, during alkaline denaturation of bovine serum albumin, in which 13 of the 19 tyrosine residues are shielded from the solvent, an intense peak with an absorption minimum at 291.1 nm appears in its SDUS as the pH increases to 12.0. As time passes, the absorption minimum shifts to 289.6 nm and its intensity decreases (Fig. 1); an isobestic point at 290.4 nm is observed.

It can be suggested that ionized tyrosine residues, like the nonionized residues, can exist in the buried and exposed forms. The tyrosine residues in which the phe-

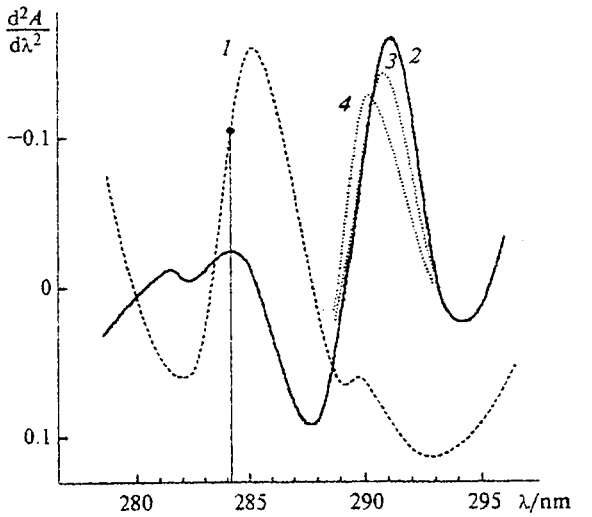

Fig. 1. Changes in the SDUS during alkaline denaturation of bovine serum albumin: pH 7.0 (*1*); immediately after adjustment of the pH to 12.0 (*2*); 40 min (*3*) and 90 min (*4*) at pH 12.

nol oxygen is accessible to the water molecules that occur near the surface of the protein globule, and the benzene ring is shielded from them by other groups, are referred to as buried ionized groups. In exposed residues, the whole group is accessible to water molecules.

The observed spectral changes can be explained by the fact that in the region of high pH values, ionization of the tyrosine residues in albumin occurs in parallel with denaturation of the protein, and dissociation of the tyrosine residues occurs much faster than denaturation of the molecule. When the pH of a solution of the protein increases from 7.0 to 12.0, the buried nonionized tyrosine residues in the albumin molecule are rapidly ionized, together with the exposed residues, and thus pass into the buried ionized state (Fig. 1, curves *1* and *2*). The slow spectral changes observed subsequently correspond to the transition of buried ionized tyrosine residues into the exposed state after alkaline denaturation of the protein (Fig. 1, curves *2* and *4*).

Our study of the alkaline denaturation of model proteins (bovine serum albumin, Carlsberg and 72 subtilisins) afforded the following wavelengths for the SDUS minima for ionized tyrosine residues: exposed groups, 289.6 nm; buried groups, 291.2 nm; and isobestic point, 290.4 nm. In addition, whereas in the case of nonionized tyrosine residues, the intensities of the absorption of the buried and exposed forms in SDUS are identical,[3] in the case of ionized tyrosine residues, absorption of the buried form is more intense than that of the exposed form.

Thus, the results that we obtained in the analysis of the SDUS of proteins in the spectral range of 275—300 nm reflect the fact that tyrosine residues in proteins can exist in solution as four spectrally distinguishable forms, namely, exposed nonionized, buried nonionized, exposed ionized, and buried ionized forms. The presence of isobestic points for the transitions between the buried and exposed forms both for ionized and for nonionized tyrosine residues indicates that there are no intermediate forms. The differences in the degree of accessibility of the tyrosine residues cannot be detected in the ordinary spectrum.

The use of the second derivative of the UV spectrum makes it possible, in principle, to observe the four forms of tyrosine in a protein and to monitor the transitions between them, in particular, ionization of the tyrosine residues, *i.e.*, to construct a titration curve of tyrosine corresponding to the increase in the pH. To ensure that the variation of the ratio of buried to exposed tyrosine residues during titration of the protein has no effect on the results, the spectral changes should be recorded at the wavelengths of the isobestic points. Moreover, titration of the tyrosine residues against the increase in the absorption intensity of the ionized residues at the wavelength of the isobestic point (290.4 nm) that corresponds to the transition between buried and exposed ionized tyrosine residues is not expedient, because this spectral region (288—292 nm) also contains the absorption maximum of tryptophan residues, whose absorption in the SDUS is approximately 3 times more intense than that of tyrosine, which can distort the results of the measurements. Therefore, to monitor the ionization of tyrosine residues, we have chosen the isobestic point that corresponds to the transition between buried and exposed nonionized tyrosine residues (284.2 nm) and recorded the decrease in the intensity of the SDUS of the protein at this wavelength.

We studied the ionization of tyrosine residues in several model proteins. The results are presented in Tables 1 and 2, and the typical titration curves are shown in Fig. 2.

