Studies on the Ultraviolet Difference Spectra of Proteins and Polypeptides*

A. N. GLAZER AND EMIL L. SMITH

From the Laboratory for the Study of Hereditary and Metabolic Disorders and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City, Utah

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Changes in the ultraviolet absorption spectra of proteins associated with denaturation or proteolysis have been intensively investigated in recent years. These studies have been, in the main, restricted to the difference spectra of proteins and model compounds in the region between 270 and 300 m\(\mu\). The changes in this region have been assigned almost entirely to the alterations in the environment of tyrosine and tryptophan residues (1). Much valuable information has been obtained concerning the environment of these chromophores in a number of proteins (2).

In a recent communication (3), we have reported on the difference spectra which are obtained on denaturation and proteolysis of proteins over the wavelength range 220 to 320 m\(\mu\). It was shown that alterations in native protein structure were associated with the appearance of a peak at 230 to 235 m\(\mu\) in the difference spectrum on comparison of the native with the denatured protein. The present paper represents both an extension of the data previously presented in preliminary form (3), and an attempt to identify the chromophore(s) responsible for the 230 to 235-m\(\mu\) peak.

EXPERIMENTAL PROCEDURE

The proteins and polypeptides used in this study are listed in Table I. The concentration of the protein solutions was generally determined spectrophotometrically. When \(E_{280}\) values were not available, the protein content was determined by dry weight (see Table I). All other materials used were of reagent grade. The urea was recrystallized from aqueous ethanol.

The Cary model 14 recording spectrophotometer was used with 1-cm silica cells. When shorter light paths were desired, 9.0-mm and 9.5-mm inserts were employed. Both the cells and inserts were matched over the range 220 to 320 m\(\mu\) with water and also with indole solutions. Determinations of pH were performed with a Radiometer TTTI titrator. All measurements were performed at 25 ± 3°C.

RESULTS

Effect of Various Denaturing Agents on Proteins

In our first communication (3), we reported that denaturation of a number of proteins was associated with the appearance of a difference peak in the region 230 to 235 m\(\mu\). It was of interest to extend this investigation to a larger number of proteins to establish the generality of this phenomenon. As shown in Figs. 1 and 2, papain, ribonuclease, human serum albumin, lysozyme, ovalbumin, chymotrypsinogen, and pep
cin all manifest the same over-all change in the 230- to 235-m\(\mu\) region on denaturation, although a variety of denaturing treatments were employed, e.g. urea denaturation for ovalbumin, acid denaturation for human serum albumin. Protamine sulfate, which is considered to exist as an open chain in aqueous solution, did not give the spectral change observed in the other cases. The \(\Delta\varepsilon_M\) values for a number of proteins are presented in Table II. These data supplement those presented earlier (3).

Autolysis of Pepsin—It was of interest to know whether changes in primary structure produced by proteolysis would also result in the appearance of the difference peak in the 230- to 235-m\(\mu\) region. Proteolysis is known to be accompanied by the appearance of the tyrosine and tryptophan difference spectra (12-14).

This point was examined by study of the difference spectra obtained by comparing pepsin at a given pH with its autolysate at different periods of time. This experiment was performed at two different temperatures and pH values. The difference spectrum showed a peak at 230 m\(\mu\), the peaks caused by tyrosine at 279 and 286 m\(\mu\), and the peak caused by tryptophan at 292 m\(\mu\). (See Fig. 2, for example.)

A reasonably good correlation was obtained between the \(\Delta\varepsilon_M\) at 230 m\(\mu\) and the amount of \(\alpha\)-amino nitrogen as determined by the ninhydrin method (15). Thus, at pH 1.45, a \(\Delta\varepsilon_M\) decrease of 13,360 was obtained after proteolysis for 2 hours at 30°C and a ninhydrin color increment per mole (\(\Delta N_{\text{molat}}\)) of 18,900, giving \(\Delta\varepsilon_M: \Delta N_{\text{molat}} = 0.71\), whereas at pH 4.5 the decrease at 230 m\(\mu\) gave a \(\Delta\varepsilon_M = 9,000\) and the ninhydrin color increment per mole, \(\Delta N_{\text{molat}} = 11,500,\) giving \(\Delta\varepsilon_M: \Delta N_{\text{molat}} = 0.78\).

