Tryptic Hydrolysis of Tropomyosin*

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ABSTRACT: Tropomyosin was digested with trypsin, and the digested materials were separated into fragments by isoelectric precipitation at pH 4.6. The fragment from the precipitate (fragment P) was soluble at neutral pH. A value of 37,000 was obtained for the molecular weight of fragment P by the Archibald technique. A helical content of approximately 60% remained in fragment P in spite of cleavage of peptide bonds, suggesting that the native molecule of rod-like shape has some structurally loose region in the molecule.

When a small amount of fragment P was added to a viscous tropomyosin solution, a marked decrease of viscosity occurred; the result indicates a strong interaction between fragment P and the native molecule. Gel filtration of the supernatant solutions of the digested materials gave two major peaks, one eluted at the front, and the other at a much slower rate. The larger component did not show significant interaction with native tropomyosin.

Tropomyosin, one of the structural proteins in muscle discovered by Bailey (1948), has recently attracted attention, because of its role in muscular contraction, and also because a new protein, similar to tropomyosin, has been found by Ebashi (1963), and this new protein can control superprecipitation of the actomyosin system (Ebashi and Ebashi, 1964). Furthermore, it has been shown that this new protein may be separated into two parts, tropomyosin and troponin, which promote polymerization of tropomyosin (Ebashi and Kuroda, 1966). At the same time, more recent reports appeared on the preparation and properties of tropomyosin with special care taken to prevent oxidation of thiol groups (Mueller, 1966; Woods, 1966), and on the molecular weight of tropomyosin (Holtzer *et al.*, 1965). These studies raise some questions regarding the purity and properties of tropomyosin, even though characteristic features of this protein, such as its high helical content and polymerization property by removal of salts, are essentially not altered by the difference of preparation (Mueller, 1966; Drabikowski and Nowak, 1965).

Another approach to the above problems is the application of chemical analysis for the determination of the purity and structure of the molecule. In this study, the method of limited proteolysis of peptide bonds was applied to tropomyosin; the extent of reaction was examined by means of an ultraviolet spectrophotometric study of tyrosine, since this protein does not contain tryptophan residues (Kominz *et al.*, 1957b; Mei-Hsüan *et al.*, 1965), whereas troponin does (S. Ebashi, personal communication). Trypsin is an enzyme suitable for this purpose, because tropomyosin is easily attacked by trypsin (Laki, 1960) despite a high helical content of this protein (Urnes and Doty, 1961). This property is convenient for structural studies of proteins, as was illustrated by the success of similar studies on myosin (Mihalyi and Harrington, 1959), and several investigations of tropomyosin along this line have been reported (Kay and Smillie, 1964). In this paper, we tried to obtain some fragments from the tryptic digest of tropomyosin, since the part of the molecule attacked by the enzyme is reasonably considered to have a looser structure than the helical parts; this result would be helpful for the determination of the molecular structure.

Experimental Section

Materials. Crude tropomyosin was extracted with 1 M KCl at neutral pH from dried rabbit skeletal muscle obtained after treating with ethyl alcohol and ethyl ether (Bailey, 1948) or after extracting actin (Tsao and Bailey, 1953). The purification process of isoelectric precipitation in the presence of 1 M KCl, followed by ammonium sulfate separation between 47 and 55% saturation, was repeated (usually three cycles for dried muscles after extraction of actin and five cycles or more for dried muscles prepared by Bailey's procedure) until all traces of nucleoprotein were removed (Kominz *et al.*, 1957b); the removal of the nucleoprotein was checked by ultraviolet absorption spectra of solutions obtained after dialysis against water. The ultraviolet spectrum of tropomyosin solution showed a typical absorption curve for tyrosine, having a maximum at 277 mμ and a minimum at 252 mμ. Accordingly, contamination of nucleoprotein in the preparation could be detected by the spectrum at the wavelengths of both maximum and minimum absorption. That is, a ratio of optical densities at both wavelengths was approximately 3 for the purified protein, whereas the ratio became smaller due to strong absorption by the nucleotide when a small amount of nucleoprotein re-

