Spectrophotometric Titration and Ultraviolet Difference Spectra of Myosin and the Meromyosins*

A. STRACHER

From the Department of Biochemistry, State University of New York College of Medicine, Brooklyn, New York

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Spectrophotometric titration studies and ultraviolet difference spectra are two techniques which have been used in recent years for determining what role, if any, the tyrosine residues in a protein play in maintaining the secondary and tertiary structure of proteins.

Crammer and Neuberger (2) utilized a spectrophotometric procedure to investigate the ionization of phenolic groups in ovalbumin and insulin. Similar investigations were carried out with plasma albumin (3, 4), ribonuclease (5, 6), and two derivatives of ribonuclease (7). From these studies it was found that some of the tyrosyl residues do not ionize freely and it was suggested that intramolecular hydrogen bonds between phenolic hydrogens and side chain carboxylate groups could exist in proteins (2). Ultraviolet difference spectra have been used for studying the anomalous tyrosyl residues and in the case of insulin and ribonuclease results which were interpreted as substantiating the hypothesis were obtained (8, 9). Wetaufer et al. (10) working with the model compounds O-methylyrosine and glyceryl-O-methyltyrosine showed that other explanations must be considered. These authors concluded that the small spectral shifts observed when the model compounds were titrated with acid or base could be attributed to a change in the state of ionization of the molecules. Blumenfeld and Perlmann (11) used this as a partial explanation for the spectral shifts observed during the autolysis of pepsin. Bigelow and Ottesen (7), with the use of two derivatives of ribonuclease, suggested that two of the anomalous tyrosyl residues of ribonuclease might be hydrogen bonded to nonionizing acceptors.

As part of a general program relating to some of the chemical and physical properties of the muscle proteins, it was felt that it would be of some interest to carry out such studies on myosin and two fragments produced by the limited tryptic digestion of myosin, L-meromyosin and H-meromyosin (12). These results have shown the existence of abnormal tyrosyl residues in myosin and L-meromyosin whereas the tyrosyl residues in H-meromyosin appear to be normal.

EXPERIMENTAL PROCEDURE

Materials—Myosin from rabbit skeletal muscle was prepared according to the method of Szent-Györgyi (13). The material was precipitated once and then dissolved in 0.6 M KCl. The turbid solution was then dialyzed for 24 hours against 4 liters of a solution containing 0.25 M KCl-0.01 M Tris buffer pH 7.50. The solution containing myosin was then centrifuged and the insoluble residue discarded. The slightly turbid solution was chromatographed on DEAE-cellulose1 according to the procedure of Brahms (14). Myosin prepared in this manner gave water-clear solutions without the necessity of ultracentrifugation. Reaction of the chromatographed material with 1, 2, 4-fluorodinitrobenzene and subsequent acid hydrolysis indicated that myosin prepared in this manner showed a greater homogeneity than a sample of myosin which had been precipitated three times and in which chromatography had been omitted. Only slight traces of dinitrophenyl-amino acids were found in the acid hydrolysate of the chromatographed myosin preparation whereas the three-times precipitated myosin gave significant amounts of dinitrophenyl-amino acids upon hydrolysis. The protein was stored in solution at 4° and was not kept for more than 10 days. Glass-distilled water was used in the preparation of all solutions.

L-meromyosin and H-meromyosin were prepared according to the procedure of Lowey and Holtzer (15) except that the tryptic digestion was carried out in 0.01 M Tris buffer, pH 7.50. Trypsin was obtained as a twice-crystallized, salt-free preparation from Worthington Biochemical Corporation. Soybean trypsin inhibitor was purchased from the same company. Solutions of trypsin and trypsin inhibitor were always prepared just before their use. DEAE-cellulose was a commercial product obtained from the California Corporation for Biochemical Research. Urea and piperidine were purchased from Fisher Scientific Company, and Tris from Sigma Chemical Company.