Alkaline Denaturation of Pepsin—Tyrosine and tryptophan residues contribute substantially to the absorption of proteins in the region of 230 m\(\mu\). It was of great importance, therefore, to determine whether the changes in the absorption spectra of these chromophores, which accompany denaturation, make a large contribution to the observed difference peak in the 230- to 235-m\(\mu\) region. As already noted (3), no correlation could be shown between the tyrosine and tryptophan content of the proteins studied and the magnitude of the 230- to 235-m\(\mu\) peak obtained on denaturation.

The spectrophotometric study of the alkaline denaturation of pepsin has provided evidence which casts some light on this problem. Fig. 3 shows the spectrum obtained when pepsin at pH 5.7 is compared to pepsin at pH 7.3. It may be clearly seen...
Table I
Proteins and polypeptides used for spectrophotometric studies

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
<th>Assumed molecular weight (MW)</th>
<th>4% E/1cm</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (bovine)</td>
<td>Squibb, Zn-free</td>
<td>5,700</td>
<td>280</td>
<td>10.0</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>Worthington Biochemicals Corporation, Lot No. 558</td>
<td>13,083</td>
<td>280</td>
<td>0.9</td>
</tr>
<tr>
<td>Chymotrypsigen</td>
<td>Armour Lot No. R357354</td>
<td>25,100</td>
<td>280</td>
<td>20.0</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>Crystallized 3 times</td>
<td>33,500</td>
<td>280</td>
<td>9.5</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Armour Lot No. 003L1</td>
<td>14,000</td>
<td>282</td>
<td>27.3</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>Nutritional Biochemicals Corporation, Crystallized twice</td>
<td>45,000</td>
<td>280</td>
<td>7.35</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Crystallized 3 times</td>
<td>35,500</td>
<td>280</td>
<td>14.3</td>
</tr>
<tr>
<td>Serum albumin (human)</td>
<td>Squibb</td>
<td>65,600</td>
<td>280</td>
<td>5.3</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>Gift of Dr. E. Katcalski</td>
<td>8,000</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Poly-γ-glutamate</td>
<td></td>
<td>150,000</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Oxidized A-chain of insulin</td>
<td>Gift of Dr. R. L. Hill</td>
<td>2,532</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Lilly Research Laboratories, Lot No. 258-234B-54-2</td>
<td>4,200</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Nutritional Biochemicals Corporation, Lot No. 7481</td>
<td>246</td>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

* Concentration was determined by dry weight.

that there are prominent difference peaks at 278, 286, and 292 μm. These peaks have been shown (16-18) to result from alterations in the environment of tyrosine and tryptophan residues. Yet, there is essentially no change in the absorption of pepsin in the region of 225 to 245 μm. This clearly demonstrates that changes in the environment of tyrosine and tryptophan residues do not necessarily produce marked changes in the absorption of pepsin in the region of 230 μm. As already noted above, however, there is no question that autolysis of pepsin does result in the appearance of a peak at 290 μm in the difference spectrum. It would seem, therefore, that the difference peak at 230 μm must be due to some factor other than, or in addition to, changes in the environment of phenolic and indole side chains, at least in the case of pepsin.

Denaturation of Ovalbumin by Urea and Guanidine Hydrochloride—Further evidence that the changes in the 230-μm region reflect alterations in native protein structure in addition to changes in the environment of the tyrosine and tryptophan residues was obtained by kinetic studies on the urea and guanidine hydrochloride denaturation of ovalbumin. Denaturation of ovalbumin by these two agents results in the appearance of peaks at 292.5, 287.5, 279, and 232.5 μm. The rate of change in optical density at 292.5, 287.5, and 232.5 μm was studied at a number of concentrations of guanidine hydrochloride and urea in the range of pH 5 to 8. In this range, the changes in optical rotation and viscosity of ovalbumin in the above mentioned denaturing agents are comparatively slow and lend themselves well to kinetic studies (19, 20). Fig. 4 shows the results obtained from a typical experiment.
TABLE II