Table 1. pK_a of the tyrosine residues in proteins determined using SDUS

Protein	pK_a	The number of residues in a group with equal pK_a
Carlsberg subtilisin	9.4	2.5
	9.6	1
	10	2.5
	10.5	2
	11	5
Subtilisin 72	9.8	3.5
	10.4	2.5
	10.7	1
	>11	3
Insulin	9.2	0.7
	9.8	0.3
	10.6	2
	>11.6	1
Bovine serum albumin	9.5	2
	9.8	2
	10.5	1
	11.3	7
	11.7	2
	>12.3	5
Ribonuclease A	9.1	1
	9.7	2
	?	3

Table 2. pK_a of the tyrosine residues in proteins determined using difference SDUS

pK_a		The number of residues in a group with this pK_a	
This study	Ref. 13	This study	Ref. 13
α-Chymotrypsinogen			
9.6	9.7	0.5	1
11.5	11.5	0.5	1
>12	13.3	3	2
α-Chymotrypsin			
9.6	—	1	—
11.4	—	0.5	—
>12	—	2.5	—

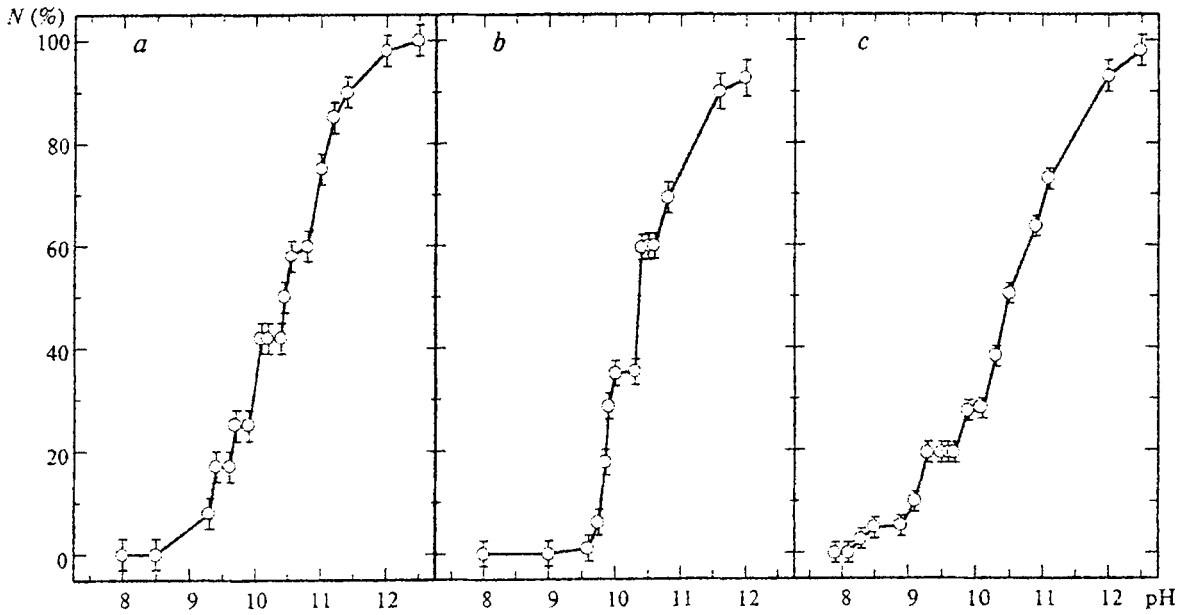

Fig. 2. Titration curves of the tyrosine residues in proteins obtained by the SDUS method: Carlsberg subtilisin (*a*); subtilisin 72 (*b*); insulin (*c*); *N* is the number of ionized tyrosine residues.

For example, it can be seen that the pK_a values obtained for the tyrosine residues in Carlsberg subtilisin (Table 1 and Fig. 2) are comparable with those reported in the literature[8] (pK_a 9.9 for six residues, 11.6 for four residues, and >12 for three residues) with the difference that conventional methods do not normally make it possible to observe the ionization of individual tyrosine residues, and the application of simulation techniques to experimental titration curves makes it possible to estimate the average pK_a values for groups consisting of three or four residues. Our method permits determination of the pK_a values and the numbers of ionizing residues directly from the experimental curve and requires no mathematical simulation.

Particular attention is attracted to the relatively low pK_a value of 9.4 that we obtained for one of the groups in Carlsberg subtilisin. According to the X-ray diffraction data for this protein,[9] one of the tyrosine residues in it is bound to a calcium ion, which results in a decrease in the electron density on the oxygen atom of the phenolic hydroxyl in tyrosine and, hence, should decrease its pK_a.

