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Amino Acid Sequence around the Single 3-Methylhistidine Residue in Rabbit Skeletal Muscle Myosin*

Gabor Huszar and Marshall Elzinga

ABSTRACT: As part of a study of the functional importance of methylated amino acids in myosin, a peptide that contains one residue of 3-methylhistidine has been isolated from rabbit skeletal muscle myosin. Enzymatically inactive subfragment-1 was prepared from white skeletal muscle myosin of adult rabbits by a modified procedure which utilizes ethanol precipitation. The subfragment-1 was reduced, S-alkylated using iodoacetamide, cleaved with cyanogen bromide, and finally digested with trypsin. After the chemical and enzymatic degradation the peptide mixture contained about 160 peptides; from this mixture the 3-methylhistidine peptide was isolated by a combination of gel filtration on Sephadex G-25 and Bio-Gel P-6, and ion-exchange chromatography using Dowex-50 and phosphocellulose. The amino acid sequence was determined to be: Leu-Leu-Gly-Ser-Ile-Asp-Val-Asp-3-methylhistidine-Gln-Thr-Tyr-Lys. The overall uncorrected recovery of the 3-methylhistidine peptide was about 40%. There was no indication of other peptides that contained 3-methylhistidine, or heterogeneity in the 3-methylhistidine peptide. The sequences around the single 3-methylhistidine residues in actin and myosin are different.

At least three unusual amino acids, methylated derivatives of histidine and lysine, are present in acid hydrolysates of myosin. Trayer *et al.* (1968) found about 2 moles of 3-methylhistidine [2-amino-3-(1-methyl-4-imidazolyl)propanoic acid] per 500,000 g of adult rabbit "white" skeletal muscle myosin. In addition to 3-methylhistidine, myosin contains methylated lysines (Hardy and Perry, 1969; Huszar and Elzinga, 1969a); adult rabbit white skeletal myosin contains both ϵ-*N*-monomethyllysine and ϵ-*N*-trimethyllysine in a ratio of about 1:2 (Kuehl and Adelstein, 1969).

The myosin molecule is generally believed to be composed of two heavy chains each having a molecular weight about 200,000 and two or three light chains with molecular weights of about 20,000. Limited proteolytic digestion of myosin liberates two large fragments, light meromyosin and heavy meromyosin. Further enzymatic cleavage of heavy meromyosin results in the formation of two molecules of heavy meromyosin subfragment-1 and one heavy meromyosin subfragment-2. Each subfragment-1 contains a portion of the heavy chain of myosin and one or two small subunits (Lowey *et al.*, 1969; see also recent reviews, Gergely, 1966, and Young, 1969). Both 3-methylhistidine and the methylated lysines have been localized in subfragment-1, which represents the globular part of the myosin molecule and contains both the ATPase and actin combining sites of myosin (Johnson *et al.*, 1967; Huszar and Elzinga, 1969a; Kuehl and Adelstein, 1969). The light chains of subfragment-1 were shown to contain no methylated amino acids (Huszar and Elzinga, 1969b; Kuehl and Adelstein, 1970).

Actin also contains 3-methylhistidine (Asatoor and Armstrong, 1967; Johnson *et al.*, 1967); there is one residue of the amino acid in the polypeptide chain and recent sequence studies have shown that it represents a single, fully methylated histidine (Elzinga, 1970, 1971; Adelstein and Kuehl, 1970). Thus actin and subfragment-1 of myosin, which are believed to interact during cross-bridge formation, each contain 3-methylhistidine, although there is no evidence that 3-methylhistidine is directly involved in this interaction.

The content of 3-methylhistidine has been found to vary depending upon the source of the myosin (Johnson *et al.*, 1969; Kuehl and Adelstein, 1970); "red" skeletal muscle myosin and cardiac muscle myosin contain essentially no methylated histidine. It has also been shown that myosin prepared from skeletal muscle of newborn rabbits has no 3-methylhistidine and has lower ATPase activity than adult myosin (Trayer *et al.*, 1968; Trayer and Perry, 1966). The appearance of 3-

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methylhistidine in the molecule during the first month of life coincides with an increase in ATPase activity. The amount of 3-methylhistidine in actin seems to be constant regardless of the source.

As a part of the study directed toward the elucidation of the functional importance of methylated amino acids in actin and myosin, we report here the isolation of a 3-methylhistidine-containing tridecapeptide in yields of about 40%, and the results of the determination of its amino acid sequence. In the light of this report the following questions are discussed. (1) Is all of the 3-methylhistidine in myosin located at a single position in the main polypeptide chain? (2) Is there any similarity between the 3-methylhistidine peptides from myosin and actin from which conclusions can be drawn concerning (a) evolutionary relationships between these two myofibrillar proteins, as suggested earlier (Laki, 1964); and (b) the nature of the recognition site for the methylating enzyme?