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH of protein</th>
<th>Denaturation conditions</th>
<th>( \lambda_{\text{max}} )</th>
<th>( \Delta \lambda )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum albumin</td>
<td>5.2</td>
<td>pH 1.4, 25°</td>
<td>233.0</td>
<td>29,250</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>5.4</td>
<td>pH 1.4, 25°</td>
<td>235.0</td>
<td>3,040</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>6.0</td>
<td>pH 1.4, 39°, 24 hours</td>
<td>None</td>
<td>231.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alkaline denaturation</td>
<td>236.0</td>
<td>1,120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heat denaturation pH 5.2</td>
<td>None</td>
<td>228.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 minutes, 90°</td>
<td></td>
<td>3,190</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>7.8</td>
<td>pH 7.8, 0.012 M phosphate buffer, 8 M urea, 24 hours at 24°</td>
<td>233.0</td>
<td>55,250*</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>5.0</td>
<td>pH 1.4, 39°, 2 hours</td>
<td>None</td>
<td>230.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alkaline denaturation</td>
<td>238.0</td>
<td>1,940</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>239.0</td>
<td>3,960</td>
</tr>
<tr>
<td>Insulin†</td>
<td></td>
<td>pH 1.6, 25°</td>
<td>234.0</td>
<td>2,400</td>
</tr>
<tr>
<td>( \beta )-Lactoglobulin†</td>
<td>pH 1.4, 24 hours, 25°</td>
<td>Alkaline denaturation‡</td>
<td>236.0</td>
<td>1,230</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>236.0</td>
<td>12,250</td>
</tr>
</tbody>
</table>

* All ovalbumin values have been corrected for urea absorbance.
† Difference peaks in the region 275 to 295 m\( \nu \) not computed.
‡ Alkaline denaturation was for 10 minutes at pH 11.4 in a water bath at 92°, followed by acidification to pH 1.4. Cooled to 25° before scanning.

In all cases, changes at 232.5 m\( \nu \) continued after changes at 292.5 and 287.5 m\( \nu \) had ceased. Table III lists the half-times of the changes at these three wave lengths obtained under a variety of conditions. The half-time of the change at 232.5 m\( \nu \) was found to be considerably longer in every case than that of the change at 292.5 and 287.5 m\( \nu \).

It is unlikely that dispersion contributes significantly to the absorbancy changes observed at 232.5 m\( \nu \). First, at the protein concentration range employed, 0.04 to 0.1%, no aggregation would be expected. This is further excluded by the fact that a plot of \( \Delta A_{232.5} \) versus concentration is linear over this range (Fig. 5). Second, the wave length dependence of the absorbancy change is not consistent with the dispersion law. This evidence indicates, therefore, contributions from chromophores other than...
tyrosine and tryptophan to the 322.5-μm peak in urea- or guanidine-denatured ovalbumin and lends further support to the conclusions based on the pepsin autolysis data presented above.

Studies on Poly-L-glutamate—The investigations on ovalbumin and pepsin, as well as the ones on the free amino acids presented earlier (3), suggested strongly that the changes in the 230-μm region might arise from disturbances of the environment of the polypeptide backbone of the protein associated with denaturation.

Poly-L-glutamic acid was chosen as a model compound. The helix-coil transition which had been clearly demonstrated for this polypeptide (21) lends itself well to spectrophotometric studies.

Doty et al. (21) have shown that at pH 4, this molecule exists as an α-helix. As the pH is raised and the carboxyl groups ionize, repulsion between adjacent carboxylate ions results in a transformation of this helix into a random coil. Doty et al. (21) have shown that the proportion of helix to random coil follows very closely the ionization curve of the γ-carboxyl of glutamic acid. Poly L-glutamic acid thus offers a model system for the investigation of the effect of ultraviolet absorption of uncoiling of an α-helix.

On comparison of a solution of poly-L-glutamic acid at pH 4.0 with a solution at the same concentration at pH 11.4, the difference spectrum showed a peak at 225 μm. Further, the height of the 225-μm peak was dependent on pH in the range 4 to 6 in an analogous fashion to that observed by Doty et al. (21) for the helical content. The experimental results are shown in Fig. 6.

Calculation of Δε per residue at 225 μm gave a value of 85. This value is somewhat lower than that observed for the proteins which gave Δε per residue values in the range 100 to 200. Moreover, some of the observed change in the absorption spectrum of poly-L-glutamate with pH may be attributed to increase in the absorption at 225 μm of the carboxyl group in going from the ionized to the un-ionized form. The Δε(COO⁻ → COOH) at 225 μm in a carboxylic acid such as citric acid was found to be approximately 15 per carboxyl group. It would appear, therefore, that the change in carboxyl ionization would account for at most 20% of the observed change in poly-L-glutamate absorption.