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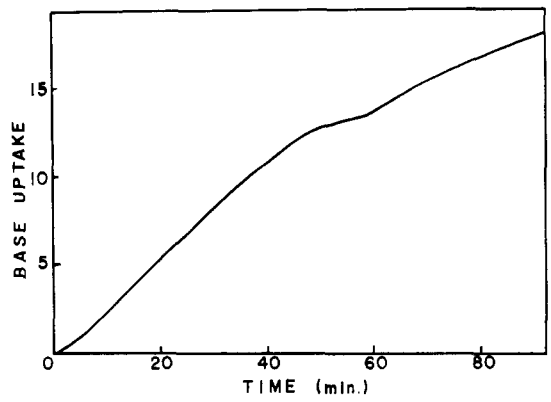

FIGURE 1: Base uptake as a function of time after addition of trypsin (1 mg) to a tropomyosin solution (60 ml of a concentration of 5 mg/ml) at 25°. Base solution: KOH (0.2 M); OH^- is represented as equiv mole/10^5 g of protein.

mained in the preparation. This was the criterion for purity of tropomyosin in this study.

Protein concentration was measured by the optical density at 277 mμ, using an extinction coefficient of 2.5 for a concentration of 10 mg/ml in 1-cm cell. This value seemed to be a reasonable one as expected from the number of tyrosines in the molecule (Kominz *et al.*, 1957a).

Trypsin was obtained in a two-times-crystallized form from Sigma Chemicals Co. (lot 123-335). The activity was checked by using benzoyl arginyl ethyl ester as a substrate according to Scwert and Takenaka (1955).

Sephadex G-75 was purchased from Pharmacia Co. Other chemicals of reagent grade were obtained from Katayama Chemicals Co. Ammonium sulfate of recrystallized form was used.

Ultraviolet absorption was measured by a Zeiss PMQ II spectrophotometer, using 1-cm quartz cells.

Sedimentation experiments were carried out with a Spinco Model E analytical ultracentrifuge. The determination of molecular weight by the Archibald method was carried out at 20,410 rpm, and the sedimentation constant (S) was obtained at 59,780 rpm. Temperature was controlled by an ATIC temperature control unit.

Diffusion constant (*D*) was estimated from the time dependence of patterns in a synthetic boundary cell at 20,410 rpm with Faxen's approximation (Faxen, 1929).

Measurements of viscosity (η) were carried out by the use of Ostwald-type viscometers, having flow times of 20 and 84 sec for the solvent at 25°.

Optical rotatory dispersion was measured by a Rudolf Model 80 polarimeter at wavelengths of 366, 414, 436, 546, and 579 mμ. The helical content was determined by the b_0 value of the Moffit equation on assuming 630° for 100% helix.

Kerr effect was measured by the instrument reported elsewhere (Kobayashi *et al.*, 1964).

Digestion of tropomyosin with trypsin was carried out in the presence of KCl (0.01–0.2 M) in a pH-Stat (Radiometer TTT1) at pH around 8 where the activity

