V. 1. CYSTEINE AND CYSTINE CONTENT OF MUSCLE PROTEIN FRACTIONS

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Muscle is our main source of fibrous α-proteins which can be brought readily into solution. Preparations which retain their biological activities and do not show obvious signs of denaturation can be easily obtained. Myosin, tropomyosin, and paramyosin are members of the KMEF-class of proteins, and films or fibers prepared from these purified proteins show the characteristic 5.1 Å meridional reflection in wide-angle X-ray studies.

Myosin is the main protein constituent of most muscles; tropomyosin is present in small concentrations in a wide variety of species; paramyosin is a major protein fraction of the peculiar muscles of annelids and molluscs, characterized by very prolonged contraction. When myosin is fragmented by controlled treatment with proteolytic enzymes into two non-equivalent portions, heavy- and light-meromyosin, these different components retain both the α-helical configuration and some of the active centers responsible for the various functions of the intact myosin. In general, the helix content of these proteins and protein fractions is high, some of them behave as a fully coiled α-helix, as judged from rotatory dispersion studies (*1*). Thus the structural proteins of muscle are particularly suitable for a study of some of the necessary requirements for the maintenance and stability of the α-helical polypeptide chain configuration in proteins.

In this study we tried to obtain information on whether cysteine and cystine residues have a major role in the stability of helical configuration of the α-proteins of muscle. Table I lists the data for the —SH content

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TABLE I

CYSTEINE AND CYSTINE CONTENT OF MUSCLE PROTEINS

	Equivalent SH in 10^5 gm.	Equivalent cysteic acid in 10^5 gm.[a]	Calculated equivalent cystine in 10^5 gm.	Calculated equivalent cystine per mole	Weight % helix[b]
Tropomyosin	3.2	6.5	1.65	1.0	94
Paramyosin	<0.5	—	—	—	91
Myosin	7.4	8.6	0.6	2.5	56
Heavy-meromyosin	8.5	10.9	1.2	2.8	45
Light-meromyosin	4.3	5.6	0.65	0.8	74
Light-meromyosin Fraction I.	0.0	—	—	—	100
Depolymerized light-meromyosin soluble	0.0	—	—	—	—
Depolymerized light-meromyosin insoluble	0.0	—	—	—	—

[a] D. R. Kominz, A. Hough, P. Symonds, and K. Laki, *Arch. Biochem. Biophys.* **50**, 148 (1954).

[b] A. G. Szent-Györgyi and C. Cohen, *Science* **126**, 697 (1957).

measured by the amperometric silver titration. The values were the same both in the presence and absence of 8 *M* urea. When silver was added in slight excess the reading reached the final value within two minutes. The reactivity of —SH groups towards silver was excellent, and there was no interfering effect of "masking" as observed with other reagents like porphyrindin, monoiodoacetic acid, or *N*-ethylmaleimide. Neither was the suppression of reactivity of the —SH groups of myosin by ammonium ions obvious with silver titration. In the presence of 1.8 *M* NH_4NO_3 over 90% of the —SH groups reacted readily.

The values listed on Table I are in good agreement with some of the values reported previously in the literature. Kominz *et al.* (*2*) measured 7.4 equivalent —SH per 10^5 gm. myosin by methyl mercury nitrate titration. For tropomyosin 3.0 —SH per 10^5 gm. protein was reported when the alcohol-ether-dried muscle was shaken with Bennett's reagent and the ammonium sulfate used for precipitating the protein was subjected to purification on a resin column (*3*). In our experiments the same value was obtained without such steps.

The —SH groups are not uniformly distributed within the myosin molecule. The —SH content of heavy-meromyosin is the highest, while the light-meromyosin Fraction 1, which represents about 25% of the intact myosin with a molecular weight of 110,000–120,000, has none. The

proportionate sum of light- and heavy-meromyosin falls somewhat below the value found for myosin.

Prolonged incubation of light-meromyosin in urea causes the disappearance of —SH groups. During this treatment the molecule falls apart into units of molecular weight somewhat below 5,000, and after removal of urea about 25% of the protein precipitates (*4*). All the —S—S— cross-linkages formed reside in this portion since this is the fraction which gives a positive nitroprusside test after reaction with cyanide. The zero titration values of the silver titration on the urea treated light-meromyosin, on light-meromyosin Fraction 1, and the nearly zero values on paramyosin, are a check on the specificity of the method indicating that the only side chain with which silver reacts under the conditions of these experiments is the —SH group.