The tentative conclusion may, therefore, be drawn that the helix-coil transition is, in fact, associated with a spectral change of the same general type as that observed on protein denaturation.

FIG. 6. pH dependence of the difference spectrum of poly-L-glutamic acid. Concentration of poly-L-glutamic acid = 0.22 mg per ml. Reference solution at pH 6, sample solutions at the pH indicated. O, in absence of salt; , in 0.16 M NaCl; light path, 10 mm.

Gluca gon—The difference spectra, obtained by comparison of glucagon in concentrated sucrose and lithium chloride solutions with glucagon in water, are shown in Fig. 7. In addition to the peaks at 288 and 292.5 μm, which are presumably due to changes in the absorption spectrum of tyrosine and tryptophan residues, a peak at 230 μm was found on comparison of the solution of glucagon in 52.5% sucrose with the solution of glucagon in water. When lithium chloride was employed, the peak was at 232 μm (see Fig. 7).

It may be noted that near neutral pH it was necessary to work in dilute guanidine hydrochloride solutions (0.5 M) to dissolve the glucagon. The details of the composition of the solutions used are given in the legend to Fig. 7.

Oxidized Insulin A-chain—The effect of sucrose on the A-chain of oxidized insulin was similar to that observed with glucagon. This is shown in Fig. 8. The optical rotatory dispersion studies of Linderstrem-Lang and Schellman (23) indicate that the oxidized A-chain is largely unfolded in aqueous solution.

Tetraglycine—Tetraglycine (10⁻⁴ M) in water was compared with the peptide in 5 M lithium chloride. No peaks were observed in the difference spectrum over the wave length range 220 to 320...
The absorption of tetruglycine in the lithium chloride solution was somewhat lower at wave lengths below 230 m. The absorption of tetruglycine in the lithium chloride solution was somewhat lower at wave lengths below 230 m.

These results indicate that a change in refractive index of the medium does bring about changes in the absorption spectrum of polypeptides in the region of 230 m similar to those observed with proteins. Since no difference spectrum was obtained with tetruglycine, it would seem that a certain minimal molecular size and, perhaps, certain types of side chains, are necessary to produce the difference peak at 230 m.

DISCUSSION

From the investigations reported in this paper, it appears that denaturation of proteins results in some conformational change which is associated with the appearance of a peak in the difference spectrum in the region of 230 m. Although the aromatic amino acids contribute to the over-all absorption of proteins in this region of the spectrum, a number of observations suggest that the 230- to 235-m difference peak cannot be attributed solely to changes in the environment of the aromatic side chains.

The most striking evidence that changes in the environment of aromatic side chains need not give rise to the difference peak at 230 m is given by the difference spectrum obtained by comparing pepsin at pH 5.7 to the protein at pH 7.3. Exposure to pH 7.3 is known to alter the native structure of pepsin and to inactivate it (10, 24). The difference spectrum shows peaks at 278, 256, and 292 m. Other investigators (16-18) have concluded that these peaks unquestionably represent changes in the environment of the tryptophan and tyrosine residues. Yet, examination of the difference spectrum of pepsin, which displays these peaks prominently, fails to reveal any significant change in the region 225 to 245 m. The conclusion may, therefore, be drawn that changes in the tyrosine and tryptophan absorption associated with protein denaturation need not be reflected in the 225- to 245-m region of the difference spectrum.

The kinetics of the denaturation of ovalbumin by urea and guanidine hydrochloride clearly indicate that the rate of change of absorbancy at 22.5 m is different from that at 287.5 and 292.5 m, whereas the changes at the last mentioned wave lengths are essentially synchronous. Finally, there is no correlation between the magnitude of the 230- to 235-m peak and the tryptophan and tyrosine content of the proteins studied (3). It should be emphasized that the examples of pepsin and ovalbumin are selected ones. In most of the proteins examined, e.g., papain and serum albumin, the changes in spectrum produced by denaturation, all occurred so rapidly that no differentiation was possible. Clearly, a study of other proteins should produce additional instances in which a separation of the spectral phenomena occurs at the different wave length regions.

The imidazole group of histidine and the carboxyl groups of the dicarboxylic amino acids absorb in the region of 225 to 230 m. However, the $\Delta m$ values obtained for the changes in ionization of the imidazole group (3) and the carboxyl group are not of the same order of magnitude as the changes observed on protein denaturation. Furthermore, as in the case of tyrosine and tryptophan, no correlation was observed between the histidine or carboxyl content of the proteins studied and the difference peak in the 230 m region.