Methods—All of the ultraviolet absorption measurements were carried out in a Beckman model DU spectrophotometer equipped with a photomultiplier attachment. Measurements were made at room temperature, 25 ± 2°. The spectrophotometric titration studies were made in a solution containing 0.4 M KCl-0.05 M piperidine buffer adjusted to the desired pH with 1 N NaOH. This procedure was essentially that used by Bigelow and Ottesen (7). AD₉₅ (the optical density at the pH in question minus the optical density at neutral pH) was determined and plotted against the pH.

Ultraviolet difference spectra on tryptic digests of myosin were determined by the addition of trypsin to a 0.2% solution of myosin in 0.01 M Tris buffer, pH 7.50 at 25°. The myosin to trypsin weight ratio was 260: 1. In a trial run it was found that the buffering strength of 0.01 M Tris was sufficient to keep the pH of the solution constant during the reaction. The digestion was allowed to proceed for varying lengths of time and then stopped by the addition of sufficient trypsin inhibitor in 0.4 M

1 The abbreviation used is: DEAE-cellulose, diethylaminoethyl cellulose (Sokal Floe).

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KCl to 4-ml aliquots of the reaction mixture. The ultraviolet difference spectra were then determined by comparing the solution in which the reaction had proceeded for varying lengths of time against one in which the reaction was terminated immediately after adding trypsin.

The low pH experiments on L-meromyosin were carried out in 0.15 M KCl. Difference spectra were determined for samples of L-meromyosin at various low pH values by reading the absorbance of the solutions against a neutral solution of equal concentration as a reference. The negative difference in optical density at the maximum of the difference spectrum (287 με) was plotted against pH.

Difference spectra were measured to follow the depolymerization of L-meromyosin in urea (16). The difference spectra were determined by comparing solutions of 0.2% L-meromyosin in a 0.4 M KCl-0.05 M phosphate buffer, pH 7.00, with urea, to solutions which were identical except that no urea was present. The observed data were corrected for the absorption due to urea. The protein solutions in urea were allowed to remain at room temperature for 3 hours before measurements were made.

Protein concentrations were determined by nesslerization (17). Nitrogen concentration was calculated by comparison with standard solutions of (NH₄)₂SO₄. The protein was assumed to contain 16.1% nitrogen (13).

pH measurements were made on a Radiometer pH meter (Model TTT1) equipped with a glass electrode (G202B). All measurements were carried out at 25°C.

Myosin ATPase activity determinations were carried out according to the procedure described by Perry (18).

**RESULTS**

**Spectrophotometric Titration of Myosin**—The spectrophotometric titration of myosin from pH 7.5 to pH 13 revealed two steps in the titration curve as can be seen in Fig. 1. One group of tyrosyl residues (approximately 65% of the total) ionizes normally at a pH of 10.5 (19), whereas the second group titrates at a pH of 12.2. Upon reaching pH 13, neutralization and retitration indicated that a marked irreversible change had occurred in the structure of the molecule by exposure to this high pH. This change can be interpreted as a rupture of tyrosyl hydrogen bonds between the phenolic groups of tyrosine and some basic acceptor group in the molecule, e.g. carboxylate residues. However, as will be discussed later, some degree of caution must be exercised in this interpretation. Other workers have shown that exposure of myosin to alkaline pH values results in irreversible structural alterations of the molecule. Kominz et al. (20) have obtained a protein of molecular weight 29,000 from treated myosin by 0.1 M Na₂CO₃ and Tsaö (21) observed that at pH 10.7 myosin was depolymerized to a large core of 170,000 molecular weight. An interesting feature of Fig. 1 is the effect of urea on the spectrophotometric titration curve of myosin. Exposure of the protein to 6 M urea, pH 7.50, for 24 hours at room temperature had no effect on the titration curve, although the ATPase activity under these conditions is completely destroyed. The titration curve was almost exactly superimposable upon that in 0.4 M KCl. Blumenfeld and Levy (22) found that in the spectrophotometric titration of ribonuclease in 8 M urea a shift of 0.7 unit toward the more alkaline region occurred in the pK_1 (is) of the phenolic groups of tyrosine compared to the pK_1 (is) of these groups in water. Such a shift was not encountered in this study. Bigelow (23) has also observed that a shift did not occur in the pK of the phenolic groups of tyrosyl residues upon titrating growth hormone in urea. At this time no explanation can be given for these differences.

