ULTRAVIOLET DIFFERENCE SPECTRA OF PEPSIN*,‡

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ABSTRACT

A shift of pH of pepsin solutions from 4.6 to 1.0 gives rise to spectral displacements in the ultraviolet. If represented as difference spectra three peaks with maxima at 2770, 2850, and 2930 Ångströms are present which can be attributed to the tyrosine and tryptophan residues in the protein. On mild autolysis of pepsin at pH 2.0 the absorbancy in the ultraviolet further decreases. Although some of these effects can be ascribed to the occurrence of hydrogen bonding between the aromatic residues and a carboxylate ion, those observed on autolysis are caused by charge effects of newly formed polar groups in the vicinity of a chromophore. No direct relation between the optical properties described here and enzymic activity of pepsin has been observed.

The ultraviolet absorption bands of protein solutions are displaced to shorter wave lengths on titration of a protein with acid (1), upon denaturation with urea or guanidine salts (2–4), and on mild proteolysis (2, 3, 5, 6). Recently, Laskowski, Scheraga, and coworkers have studied the spectral shifts that occur if the pH of solutions of insulin and ribonuclease is altered from neutrality to pH 1.0 (6, 7). On the basis of their results these investigators proposed the hypothesis, at present widely accepted, that these small spectral displacements reflect changes in the hydrogen bonding between the tyrosyl hydroxyls and neighboring basic groups of the protein; *e.g.*, carboxylates.

An alternative explanation, however, follows from the work of Wetlaufer, Edsall, and Hollingworth, who observed spectral changes of the same order of magnitude if solutions of tyrosine, *O*-methyltyrosine, and glycyl *O*-methyltyrosine of pH 7.0 are compared with those of pH 1.0 (8). Since the occurrence, in these materials, of intramolecular hydrogen bonds can be excluded these

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effects are ascribed to the ionization of groups adjacent to the benzene ring. Thus in a protein with tightly folded peptide chains the extent of ionization of a dissociable group in close vicinity to a chromophore may influence the spectral properties.

During the past 4 years, we have initiated a study to correlate the chemical and physicochemical properties of pepsin with its enzymic activity. Pepsin is unique in that it has a great predominance of acidic amino acids over the basic ones and a high tyrosine content. It, therefore, seemed of interest to test whether or not pepsin would manifest spectral shifts in the acid pH range similar to those found for insulin and ribonuclease. The results presented in this paper indicate that this is the case but that no direct relation to the enzymic activity exists.

Experimental

The crystalline pepsin used in this research was the Worthington preparation 617 with a nitrogen content of 14.83 per cent and the molar extinction coefficient, $\epsilon = 52.47 \times 10^3$ at λ 2780 Å.

The spectroscopic measurements were made at room temperature with a Beckman DU spectrophotometer equipped with a photomultiplier attachment in cells of 1.5 ml. capacity and a light path of 10 mm. The instrument was operated with a slit width of 0.05 to 0.3 mm. As in the work of Scheraga and coworkers the technique of differential spectroscopy was employed.

Two per cent pepsin solutions in 0.1 N sodium acetate buffer of pH 4.6 were prepared and the concentration determined by the Pregl microKjeldahl method, using the factor 6.7_6 for conversion to a dry weight basis. Aliquots of the stock solutions were diluted to 0.16 per cent protein with the solvent of the desired composition and pH. In the pH range of 3.6 to 5.6 sodium acetate buffers of 0.1 ionic strength were used. In some cases 80 per cent of the sodium acetate of the mixture was replaced by sodium chloride. The acid pH values were obtained with hydrochloric acid to which sodium chloride had been added to attain a final ionic strength of 0.1. In some of the experiments an ionic strength of 0.02 was used. Unless stated otherwise the optical density of the test solution was recorded against a blank consisting of 0.16 per cent pepsin in 0.1 N sodium acetate buffer of pH 4.6.

The pH measurements were made at 25°C. with a MacInnes type glass electrode (9) and the pH meter was calibrated with the standard buffers recommended by Bates (10).

The pepsin solutions were assayed with the aid of the hemoglobin method (11). In some experiments the hydrolysis of the synthetic substrate, *N*-acetylphenylalanine diiodotyrosine, was tested (12).

