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Second-Derivative Spectroscopy of Proteins: Studies on Tyrosyl Residues

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Ionization of the phenolic group of *N*-acetyltyrosynamide has been studied using second-derivative spectroscopy. At pH 12.5 the second-derivative spectrum of the model compound revealed the presence of derivative bands in a spectral region (between 250 and 270 nm) where interference coming from other ultraviolet-absorbing chromophores is negligible. One of these peaks (260-nm peak) has been employed for the determination of tyrosyl groups in mixtures containing the aromatic amino acids.

The advantages of directly recording a curve of the second-order differential coefficient of absorbance A with respect to wavelength, i.e., $d^2A/d\lambda^2$, have been recently pointed out by Balestrieri *et al.* (1). They were able to isolate the contributions of the phenylalanine and tryptophan residues from the complex absorption spectrum of a protein and to carry out a very simple method for the determination of these two amino acids in the near ultraviolet range.

Any attempt made to isolate the contributions of the tyrosyl residues failed because of the large overlapping degree between the spectral bands of the major aromatic chromophores at neutral pH (2,3). Since it is well known that the ionization of tyrosyl residues produces large changes of its absorption properties (4,5), we have examined whether the use of second-derivative spectroscopy could be useful in following the formation of phenolate ion. The results reported in this paper indicate that the formation of phenolate ion may be easily followed by the appearance of new derivative bands in a spectral region where the contributions from the other aromatic chromophore are negligible.

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MATERIALS AND METHODS

N-Acetyl-L-tryptophanamide (*N*-acTrp-NH_2),[2] *N*-acetyl-L-tyrosynamide (*N*-acTyr-NH_2), and *N*-acetyl-L-phenylalanine ethyl ester (*N*-acPheOet) were purchased from Sigma Chemical Company. Concentrations were estimated by absorption measurements using $E_{280.8} = 5690\ M^{-1}cm^{-1}$ for *N*-acTrp-NH_2 (6), $E_{275.5} = 1490\ M^{-1}cm^{-1}$ for *N*-acTyr-NH_2, and $E_{257.7} = 195\ M^{-1}cm^{-1}$ for *N*-acPheOet (7). Chemicals not mentioned above were reagent grade.

Instruments

All the normal and second-derivative spectra were recorded with a Perkin–Elmer spectrophotometer, Model 575, equipped with an electronic derivative accessory (Hitachi 200-0507 derivative spectrum unit) inserted between the signal output and the recorder input. The second-derivative accessory actually differentiates the changing absorbance signal against time rather than wavelength, i.e., d^2A/dt^2 and not $d^2A/d\lambda^2$.

[2] Abbreviations used: *N*-acTrpNH_2, *N*-acetyl-L-tryptophanamide; *N*-acTyrNH_2, *N*-acetyl-L-tyrosynamide; *N*-acPheOet, *N*-acetyl-L-phenylalanine ethyl ester.

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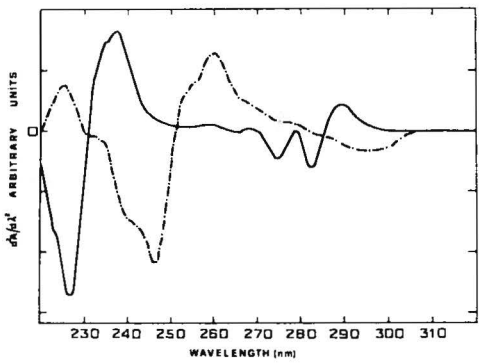

FIG. 1. Second-derivative spectra of *N*-acTyrNH$_2$ at pH 7.0 (——) and at pH 12.5 (– · – · –). Chromophore concentration was 0.05 mM; solvent: 0.05 M phosphate containing 0.15 M KCl.