Table I lists for comparison the cysteic acid content of some of the proteins obtained after performic acid oxydation by Kominz *et al.* (*2*). The difference between the values of SH and cysteic acid may be used to estimate the upper limit of cystine content. The calculations indicate that there are relatively few —S—S— cross-linkages present. None of the proteins studied have more than two cystine per 100,000 gm. With a molecular weight of 60,000 tropomyosin is the richest in cystine, containing one cross-linkage per molecule.

The helix content obtained previously from rotatory dispersion studies is shown in the last column of Table I. The highly helical molecules of tropomyosin, paramyosin, and the "crystalline" component of light-meromyosin are of particular interest. The low —SH titration values of paramyosin and the zero values of light-meromyosin Fraction 1 allow an easy way to check for the presence of —S—S— cross-linkages with the aid of the nitroprusside test. The lack of color development after 15 minutes' incubation in 0.5 *M* NaCN indicates the absence of cystine as well as cys-

TABLE II

NITROPRUSSIDE TEST

	5 *M* urea	15 min. in 0.5 *M* NaCN	15 min. in 0.5 *M* NaCN and 5 *M* urea
Depolymerized light-meromyosin soluble	negative	negative	negative
Depolymerized light-meromyosin precipitate	negative	strongly positive	strongly positive
Paramyosin	traces?	traces?	traces?
Light-meromyosin Fraction I	negative	negative	negative

teine in paramyosin and light-meromyosin Fraction 1 (Table II). Thus the presence of —S—S— cross-linkages is not a necessary requirement for the stability of the α-helical configuration in proteins. In fact, those α-proteins which have the highest helix content contain no, or maximally one, —S—S— cross-linkage on a mole basis.

Methods and Materials

The amperometric silver titration was performed according to the procedure of Benesch *et al.* (*5*) using 2 *N* KNO_3 in place of saturated KCl in the bridge connecting the electrode fluid with the protein solutions.

All reagents used were Analytical Grade.

Urea was purified with mixed cation-anion exchange resin (*6*).

Myosin was prepared according to Szent-Györgyi (*7*), omitting the bicarbonate treatment necessary for the removal of actin contamination. Actin content was below 1% as determined viscosimetrically in the presence and absence of ATP.

Heavy-meromyosin and light-meromyosin were prepared as described previously (*8*). Lipids were removed from myosin, light-meromyosin, and heavy-meromyosin by centrifuging the protein solutions for 2 hours at 35,000 r.p.m. The lipid particles accumulated at the top of the centrifuge tubes and were separated by filtration through several layers of gauze.

For the preparation of tropomyosin Bailey's procedure (*9*) was closely followed. The crystallization and final drying were omitted.

Light-meromyosin Fraction 1 was obtained from light-meromyosin which was precipitated with three volumes of 95% ethanol. The precipitate was resuspended in 0.6 *M* KCl, and the suspension dialyzed overnight against 10 volumes of 0.6 *M* KCl. After centrifugation the protein was crystallized by dialyzing the supernate against 12 volumes of water at 0°. The crystals were collected by centrifugation, redissolved in 0.6 *M* KCl, and recrystallized by reducing the salt concentration. Before lyophilization, KCl was removed by extensive dialysis against water. Before measurements, the lyophilized protein was dissolved in 0.6 *M* KCl.

Paramyosin was obtained from the tinted and white portions of the adductor muscle of *Venus mercenaria.* The muscle was homogenized in a Waring blendor in 0.1 *M* KCl and washed three times in 10 volumes of 0.1 *M* KCl. The residue was extracted with 0.6 *M* KCl in the presence of 0.01 *M* tris buffer of pH 7.4 for 10 minutes. To the extract 2 volumes of 95% ethanol was added, the precipitate was resuspended in 0.6 *M* KCl and dialyzed against 10 volumes of 0.6 *M* KCl, containing 0.01 *M* pH 7.4 tris buffer. The precipitate was removed by centrifugation and the paramyosin crystallized from the supernate by dialysis against 6 volumes of 0.01 *M* phosphate buffer, pH 6.5. The crystals were redissolved in 0.6 *M*

KCl at pH 7.4 and crystallization repeated. Paramyosin was lyophilized from 0.6 *M* KCl. Presence of salt during lyophilization facilitated redissolution. 0.6 *M* KCl was added to the dried protein and it was gently shaken at 0° overnight. The small amount of insoluble portion was removed by centrifugation and the supernate dialyzed against 0.6 *M* KCl. This procedure is a combination of Bailey's "wet method" and "alcohol method" (*10*).

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