The pK_a value of 9.2 found for insulin, which is abnormally low for tyrosine, might be due to the fact that, according to X-ray diffraction analysis, one of the tyrosine residues is bound to a zinc ion[10] (similarly to the situation observed for Carlsberg subtilisin). The resolving power of our method makes it possible to determine pK_a for individual residues, whereas earlier it was found[11] that all four tyrosine residues are characterized by pK_a values of ~9.9. The non-integer values found for the numbers of the residues in groups might be due either to the participation of tyrosine residues belonging to the same group in several donor-acceptor interactions in the protein molecule or to the existence of various conformations of the protein in the solution. Similar non-integer values for the number of residues have also been obtained before.[2]

The pK_a values for the tyrosine residues in ribonuclease A that we found (pK_a 9.1 for one residue and 9.7 for two residues) are somewhat lower than those reported in the literature;[12] according to the published results, three tyrosine residues in this protein have pK_a 9.9. It should be noted that we were not able to determine the exact pK_a values for the last three tyrosine residues (pK_a > 11.5) at all,[12] since at pH above 10.5, an intense minimum at 287.3 nm arises in the SDUS of this protein. This may be due to the fact that in this region of pH, the three exposed tyrosine residues in the ribonuclease have been already ionized, whereas the three buried residues have not yet started to ionize. The peaks corresponding to the buried nonionized (λ_{min} = 286.1 nm) and exposed ionized (λ_{min} = 289.6 nm) forms of the tyrosine residues have approximately equal intensities and overlap giving an intense peak at λ_{min} = 287.3 nm in the SDUS. The displacement of the absorption maximum of the nonionized tyrosine residues in this protein to 287 nm at high pH values has also been noted in a previous study in which the fourth derivative of the spectrum was used.[5] The situation in which all the ionized tyrosine residues are exposed to the solvent, while all the nonionized residues are buried, apparently seldom occurs in the titration of tyrosine residues of proteins; therefore, the complications arising

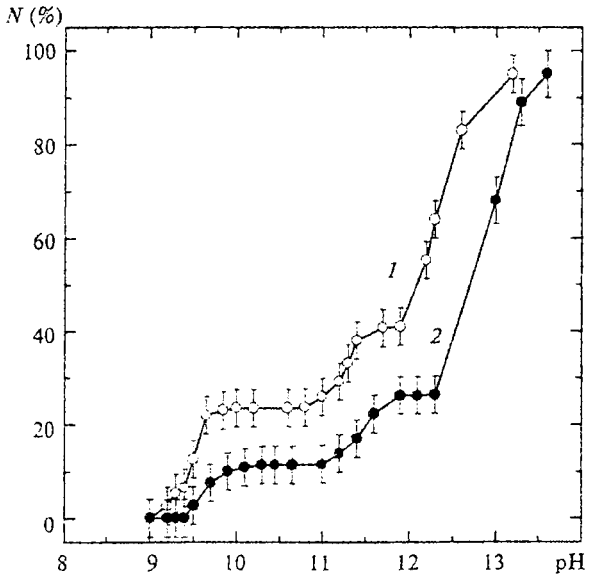

Fig. 3. Titration curves of the tyrosine residues in chymotrypsin (*1*) and chymotrypsinogene (*2*) obtained using difference SDUS.

in this case do not likely create serious limitations to this method.

To study proteins with high amounts of tryptophan, modification of the method is needed. This is due to the fact that the absorption minimum of tryptophan in the SDUS lies in the 278—283 nm range and its intensity is similar to that of the minimum corresponding to the absorption of nonionized tyrosine residues. At a Tyr/Trp ratio in the protein under study of < 2/1, the accuracy of the determination of pK_a of tyrosine residues by the SDUS diminishes due to the high intensity of the background. This obstacle can be eliminated by using difference SDUS, *i.e.*, by subtracting the spectrum of the same protein at the same concentration and at neutral pH from the spectrum recorded at a current pH.

Owing to this modification, our technique becomes applicable to studies of a wide variety of proteins. The pK_a values of tyrosine residues can be determined with an accuracy of 0.1 pH units, and the number of tyrosine residues with a particular pK_a can be found with an error of 5—7% of the total number of these groups in the protein. For proteins with high proportions of tryptophan (Tyr/Trp < 2/1), the error of the determination of the number of tyrosine residues with a particular pK_a increases to 10%.

Using the above-described modification of the method, we determined the pK_a values for tyrosine residues in α-chymotrypsin and α-chymotrypsinogen (see Table 2 and Fig. 3). Our results are close to the published data.[13] It can be seen that when chymotrypsinogen is activated to chymotrypsin, the number of residues with pK_a 9.6 increases from 0.5 to 1. This can be easily explained, since during the activation of chymotrypsinogen, the peptide bond near Tyr 146 undergoes proteolysis, and Tyr becomes a terminal group and is thus completely accessible to the solvent.[14]

Thus, while analyzing the SDUS of proteins in the range of 275—300 nm, we found that tyrosine residues in proteins can exist in solution in four states: exposed nonionized, buried nonionized, exposed ionized, and buried ionized states.

In this study, we propose a method for studying the ionization of tyrosine residues in proteins based on measuring the intensity of the second derivative of the UV spectrum of the protein at 284.2 nm, *i.e.*, at the wavelength of the isobestic point corresponding to the transition between buried and exposed nonionized tyrosine residues in an aqueous solution.

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