If the spectrophotometer has a constant scanning speed the two functions are equivalent, but choice of scanning speed as well as slitwidth and time constants affect the intensity of the second-derivative spectra. In all the experiments we have used a scanning speed of 50 nm/min, a slitwidth of 1 nm and the highest sensitivity (corresponding to the time constants) of the derivative unit (mode 6). Due to the electronic differentiation process, the position of the minima in the second-derivative spectra are slightly red shifted if compared to the positions of the absorption maxima or shoulders in the corresponding normal spectra. We have observed that the wavelength shift depends upon the scanning speed, i.e., the shift becomes higher at higher scanning speed values.

Using a scanning speed of 50 nm/min, the wavelength shift was 2 nm; the wavelength values reported in the text have been corrected for the above mentioned shift but the correction was not made for the spectra reported in the figures, since they were taken directly from the instrumental chart. The only exception is Fig. 1, the shift being assessed comparing the second-derivative minima and the corresponding zeroth maxima over a large number of determinations.

A Zeiss PMQII spectrophotometer was used for absorption measurements at specific wavelengths. pH measurements were made with a Radiometer M 62 pH meter. Alkaline titrations were carried out keeping the model compounds solutions at 25°C under a stream of nitrogen and stirring continuously. Small additions of a concentrated KOH solution (10 M) were made using an agla syringe. All solutions contained 0.15 M KCl.

RESULTS

The ultraviolet absorption spectra of the ionized and the un-ionized form of *N*-acTyrNH$_2$ are known to be largely different (4,5). The most significant changes observed with phenolic ionization are a large red shift of the absorption maxima (from 222 to 242 nm and from 275 to 295 nm) and a considerable hyperchromic effect ($\Delta E_{243} = 11.10 \times 10^3$ $M^{-1}cm^{-1}$ and $\Delta E_{295} = 2.33 \times 10^3$ $M^{-1}cm^{-1}$ (4)). Consequently changes in the second-derivative spectrum of *N*-acTyrNH$_2$ are to be expected if the chromophore undergoes ionization. Figure 1 shows the second-derivative spectra of *N*-acTyrNH$_2$ at neutral and alkaline pH. The second-derivative spectrum obtained at neutral pH can be divided into three distinct regions: the first one, going from 215 to 245 nm, shows two main bands opposite in sign, centered at 227 and 238 nm. The second region, from 245 to 270 nm, is essentially flat, whereas the third region, between 270 and 300 nm, shows two negative peaks at 275 and 283 nm and a positive one at 288 nm. At pH 12.5 the main absorption peak in the far uv is about 20 nm red shifted; therefore the two main bands seen at neutral pH in the far uv are both red shifted and they occur at 246 and 260 nm at pH 12.5. In addition the fine-detailed structure observed at neutral pH between 270 and 295 nm is essentially lost, because of the broadness of the phenolate absorption peak at 292 nm. Thus the second-derivative spectrum of the phenolate ion shows a detailed structure between 235 and 270 nm, where the un-ionized form does not present any significant feature. *N*-acTrpNH$_2$ and *N*-acPheOet are not expected to show changes in their absorption

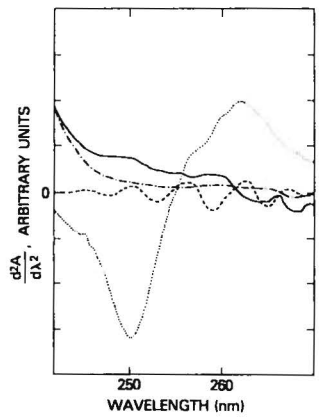

FIG. 2. Comparison of the second-derivative spectra of N-acTrpNH$_2$, N-acTyrNH$_2$ and N-acPheOet at neutral and alkaline pH. ——, N-acTrpNH$_2$, pH 7.0 and pH 12.5; –·–·, N-acTyrNH$_2$, pH 7.0; ·····, N-acTyrNH$_2$, pH 12.5; – – –, N-acPheOet, pH 7.0 and pH 12.5. The concentration of each chromophore was 0.05 mM; solvent: 0.05 M phosphate, 0.15 M KCl.

spectra since they do not contain any ionizable group. We have verified this assumption by recording the second-derivative spectra of the N-acTrpNH$_2$ and the N-acPheOet at neutral and alkaline pH. No change was observed between 220 and 270 nm after alkalinization of model compound solutions. The second-derivative spectra of the three chromophores at neutral and alkaline pH are shown in Fig. 2.