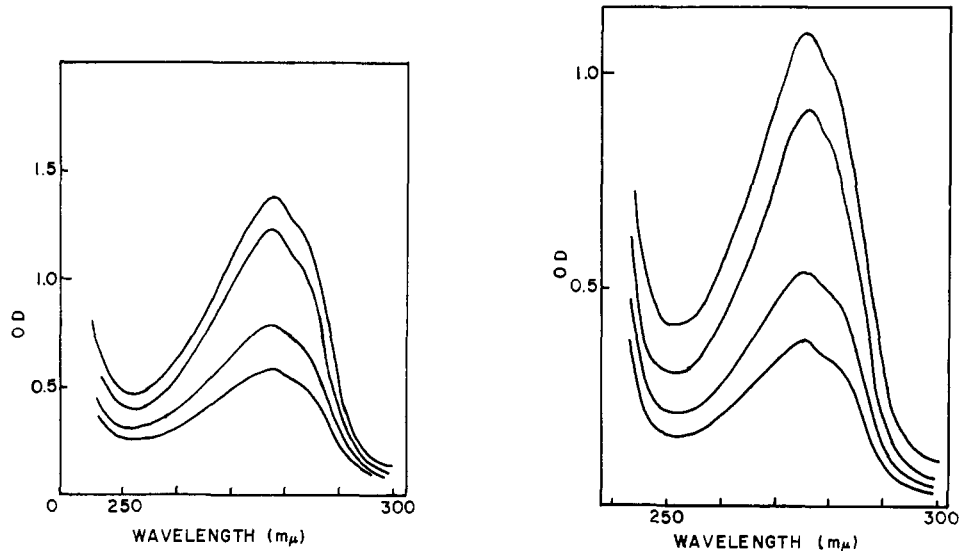

FIGURE 2: Ultraviolet spectra for solutions after separation of the precipitate and the supernatant by isoelectric separation at pH 4.6. The curves were obtained for various time intervals of digestion. (a) (left) Solutions of the precipitates dissolved in 0.01 M KCl–0.05 M phosphate buffer (pH 6.9). Time intervals: 12, 35, 80, and 125 min, from top to down, 8 ml of the reacted solution was drawn and the precipitate was dissolved in 5 ml of phosphate buffer, so that the concentration increased by a factor of 8:5. (b) (right) Supernatants at pH 4.6. Time intervals: 12, 60, 80, and 125 min, from down to top.

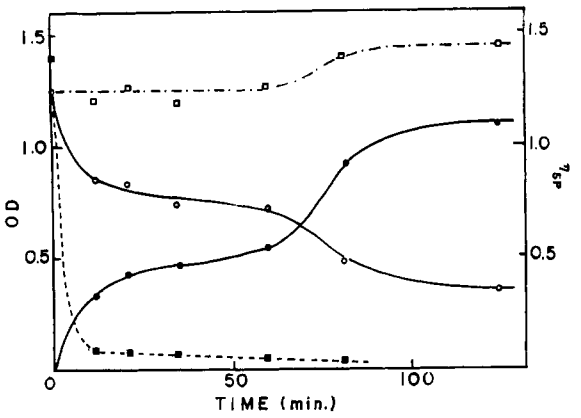

FIGURE 3: The amounts of the fragments in the precipitate (○) and in the supernatant (●) expressed in optical density at 277 mμ at various time intervals after addition of trypsin. The optical density for the precipitate was corrected by a factor of 5:8 to match the original solution scale. The addition of both curves is shown by a chain line (□). The viscosity for solutions of the precipitate (■) is represented by a broken line.

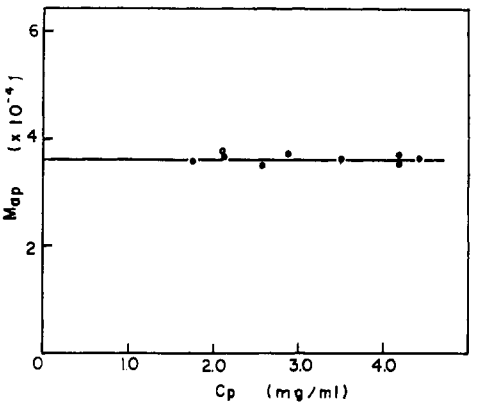

FIGURE 4: Apparent molecular weight (M_w) of the fragments obtained by Archibald technique. KCl (0.05 M)–phosphate buffer (0.01 M) (pH 6.9). Temperature, 17°. (○) 12-min digestion, (⊙) 35-min digestion, and (●) 60-min digestion.

of trypsin is optimum. Usually, a ratio of enzyme to protein of approximately 1:300 was selected. A time course of digestion was recorded by the amount of uptake of a base solution; the proper concentration of KOH used depended on the amount of the protein in the solution. In order to stop the reaction, the pH was lowered by the addition of acetate buffer (pH 4.6) to a final concentration of 0.01 M, instead of employing soy bean trypsin inhibitor, since trypsin has no activity at this pH. When the pH was lowered, isoelectric precipitation of tryptic fragments occurred, as in the case of tropomyosin, and the precipitate was spun down, followed by washing twice with acetate buffer (0.01 M) to remove a trace of trypsin remaining in the solution. The precipitate was dissolved in water by raising the pH to neutrality by the addition of an alkaline solution. The solutions were dialyzed against water. The final solution, after dialysis, was used immediately for the measurements or stored in dried form after lyophilization. The supernatant solution was put on a Sephadex G-75 column of 2 × 70 cm employing 0.05 M ammonium acetate as a supporting solvent, and the eluted solutions were collected into tubes of a fraction collector. After reading the optical density of the tubes at 276 mμ, those at the peaks were combined and dried by repeated lyophilization until the ammonium acetate was removed.