RESULTS

In Fig. 1 are recorded typical differential spectra for pepsin solutions of pH 2.0_4 and 3.3_9, respectively, referred to pH 4.6. It will be noted that three peaks are present with absorption maxima at 2780, 2850, and 2940 Å. Maxima of

wave lengths of 2780 and 2850 Å were found by Laskowski and Scheraga whereas in their experiments that at 2940 Å was absent. It has been ascertained in this laboratory that this peak is due to the presence of tryptophan residues in pepsin, an amino acid which does not occur in insulin and ribonuclease (13, 14).[1,2] As indicated in Fig. 1, the difference of the molar extinction coefficient, $\Delta\epsilon = \epsilon_{pH} - \epsilon_{4.6}$, is in all cases negative thus revealing that the absorbancy of pepsin at acid pH values is smaller than that recorded above pH 4.0. In the pH range of 4.6 to 6.0 the absorption spectra of the protein are indistinguish-

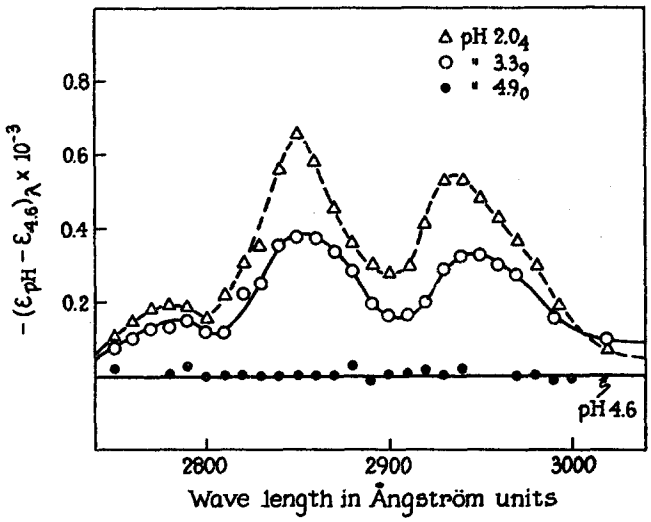

FIG. 1. Difference spectra of 0.16 per cent pepsin at various pH values referred to pH 4.6.

able. Moreover, $\Delta\epsilon$, is independent of the protein concentration and, at constant ionic strength, does not vary with ionic composition of the solvent.

Having thus demonstrated that addition of acid to pepsin solutions gives rise to differential spectra, the question arises as to the significance of the change, $\Delta\epsilon$, of the molar extinction coefficient. If, as illustrated with the aid of Fig. 2, $\Delta\epsilon$ at 2850 Å is plotted against pH a steady decrease in absorbancy is obtained

[1] In experiments with tryptophan, mixtures of tyrosine and tryptophan, it was established that the peak at 2920 to 2940 Ångström can be ascribed to tryptophan. A similar result has also been obtained by Scheraga and coworkers (*Biochim. et Biophysic. Acta*, 1958, **29**, 455).

[2] We are much indebted to Dr. Scheraga for having communicated the results of his work on tryptophan, tryptophan derivatives and lysozyme prior to their publication.

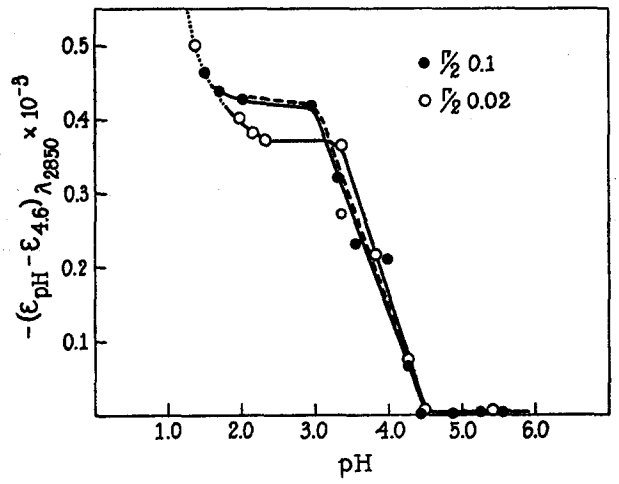

FIG. 2. Difference in molar extinction at 2850 Ångström between pepsin at pH 4.6 and other pH values and varying ionic strength.