The alkaline titration of solutions containing mixtures of the three aromatic chromophores also showed the appearance of the couplet at 246 and 260 nm due to the formation of the phenolate ion. Figure 3A shows the second derivative spectrum at neutral pH of a mixture containing N-acPheOet, N-acTrpNH$_2$, and N-acTyrNH$_2$ in the molar ratio 3:2:1, respectively. The positive peak centered at 260 nm arises from phenylalanine contribution since the second-derivative spectra of the other two chromophores are essentially flat around 260 nm at neutral pH (1). Alkalinization of the same solution to pH 12.5 produces an increase of the intensity of the positive peak at 260 nm. Since N-acPheOet and N-acTrpNH$_2$ do not change their second-derivative spectra with pH, the increase at 260 is exclusively due to the formation of phenolate ion; therefore the second-derivative value at 260 nm is the sum of two different contributions, i.e., the contribution of N-acPheOet indicated as a in Fig. 3A, and the contributions of N-acTyrNH$_2$ equal to $d_0 - a$. If the concentration of phenolate ion is increased, the intensity of the peak at 260 nm also increases. We have verified whether the value of the second derivative at 260, corrected for the small contribution of N-acPheOet, i.e., $d - a$, followed the Beer–Lambert law. The plot of $d - a$ versus N-acTyrNH$_2$ concentration gave straight lines for all assayed mixtures. Figure 4 shows the linear correlation existing between $d - a$ and N-acTyrNH$_2$ concentration for a mixture, the molar ratio of which (Phe/Trp/Tyr) varied from 3/2/1 to 3/2/10. The intercept (i) between the straight line and the ordinate axes is related to the initial concentration (C_0) of N-acTyrNH$_2$ by the equation: $i = \alpha C_0$, where α is the slope. Table 1 shows the results obtained on three mixtures containing different initial molar ratios of the three aromatic chromophores at pH 12.5. The concentration of N-acTyrNH$_2$ varied by adding small amounts of a more concentrated solution. The value of i and α were calculated by least-square

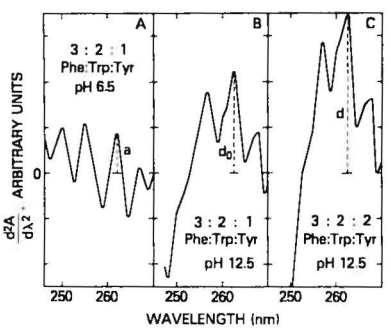

FIG. 3. Details of the second-derivative spectra of a series of mixtures containing the same concentration of N-acTrpNH$_2$ (0.10 mM) and N-acPheOet (0.15 mM) and increasing concentrations of N-acTyrNH$_2$: (A) 0.05 mM, pH 6.5; (B) 0.05 mM, pH 12.5; (C) 0.10 mM, pH 12.5. Solvent: 0.05 M phosphate, 0.15 M KCl.

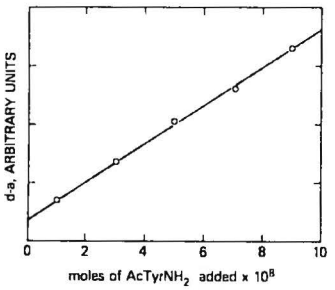

FIG. 4. The dependence of the second derivative at 260 nm on *N*-acTyrNH$_2$ concentration at pH 12.5. The abscissa is the moles of chromophore added to 3.0 ml of a solution composed of 0.05 mM *N*-acTyrNH$_2$, 0.10 mM *N*-acTrpNH$_2$, and 0.15 mM *N*-acPheOet. Solvent: 0.05 M phosphate, 0.15 M KCl.

method. The values of the initial concentrations of *N*-acTyrNH$_2$ calculated by this procedure were consistent with the taken values.