**Spectrophotometric Titration of Meromyosins**—In Fig. 2 can be seen the spectrophotometric titration curve of L-meromyosin. It is apparent that approximately 90% of the tyrosyl residues titrate at a pH near 12.2. The remaining 10% seem to titrate normally. Exposure of L-meromyosin to high pH, neutralization, and retitration results in a normal titration curve, as can be seen in Fig. 2. If L-meromyosin is allowed to remain in a solution of 5 M urea for 24 hours at room temperature, the spectrophotometric titration curve indicates that all of the abnormal tyrosyl residues have been normalized, as shown in Fig. 2. The normalization of these tyrosyl residues in 5 M urea may be associated with the depolymerization of L-meromyosin into smaller subunits under these conditions (18). Whether the effect of high pH results in a depolymerization or a change in the asymmetry of the molecule is currently under investigation.

In Fig. 3 the spectrophotometric titration curve of H-meromyosin shows that all of the tyrosyl residues in this molecule titrate normally with a pK' of 10.4.

**Ultraviolet Difference Spectra Studies**—When the ultraviolet difference spectra of tryptic digests of myosin were studied, only a very small change in -ΔΔabs occurred for the first 20 minutes. Between 20 and 40 minutes a large change took place and no further change occurred after 60 minutes. These results can be seen in Figs. 4 and 5. In Fig. 4 the ultraviolet difference spectra can be seen to have three maxima at 279, 287, and 294 με. The

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changes in the immediate environment of these residues take place with a resultant shift in the absorption spectrum. Longer hydrolysis times produce no further change. These findings are in accord with the results of Mihalyi and Harrington (23), who have shown that only minor changes in optical rotation take place during the formation of the meromyosins by tryptic hydrolysis.

**Low pH Titration of L-meromyosin**—It was of interest to de-

first two peaks have been attributed by others as a shift to shorter wave lengths of the tyrosine absorption maximum as a result of changes in the hydrogen bonding between the tyrosyl hydroxyls and neighboring acceptor groups in the protein (8). The peak at 204 mp has been shown to occur in the difference spectra of proteins which contain tryptophan (11). Fig. 5 shows the results plotted in a different manner. It would seem from these results that during the formation of the meromyosins by limited tryptic hydrolysis no change occurs in the immediate environment of the chromophoric tryptophanyl and tyrosyl residues present in the molecule. When the fragments produced are disrupted by further tryptic hydrolysis,
termine whether the addition of acid to solutions of this protein gives rise to a difference spectrum since this has been shown to occur with other proteins (4, 7–9). When this experiment was carried out, a typical three-peaked difference spectrum was observed and Fig. 6 shows a plot of $-\Delta D_{287}$ against pH. A steady decrease in the absorbancy was observed as the pH was varied from 4.7 to 2.3 and the change was irreversible. Changes similar to this with other proteins have been taken as an indication of the occurrence of hydrogen bonds between the carboxylic groups of the dicarboxylic acid residues in the protein and the phenolic groups of the tyrosyl residues (8, 9).

Although not shown in Fig. 6, myosin also gives rise to a difference spectrum when titrated to low pH. The effect of acid on myosin seem to be irreversible and the ATPase activity is rapidly lost.3

Depolymerization of L-meromyosin in Urea—Since the preceding results had indicated that the tyrosyl residues of L-meromyosin were anomalous for some reason, perhaps due to hydrogen bonding of the phenolic hydrogens to some, as yet, unidentified acceptor groups, it was of interest to examine the effect of urea in producing a difference spectrum. The results are shown in Fig. 7. It can be seen that urea has little effect up to 2.5 M. Between 2.5 M and 5.0 M urea a difference spectrum is produced with an increase in $-\Delta D_{287}$. These results are very similar to those obtained by Szent-Györgyi and BORBRO (16) on the changes in viscosity produced by varying the urea concentration.