Results

A time course of digestion, after addition of trypsin to the solution, is illustrated in Figure 1, where the base uptake/10^5 g of tropomyosin is represented as a function of time. After the addition of trypsin, digestion proceeded almost linearly with time for about 50 min, and the rate became smaller around 60 min. Then, further base uptake occurred beyond this region, suggesting that some change in molecular state took place during digestion. When the ratio of the protein to the enzyme was increased, the rate of digestion increased, but the shape of the curve was similar to that shown in Figure 1. Also the features of the curve were not changed at various salt conditions in the range from 0.01 to 0.2 M KCl, although the initial viscosity of the solution decreased markedly with the raising of the salt concentration.

The pH of aliquots drawn out from the reacting solutions at various time intervals was lowered to the isoelectric point (4.6), and precipitates and supernatants were separated by centrifugation. The precipitates were dissolved in a given volume of solvent which contained 0.01 M KCl and 0.05 M phosphate buffer (pH 6.9). The ultraviolet absorption spectra of the solutions in which the precipitates were dissolved, and those of the supernatants at various intervals are shown in Figure 2a,b. The spectra have a maximum at 277 mμ for the precipitates and at 276 mμ for the supernatants, both having typical absorption spectra for tyrosine.

The amount of components, which corresponds to the degree of digestion, as a function of time could be estimated by the peak heights of the spectra of the solutions because the optical density at the peak is considered to represent the concentration (see next section). The result is shown in Figure 3. As expected, the amount of precipitates decreased and that of the supernatants increased with increasing digestion time for about 10 min, and then production of the precipitate became slower until 60 min, forming a relatively flat plateau region. After 60 min, where the rate of the base uptake became greater again as shown in Figure 1, the amount of the precipitate decreased and the corresponding amount in the supernatant increased sig-

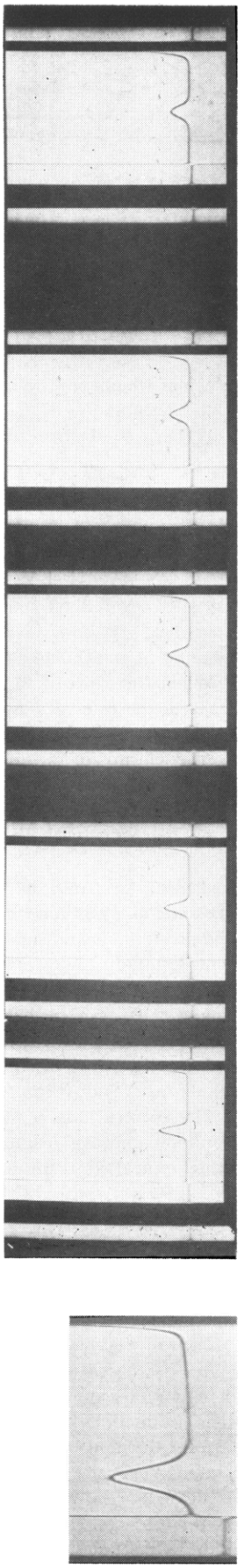

FIGURE 5: Sedimentation and schlieren patterns. (a) Sedimentation pattern for fragment P obtained by the isoelectric precipitation (35-min digestion). Photograph was taken 73 min after reaching maximum speed of 59,780 rpm; bar angle, 70°. Protein concentration, 12.8 mg/ml in 0.05 M KCl–0.01 M phosphate buffer (pH 6.9). (b) Schlieren patterns of diffusion experiments at 20,410 rpm. Protein concentration, 4.0 mg/ml in 0.05 M KCl–0.01 M phosphate buffer (pH 6.9); temperature, 13.5°. Time interval, 8 min; bar angle, 70°.

nificantly. The sum of both curves remained constant from the beginning to 60 min, but an appreciable increase was observed between 60 and 70 min. These results suggest that the digestion occurs in two steps. The yield of the precipitate between the time range of 10–60 min estimated from the optical density is approximately 60%.