Among the titratable groups of proteins sulfhydryl groups have p*K* values slightly lower than those of normally ionizing phenol groups. Mercaptoethanol was determined to have a p*K* of 9.5 in both water and 4.0 M urea (8), while the p*K* of *N*-acetylcysteine was found to be 9.8 (9). Benesh and Benesh (10,11) first demonstrated that large changes in absorptivity take place upon ionization of thiol group of cysteine; from the amplitude of the optical changes the same authors were also able to obtain the p*K* values of cysteine thiol group in presence of charged and uncharged amino group (10). Since the peak of mercaptide ion (235 nm) is very close to that of phenolate, we have examined whether the second-derivative peak at 260 was affected by a concomitant sulfydryl ionization. Unfortunately the second-derivative spectrum of mercaptide ion is almost completely superimposable to that of the phenolate, except that its intensity is lower. The phenolate peak at 260 nm is increased of 22% when the ratio between phenolate and mercaptide is 1:1, but a higher ratio values this increase becomes much smaller (4% for solution containing phenolate and mercaptide in the molar ratio 4:1).

DISCUSSION

Tyrosyl residues have been extensively investigated in proteins because of the large absorbance change due to the formation of the phenolate ion that occurs at alkaline pH values (4,5). The 295-nm peak is usually employed for measuring the degree of tyrosyl ionization even though the absorptivity change at 243 nm is five times as large as at 295 nm (4,5). Only in few cases, especially when some conjugated groups overlap the 295-nm peak, the changes at 243 nm are used (12,13). From the magnitude of the optical changes at 243 nm or at 295 nm, the number of titratable phenolic groups can be determined (5). However this method is complicated by the fact that absorptivity changes may be also produced from conformational changes af-

TABLE 1

DETERMINATION OF *N*-acTyrNH$_2$ CONTENT IN MIXTURES CONTAINING ALL THREE AROMATIC AMINO ACIDS WITH BLOCKED AMINO AND CARBOXYL GROUPS[a]

Composition of mixtures (nmol)			Number of determinations	Estimated initial concentration of *N*-acTyrNH$_2$ (nmol)
N-acPheOet	*N*-acTrpNH$_2$	*N*-acTyrNH$_2$		
150	150	150	4	149 ± 2
600	150	150	4	152 ± 2
150	600	150	3	151 ± 3

[a] The compositions of the mixtures as well as the estimated initial concentrations of *N*-acTyrNH$_2$ ± SD are given as the amount of chromophore dissolved in 3.0 ml of 0.05 M phosphate, 0.15 M KCl, pH 12.5.

10. Benesh, R. E., and Benesh, R. (1955) *J. Amer. Chem. Soc.* **77**, 5877–5881.
11. Benesh, R., and Benesh, R. E. (1959) *in* Sulfur in Proteins (Benesh, R., Benesh, R. E., Boyer, P. D., Klotz, I. M., Middlebrook, W. R., Szent-Györgyi, A. G., and Schwarz, D. R., eds.), p. 15, Academic Press, New York.
12. Hermans, J. (1962) *Biochemistry* **1**, 193–196.
13. Stellwagen, E., and Van Rooyan, S. (1967) *J. Biol. Chem.* **242**, 4801–4805.
14. Donovan, J. W. (1969) *in* Physical Principles and Techniques of Protein Chemistry (Leach, S. J., ed.), part A, p. 101, Academic Press, New York.
15. Tanford, C., and Wagner, M. L. (1954) *J. Amer. Chem. Soc.* **76**, 3331–3336.
16. Dayhoff, M. O. (1972) Atlas of Protein Sequence and Structure, Vol. 5 (Suppl. 2, 1976), National Biomedical Research Foundation, Washington, D. C.
17. Giese, A. T., and French, C. S. (1955) *Appl. Spectrosc.* **9**, 78–86.
18. Hager, R. N., Jr. (1973) *Anal. Chem.* **45**, 1131 A.