**DISCUSSION**

The results presented here indicate that approximately 35% of the tyrosyl residues of myosin and 90% of those present in L-meromyosin are so situated in these proteins as to be accessible to titration with base only when a high pH (above 12) is reached. These facts suggest the existence of intramolecular hydrogen bonds between the phenolic residues of tyrosine and some acceptor group in the molecule. However, as pointed out by Williams and Foster (4) extreme caution must be exercised in accepting the hypothesis that the phenolic residues of tyrosine are hydrogen bonded at all. Other workers have suggested as an alternative explanation that the aromatic chromophores take part in hydrophobic bonding and that upon breaking such bonds these chromophores are exposed to the aqueous environment (4, 6, 26–28).

Since the chemical and physical properties of myosin seem to be an additive function of the properties of the meromyosins (12) it seems likely to assume that the abnormal tyrosyl residues of myosin are associated with the L-meromyosin fragment. In addition, the fact that 6 M urea is not effective in normalizing the abnormal tyrosyl residues in myosin whereas 5 M urea does effect a normalization of these residues in L-meromyosin, would seem to imply that the L-meromyosin fragment is shielded from urea depolymerization in the native molecule. The use of higher urea concentrations than those used in this study may be necessary for normalizing the abnormal tyrosyl residues of myosin. Wetlaufer (29) has shown that in 8 M urea myosin forms three fragments which differ in sedimentation constant. Cohen and Szent-Györgyi (30) have demonstrated a 5-fold increase in optical rotation in 9.5 M urea.

In conclusion it must be emphasized that the spectral changes observed in this study with both myosin and L-meromyosin may simply provide an index of certain other structural rearrangements that are occurring, and the role of the tyrosyl residues in

3 Holtzer and Lowey (24) have shown that at pH 2.9, myosin aggregates extensively and shows polydispersity in the ultracentrifuge. It is possible, therefore, that the spectral changes at the low pH value indicate that the pH-sensitive tyrosyl residues are intermoleculare hydrogen bonded and are a reflection of aggregation. Bonds of this type might well be important in stabilizing molecular aggregates as suggested by Laskowski et al. (25). General light scattering from large molecular aggregates may also contribute to the changes in $-\Delta D_{287}$, as suggested by these authors. Further data would be necessary to decide the contribution of each of these factors in the present investigation.
stabilizing the native configuration may be only a relatively minor one. Indeed, it has been found that the ATPase activity of myosin is rapidly lost upon exposure of this molecule to pH 10.30 at 0° whereas full activity is retained when myosin is exposed to pH 10.15. The fact that the ATPase activity of myosin is irreversibly lost at a pH value less than that necessary for ionization of the anomalous tyrosyl residues would suggest that these residues do not play a significant role in stabilizing the configuration necessary for enzymatic activity. Further evidence comes from a study of the effect of urea on the ATPase activity of myosin. Enzymatic activity is lost rapidly in concentrations of urea even as low as 3 M, whereas the anomalous tyrosyl residues have not been normalized in 6 M urea.

SUMMARY

Spectrophotometric titration and ultraviolet difference spectra studies have provided evidence for the involvement of the tyrosyl and tryptophanyl residues of myosin and L-meromyosin in hydrogen or hydrophobic bonds. No bonding of this type could be found for the H-meromyosin fragment. The abnormal tyrosyl residues of myosin do not become normalized in the presence of 6 M urea, whereas those in L-meromyosin are normalized in 5 M urea.

Digestion of myosin with trypsin produces a difference spectrum only after the digestion has proceeded for a time sufficient to produce the meromyosins.

The possible importance of these abnormal tyrosyl residues in maintaining the configuration necessary for enzymatic activity is discussed.

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A. Stracher


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