Molecular Characteristics of the Fragment. The concentration of the precipitate (fragment P) was determined by the optical density at 277 mμ. A value of 0.27 was obtained for the extinction coefficient with 1-cm path length at 277 mμ for a concentration of 1 mg/ml of fragment P by calibration with the dry weight. As shown in Figure 4, extrapolated values of the apparent molecular weights of the precipitates obtained by the Archibald technique, from short-time digestion (15 min) and from medium-time digestion (35 and 60 min) were the same (37,000). The concentration dependence of the apparent molecular weight was not significant. Sedimentation constants for precipitates of short and medium digestion were also the same value of 2.3 S, showing no appreciable concentration dependence. The sedimentation pattern shown in Figure 5a seems to be approximately symmetrical, indicating homogeneity of the precipitate, and the material obtained by isoelectric precipitation is considered to be nearly monodisperse and to have the same order of molecular weight regardless of the digestion time.

In contrast with the high viscosity of the original tropomyosin, fragment P has low viscosity in the same salt concentration, and a value of 0.12 dl/g was obtained for the intrinsic viscosity. Since the sedimentation constant and the intrinsic viscosity are known, a molecular weight and an axial ratio can be estimated by measuring a diffusion constant which could be obtained by the use of a synthetic boundary cell in the sedimentation experiments (Schachman, 1959). Sometimes, hypersharpening of the ultracentrifuge pattern is a problem in diffusion calculations, but such hypersharpening was not observed for solutions of fragment P as shown in Figure 5b. A diffusion constant was estimated from the extrapolation of D to infinite dilution. Assuming a partial specific volume of 0.73 for fragment P, we obtained the average molecular weight of 38,000 by combination of the values, s_{20} = 2.3 S, $[\eta]$ = 0.12 dl/g, and $D_{20} = 5.35 \times 10^7$ cm²/sec. This value is in agreement with that described previously. An axial ratio of 1:7 for an ellipsoidal molecule is estimated by the β function (Scheraga and Mandelkern, 1953).

Since tropomyosin is a typical molecule which has a high helical content, measurements of optical rotatory dispersion were performed for the purpose of examining the extent of helical content remaining in the fragment. From the b_0 value of Moffitt–Yang plots for a solution of fragment P, the helical content was calculated as 60%, indicating that a part of the native structure remained in fragment P.

Separation of Small Components from the Supernatant. As seen in Figure 3, the amount of an acid-soluble

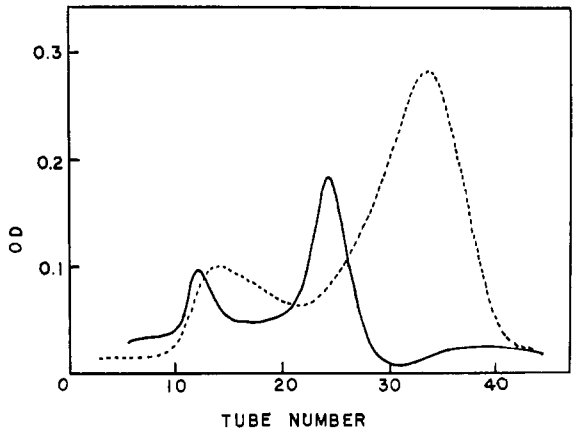

FIGURE 6: Chromatograms on Sephadex G-75 column for the supernatant of short-time digestion (full line) and of medium-time digestion (broken line).

part of the digested materials at pH 4.6 increased with the progress of digestion. Probably further digestion of acid-soluble components in the solution occurred. Separation of two (large and small) components of the supernatant by gel filtration, is shown in Figure 6 for the supernatants of short- and medium-time digestions. A larger component appeared at the front of elution, and a smaller component was eluted at a retarded position in both cases. The position of the smaller component for medium-time digestion is around the middle of the chromatogram, whereas that for short-time digestion is almost at the end of the chromatogram, providing evidence of cleavage of smaller fragments released from parent molecules. Also the asymmetrical shape of the peak suggests a wide distribution of several sizes of the produced components.