fecting the electrostatic environment of indole and phenolic rings (14). Moreover, even in absence of tryptophan, interference may come out from the phenylalanine and cystine contributions in the spectral region between 240 and 270 nm (14). The ionization of cysteinyl residues also affects the optical changes at 243 nm since the peak of mercaptide ion, centered at 235 nm, is very close to the phenolate maximum (5,10,14). In such cases measurements at several wavelengths indicate whether the titration curves reflect only tyrosine ionization or some other phenomenon (15).

One of the most interesting features of the second-derivative spectrum of the phenolate ion is the presence of a positive peak at 260 nm arising from an inflection point present in the normal spectrum at the same wavelength. This peak is far enough from the spectral region in which absorptivity changes, arising from conformational changes of the indole or phenolic microenvironment (14), may take place. In addition any contribution from phenylalanine residues can be easily corrected as indicated under Results. Unfortunately the use of the second derivative cannot reduce or eliminate the interferences produced by cysteine thiol ionization, since the second-derivative spectrum of the mercaptide ion overlaps that of the phenolate. However, in proteins containing cysteine–tyrosine ratios lower than 0.5 the increase of the 260 nm peak might be considered negligible. We have calculated from Dayhoff data (16) that the average ratio value between cysteinyl and tyrosyl residues is 0.77 over a large number of proteins, but the number of cysteinyl residues taken for this evaluation also includes the disulfide-linked residues, which do not undergo ionization.

The maximal tyrosyl ionization in proteins is usually estimated in denaturing solvents, which normalize the ultraviolet spectral properties of proteins to those of low molecular weight model compounds (5,18). In such case particular attention should be given to the guanidine solution, the absorbance of which becomes very large in strongly alkaline solutions especially at wavelengths lower than 250 nm (5,14). The second-derivative peak of the phenolate ion at 260 nm is not affected by absorbance change in guanidine solutions; therefore the experimental values do not necessitate any correction. Moreover, it has been shown that the optical interference of any other ultraviolet-absorbing material are completely eliminated when it produces a constant or a gradual background variation upon which the spectrum of a given chromophore is superimposed (1,3,23,24). This feature makes the second-derivative spectroscopy particularly useful in following tyrosyl ionization even in solution, the turbidity, or the background absorbance of which is very high.

The linear correlation between the intensity of the second-derivative peak at 260 nm and the phenolate concentration could be used for the quantitative determination of tyrosyl chromophores in proteins when the most common methods fail.

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REFERENCES

1. Balestrieri, C., Colonna, G., Giovane, A., Irace, G., and Servillo, L. (1978) *Eur. J. Biochem.* **90,** 433–440.
2. Balestrieri, C., Colonna, G., Giovane, A., Irace, G., and Servillo, L. (1978) *in* 12th Febs Meeting, Dresden, Communication 3325.
3. Irace, G., Colonna, G., Giovane, A., Servillo, L., Russo-Spena, F., and Balestrieri, C. (1979) *Rend. Atti Accad. Sci. Med. Chir.* **123,** in press.
4. Beaven, G. H., and Holiday, E. R. (1952) *Advan. Protein Chem.* **7,** 319–386.
5. Wetlaufer, D. B. (1962) *Advan. Protein Chem.* **17,** 303–390.
6. Edelhoch, H. (1967) *Biochemistry* **6,** 1948–1954.
7. Mihaly, E. (1976) *in* Handbook of Biochemistry and Molecular Biology (Fasman, G. D., ed.), 3rd ed., Vol. 1, CRC Press, Cleveland, Ohio.
8. Edsall, J. T., and Wyman, J. (1958) Biophysical Chemistry, Vol. 1, Academic Press, New York.
9. Friedman, M., Carins, J. F., and Wall, J. S. (1965) *J. Amer. Chem. Soc.* **87,** 3672–3682.