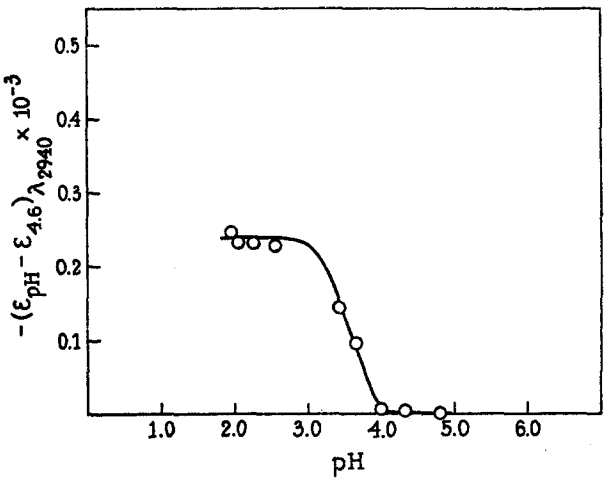

FIG. 3. Difference in molar extinction at 2940 Ångström between pepsin at pH 4.6 and other pH values at 0.1 ionic strength.

until a plateau is reached between pH 3.0 and 1.6. On further acidification to pH 1.0 $\Delta\epsilon$ decreases. If the change in $\Delta\epsilon$ is due to a shift in acid-base equilibrium, as the shape of Fig. 2 suggests, the apparent pK of the responsible group (or groups) is 3.70 at 0.1 ionic strength and 3.95 at 0.02 $\Gamma/2$; *i.e.*, the pH of half maximal $\Delta\epsilon$. The broken line of Fig. 2 which closely follows the solid curve

indicates that the change in absorbancy is fully reversible over the pH range of 2.0 to 6.0.

A similar picture is obtained if the difference in extinction recorded at 2940 Å is expressed as a function of pH. The apparent pK in solvents of 0.1 ionic strength, however, is 3.55 (Fig. 3). Since in the pH range of 3.0 to 4.6 the carboxylic groups of the dicarboxylic acids in proteins can exist as carboxylate ions the apparent pK values given above can be taken as indication of the occurrence of bonds between such groups and the aromatic amino acid residues as postulated by Laskowski (6) and Scheraga (7).

TABLE I

*Effect of Storage in Solvents of Various Composition and pH on the Activity of Pepsin**

Composition of solvent	pH	Relative specific activity per nitrogen unit‡	
		Hemoglobin	Synthetic substrate
0.1 N NaAc–0.01 N HAc	5.6_4	100	100
0.1 N NaAc–0.3 N HAc	4.3_4	100	92
0.1 N NaAc–1.0 N HAc	3.6_0	92.4	85
0.1 N NaCl–0.01 N HCl	2.3_9	87.8	Not tested
0.1 N NaCl–0.1 N HCl	1.8_3	Not tested	83
0.1 N HCl	1.0_8	78.6	—

Ac = acetate.

* Assay carried out at pH 2.0.

‡ The activity of a freshly prepared solution of pepsin in 0.1 N acetate buffer of pH 4.6 is taken as 100.

In the second series of experiments the activity of the protein was tested after storage in the same solvents as those used in the spectroscopic measurements. The solutions were kept at the pH chosen for 1 to 3 hours prior to the assay which was carried out in the manner described, at pH 2.0, the optimum pH for the hydrolysis of hemoglobin and *N*-acetylphenylalanine diiodotyrosine. Although the relative specific activity decreases with the pH, only the loss of activity below pH 2.0 is significant and most likely due to denaturation of the protein. In view of the reversibility of $\Delta\epsilon$ it is felt that no direct relation exists between the changes in the configuration of the protein as reflected in the spectral displacements and its enzymic activity.[3]

Since pepsin readily undergoes autodigestion and preferably hydrolyzes bonds adjacent to aromatic amino acids (15, 16), on autolysis new groups are formed in the vicinity of a chromophore residue which at certain pH values will be ionized. If, as illustrated with the aid of Fig. 4, the differential spectrum of a pepsin solution of pH 2.0_4, kept at 37°C. for 24 hours and referred to pH 4.6,

[3] The apparent inactivation of pepsin stored at pH 1.0 is partly reversible.