Interaction of Fragments with Tropomyosin. As described above, fragment P retains the native structure of tropomyosin to some extent, as judged from the helical content, so that some interaction is expected between this fragment and tropomyosin. Since polymerization of tropomyosin is very sensitive to salt concentration, especially at low ionic strength, a salt condition was chosen around 0.01 M so as to distinguish the effect of fragment P from a salt effect. In Figure 7, the viscosity of the mixture of tropomyosin and fragment P is plotted against the ratio of the added fragment to tropomyosin. The viscosity falls rapidly upon increasing the ratio and reaches the value for a depolymerized molecule at an equimolar ratio. The measurement of the Kerr effect on the same solutions gave a similar curve for the relaxation time, which corresponds to the particle length. These results indicate that depolymerization occurs on the addition of fragment P. On the other hand, the larger component from the supernatant which appeared at the elution front did not have such a remarkable effect on depolymerization. However, it is difficult to conclude whether some depolymerization, observed by the addition of the component, was due to the presence of interaction between the component and tropomyosin or simply to the salt effect.

Discussion

From the result described above, the sequence of tryptic digestion could be interpreted as follows. First, cleavage of peptide bonds in the structurally loose region occurs and separates the molecule into two parts, fragment P which can be precipitated by lowering the pH, and the other small fragment which is acid soluble. Second, further cleavage takes place on the smaller fragment producing several components

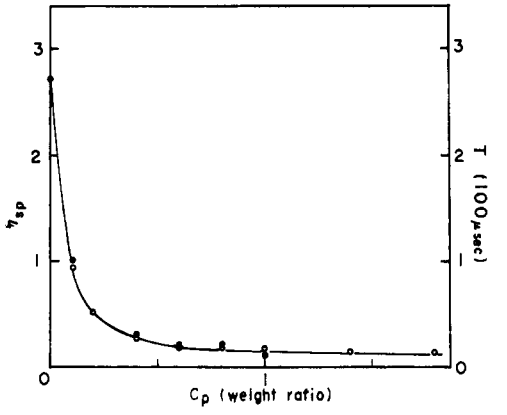

FIGURE 7: Effect of fragment P on polymerization of native tropomyosin. (○) Specific viscosity. (●) Relaxation time from Kerr effect. Tropomyosin concentration: 1.5 mg/ml, 0.007 M phosphate buffer (pH 6.9) at 25°. The fragment was added to the tropomyosin solution so as to give the constant tropomyosin concentration of 1.5 mg/ml, *i.e.*, C_p corresponds to the amount of the fragment added.

(also presumable on fragment P at a much slower rate), and finally fragment P is digested at a faster rate. Therefore, the linear increase in base uptake observed in the earlier region shown in Figure 1 does not represent the production of fragment P directly, but comes mainly from further digestion of smaller fragments, as suggested by the results in Figure 6.

The spectra shown in Figure 2 for the precipitate and the supernatant have maxima at 277 and 276 mμ, respectively. Since the native tropomyosin has a spectral maximum at 277 mμ, the environment of tyrosine residues for fragment P does not seem to be different from that of the native molecule, whereas the smaller fragments in the supernatant have more exposed tyrosines as judged from the blue shift of the tyrosine spectrum. This blue shift of the spectrum for the smaller fragments suggests that the tyrosine residues in the native molecule or fragment P may be buried to some extent in the hydrophobic region or are hydrogen bonded to another part of the molecule, although no buried tyrosine is suggested by spectrophotometric titration (Lowey and Kucera, 1964).

It might be of interest to do similar experiments at a low salt concentration where tropomyosin molecules polymerize to long fibrous ones in order to examine the difference of digestion rate on degree of polymerization. However, such an experiment is difficult because the solution is too viscous to get uniform mixing after addition of the enzyme. When the solution is diluted to avoid this difficulty, the molecules depolymerize on dilution (Maruyama, 1959; Ooi *et al.*, 1962). Therefore, the dependence of the digestion rate on salt concentration is limited to examination by the aforementioned technical problem, and an appreciable difference was not observed in the range of 0.01–0.2 M KCl.