The finding of a difference peak associated with the helix-coil transformation of poly-L-glutamic acid at 225 m suggests that changes in the conformation of the peptide backbone are reflected in this region of the spectrum.

The fact that a difference peak at 230 to 235 m is obtained on comparing glucagon and the A-chain of oxidized insulin in media of high refractive index with these polypeptides in water also indicates that this region of the spectrum of these polypeptides is influenced by the refractive index of the medium. Deuterium exchange studies on a number of the proteins used in this investigation (25-27) indicate that some of the hydrogen atoms of native proteins exchange very slowly with deuterium. In general, the ratio of slowly exchanging hydrogen atoms to peptide bonds in these proteins is less than 1:2. Even if it is assumed that all of these slowly exchanging hydrogen atoms represent peptide N—H hydrogen atoms, no more than half of the peptide N—H groups could be involved in hydrogen-bonded $\alpha$-helices. On the other hand, little is known about possible conformations in the so-called "random" sections of polypeptide chains in proteins in which hydrogen and hydrophobic bonding could result in the formation of large ring structures with strong absorption in the far ultraviolet. It is likely that disruption of such arrangements could produce the observed spectral changes. Although it is not as yet possible to define in structural terms the exact changes responsible for the observed spectral shift, the available evidence would seem to preclude changes in the conformation of the peptide backbone of the proteins as a possible source of the effects observed.

Support for this interpretation is provided by the work of Simmons and Blout (28) on the ultraviolet rotatory dispersion of the protein subunits of tobacco mosaic virus. The rotatory dispersion of the protein subunits shows the beginnings of a large negative Cotton effect with a trough at 233 m. If the protein is denatured with 8 M urea, this Cotton effect disappears and the optical rotation in the 233-m region becomes much less negative. Blout (29) has reported that this Cotton effect is characteristic of the helical form of polypeptides and proteins as indicated by investigations of the rotatory dispersion of known helical forms of poly-$\gamma$-benzyl-L-glutamate, poly-L-methionine, and poly-$\gamma$-glutamic acid.

It appears very likely that the peak in the 230-m region obtained by us in the difference spectrum on comparing native with denatured proteins represents changes in the position and intensity of the absorption band which is the locus of the Cotton effect in the rotatory dispersion measurements reported by Simmons and Blout (28).

Studies on the far ultraviolet spectra of amides (30), peptides (31, 32), polyaminosacids (33), and proteins (32), both in the solid state and in solution, have indicated that the main absorption band of the peptide group is at about 190 m. This band cannot account for the Cotton effect reported by Simmons and Blout (28). It is also unlikely that changes in this band would be detected in the difference spectrum at 230 m. On the other hand, Peterson and Simpson (34) have concluded from a study of the polarized electronic spectrum of crystalline myristamide that a weak n-II transition of the peptide group is situated at 220 m. Such a band may contribute to the observed effects.

Finally, it should be emphasized that we do not feel that the difference peak in the region of 230 m produced by denaturation of proteins can be attributed to a single phenomenon. There are, undoubtedly, contributions produced by changes in carboxyl group ionization and in aromatic absorption; however, some of the changes appear to represent contributions from alterations in protein conformation other than those mentioned. Clearly, further study on model compounds is necessary before the struc-
tural features giving rise to the difference peak in the 230 m\(\mu\) region can be unequivocally identified.

**SUMMARY**

It has been established that denaturation or proteolysis of proteins results in some conformational change associated with the appearance of a peak in the difference spectrum in the region of 230 to 240 m\(\mu\). Studies on the alkaline denaturation of pepsin have demonstrated that changes in the environment of tyrosine and tryptophan residues as manifested by spectral changes at 278, 286, and 292 m\(\mu\) are not accompanied by a change in the region of 230 to 240 m\(\mu\). These observations show that the conformational change reflected by the spectral difference at 230 to 240 m\(\mu\) involves structural alterations other than those associated with tyrosine and tryptophan.

Studies on the difference spectra of glucagon and the A-chain of oxidized insulin produced by varying the refractive index of the medium are associated with spectral changes in the 230-m\(\mu\) region. Recent evidence indicates that the peak in the 230-m\(\mu\) region in the difference spectrum of proteins is related to changes in the peptide backbone conformation.

**REFERENCES**

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