is compared with that of a freshly prepared solution in the same solvent, a six-fold decrease in $\Delta\epsilon$ has occurred. Moreover, the maximum of the tryptophan peak has been displaced from 2940 to 2920 Å. The decrease in absorbancy is rapid during the first 6 hours but continues steadily as autodigestion proceeds. It is further indicated in Fig. 4 that, on removal by dialysis of the low molecular weight peptides, this differential spectrum is abolished. Since in these experiments conditions similar to those chosen by Wetlaufer and coworkers in their work with tyrosine derivatives prevail, it can be inferred that charge

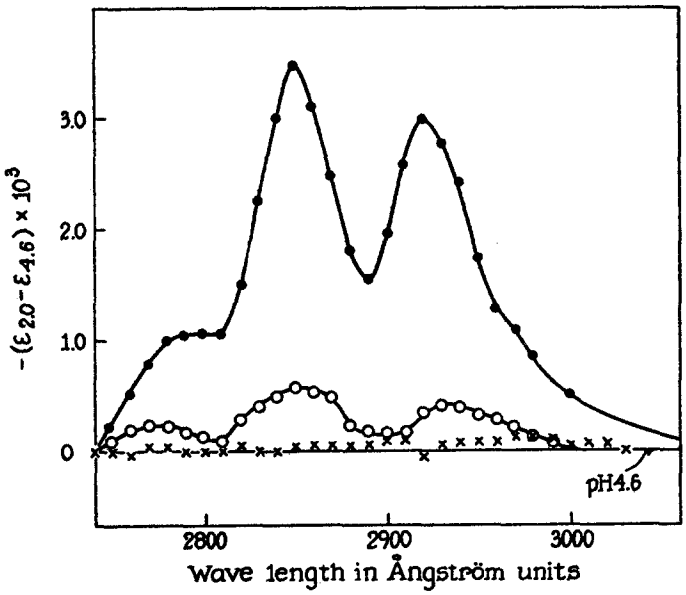

FIG. 4. Difference spectra of 0.16 per cent at pH 2.0_4 and 0.1 ionic strength referred to pH 4.6 before (○) and after (●) autodigestion at 37°C. for 24 hours, and after dialysis of autodigested sample (×).

neutralization adjacent to an aromatic amino acid may arise on mild proteolysis and thus influence the chromophore.

DISCUSSION

In the present investigation it has been shown that spectral shifts occur if the pH of a pepsin solution is altered from 4.6 to 1.0. If these changes are represented as difference spectra three peaks with maxima at 2770, 2850, and 2940 Å are present which can be attributed to the presence of tyrosine and tryptophan in the protein. In view of the fact that the pepsin preparation used in this research contains 18 tyrosines, 6 tryptophans, 71 dicarboxylic acids with 36 amides, and 35 free carboxyl groups (17), hydrogen bonds of the type sug-

gested by Scheraga and coworkers could exist between the phenolic hydroxyls of tyrosine and the imino group of tryptophan on one hand and a basic group; *i.e.*, carboxylate on the other.

If the change of absorbancy at 2850 Å is expressed per tyrosine residue the value is of the same order of magnitude as that obtained for the same pH range by Scheraga for ribonuclease (7) and by Wetlaufer, Edsall, and Hollingworth, for free tyrosine (8). In view of the fact that the relation between hydrogen bond strength and ultraviolet spectral shifts is not known it cannot be stated how many groups of the protein will be involved in bonds as those postulated above. This is further complicated by the fact that not all the tyrosine residues of pepsin are of the same reactivity (18, 19) and that the pK values of the carboxyls may vary considerably and depend on the type of the surrounding amino acids.

Although the effects described above depend, in part, on the breaking of hydrogen bonds, the decrease in absorbancy which occurs during autodigestion may be explained as follows: Since pepsin preferably acts on peptide bonds adjacent to an aromatic residue (15, 16) new polar groups in close proximity to the chromophore are formed. Thus a relatively small configurational change takes place that influences the spectral properties of the protein in the manner postulated by Edsall and collaborators. Moreover, it is of interest to note that the decrease in absorbancy precedes somewhat the release of non-protein nitrogen material. It appears, therefore, that we actually observe during the early stages of the autolysis the breaking of a few peptide linkages before the appearance of dialyzable peptides.

In the light of the results presented in this investigation we have arrived at the conclusion that although hydrogen bonds between tyrosine hydroxyls and carboxylate ions undoubtedly exist in pepsin and may account for some, but not all, of the changes in the optical properties described here, they are not the determining factor in establishing the specific configuration of the peptide chain necessary for the enzymic activity of the protein.

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