From the molecular weight of fragment P, it is suggested that the structurally loose regions for enzymatic attack are located two-thirds apart from the end of the molecule when the molecular weight of tropomyosin is 54,000 (Tsao *et al.*, 1951; Kay and Bailey, 1960; Ooi *et al.*, 1962), and one-half when a value of 74,000 (Holtzer *et al.*, 1965) is accepted for a correct molecular weight. Since the yield of fragment P was nearly two-thirds of the material, the former value is rather likely for the molecular weight of tropomyosin used in this study, which was purified on the basis of the ultraviolet spectrum for tyrosine residues. However, further study seems to be necessary for molecular characteristics of purified tropomyosin.

The result that fragment P can depolymerize tropomyosin polymers may permit the following interpretation for the polymerization mechanism of tropomyosin. A high helical content of this protein indicates a stable structure of a monomer molecule, so that little conformational change would be expected during polymerization.

In addition, quick depolymerization by the addition of salt suggests that the force responsible for polymerization is mainly electrostatic in nature as proposed by Bailey (1954). Since polymerization of three monomers results in an increase of monomer length, one-half of the molecules would be overlapping on polymerization (Ooi *et al.*, 1962). From these considerations it is deduced that heterogeneous charge distribution on the monomer molecule plays an important role in polymerization. Therefore, fragment P which retains some binding capacity, despite the loss of a part of the native molecule, would bind to monomers to terminate polymerization because another polymerization site may be lost in fragment P through cleavage with tryptic hydrolysis. The preliminary sedimentation experiments for the mixture of tropomyosin and fragment P showed that the mixture moves almost as a single peak and its molecular weight is estimated to be about 100,000 by the Archibald technique, a result which is consistent with the interpretation described above. It is hoped that further studies by chemical analysis, such as amino acid analysis on fragment P and on tropomyosin now in progress, will clarify the ambiguity about the molecular weight, the structure of the tropomyosin molecule and its polymerization mechanism.

Finally it is added that the results were not altered on tropomyosin prepared with a special care for preventing thiol groups from oxidation (Mueller, 1966).

Acknowledgment

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Cross-Linking of Bovine Pancreatic Ribonuclease A with Dimethyl Adipimidate*

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ABSTRACT: Modification of bovine pancreatic ribonuclease A with [^{14}C]dimethyl adipimidate, a bifunctional imido ester, resulted in the cross-linking of lysine ϵ-amino groups with a concomitant increase in enzymic activity. Dimers and higher molecular weight derivatives arising from intermolecular reactions were separated from intramolecularly cross-linked monomeric species by gel filtration.

The isolated monomeric components were characterized in terms of molecular weight homogeneity, the extent and specificity of the reagent incorporation, and enzymic activity. A monomeric derivative with a specific activity of 160, compared to 100 for the native enzyme, was degraded by performic acid oxidation and trypsin digestion, and the tryptic peptides were fractionated by preparative paper electrophoresis and chromatography. Two cross-linked peptides, representing at least 50% of the total cross-links in the derivative, were isolated and their structures demonstrated the presence of Lys 31–Lys 37 and Lys 7–Lys 37 cross-links in the enzymatically superactive amidinated ribonuclease.

The introduction of covalent, intramolecular cross-links into proteins of known amino acid sequence by reaction with bifunctional reagents and the subsequent identification of the location of the cross-link provide a direct chemical method for determining interresidue distances in biologically active proteins in dilute aqueous solution (Zahn and Meienhofer, 1958; Hiremath and Day, 1964; Marfey *et al.*, 1965a,b; Fasold, 1965). Although it is highly improbable that the complete three-dimensional structure of any protein can be solved by this method, the determination of distances between several pairs of residues in a given protein should greatly reduce the number of possible conformations one needs to consider in constructing three-dimensional models of that protein. The kind of information one can obtain from the characterizations of cross-linked proteins should have a very immediate significance in providing answers to the question of whether or not there are significant differences in the structure of a protein in solution as compared to its structure, determined by X-ray diffraction, in the crystalline state.

A wide variety of bifunctional protein reagents, each with its own set of advantages and disadvantages in terms of specificity, reactivity, solubility, and stability, have been studied (for a review, see Wold, 1967). The diimido esters represent a relatively new and attractive addition to the list of such reagents. Hunter

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