Methods

Preparation of Myosin. Myosin was prepared by a modification of the procedure in which ammonium sulfate fractionation is used for purification (Tsao, 1953; Sreter *et al.*, 1966a). Adult white rabbits of the New Zealand White strain were used in all experiments described in this work. Myosin preparations were carried out at 0–4° and all solutions contained 1 mM EDTA.

A mince of rabbit thigh and back muscle was washed with 0.5 M phosphate buffer, pH 6.5. It was then extracted with 3 volumes of Guba-Straub solution (0.3 M KCl–0.1 M KH_2PO_4–0.05 M K_2HPO_4, pH 6.85 adjusted with 50% KOH) and after 13 min an equal volume of H_2O was added. The diluted extract was filtered through cheesecloth and within minutes 6.5 volumes of distilled water was added. The crude precipitate was centrifuged at 1300*g* and then redissolved in 0.6 M KCl–10 mM Tris, pH 7.0. The solution was then brought to 4 mM ATP and 6 mM $MgCl_2$ to dissociate any actomyosin present and was centrifuged at 100,000*g* for 40 min to remove F-actin (Weber, 1956). Saturated ammonium sulfate was added to the supernatant and the fraction that precipitated between 37 and 50% saturation was collected. The precipitate was dissolved in 0.5 M KCl–10 mM Tris, pH 7.0, and dialyzed against several changes of the same solvent. The myosin was finally clarified by centrifugation at 120,000*g* for 40 min. The yield was 1.2 g of myosin per 100 g of wet muscle. Potassium-activated (EDTA) ATPase was about 3.0 μmoles of P_i/mg of protein per min and Ca-activated ATPase (10 mM Ca^{2+}, low K^+) was about 0.6 μmole of P_i/mg of protein per min when measured by the methods of Sreter *et al.* (1966b). In the presence of 5 mM Mg^{2+} no ATPase activity was detectable.

Preparation of Heavy Meromyosin. Heavy meromyosin (HMM) was prepared by limited tryptic digestion essentially by the method described by Lowey and Cohen (1962). Myosin (15–20 mg/ml in 0.5 M KCl–50 mM Tris, pH 7.5) was digested with trypsin at a myosin:trypsin ratio of 200:1 (w/w) at 20° for 10 min. The digestion was stopped by addition of soybean trypsin inhibitor, in twofold excess by weight over trypsin. The digest was then dialyzed against 10 volumes of water; the light meromyosin (LMM) and undigested myosin precipitated and were removed from the digest by centrifugation.

Preparation of Subfragment-1. The usual procedure for preparing subfragment-1 involved gel filtration on a column of Sephadex G-200 (Lowey *et al.*, 1969; Young, 1969), and it soon became apparent to us that it would be very time consuming to prepare sufficient amounts (*i.e.*, 1–2 g) of subfragment-1 by this technique. However, we took advantage of the fact that for purposes of peptide isolation it was not necessary for the subfragment-1 to be enzymatically active. Thus a method was developed by which large amounts of denatured subfragment-1 could be prepared from HMM. HMM (1.0–2.0% in 50 mM KCl–50 mM Tris, pH 7.5) was digested with trypsin (HMM–trypsin, 64:1) for 40 min at 25°. Three volumes of cold ethanol were then added to the digest and the precipitate was collected by centrifugation at 13,000*g*. The precipitate contained a mixture of HMM subfragment-1 and HMM subfragment-2. After precipitation with ethanol the supernatant contained about 5% of the total protein, and this presumably represented tryptic peptides released during the formation of subfragment-1 and subfragment-2.

Subfragment-2 had been shown previously to be reversibly denatured by ethanol (Lowey *et al.*, 1969). Thus subfragment-2 was removed from the ethanol precipitate by extracting it three times with a solution containing 0.1 M KCl–10 mM Tris, pH 7. The residue after the extraction was denatured subfragment-1 and the amino acid composition of this material is shown in Table I; it contains essentially all of the methylated amino acids, and the overall composition corresponds well with the literature values for subfragment-1. In a typical preparation 8.4 g of myosin yielded 5.4 g of HMM and from this, 2.8 g of subfragment-1 was isolated. This denatured subfragment-1 was the starting material for the peptide isolation, and it will be referred to subsequently as subfragment-1. HMM subfragment-2 could be isolated from the combined extract by lowering the pH to 4.5; the amino acid composition of this product is also shown in Table I and it agrees with the literature value.

Alkylation of Subfragment-1. Reaction of subfragment-1 with iodoacetamide (IA) was carried out following the general procedure described by Crestfield *et al.* (1963). In a typical experiment 1.6 g (about 15 μmoles) of subfragment-1 were dissolved in 150 ml of a solution containing 5 M guanidine·HCl, 0.1 M Tris, pH 8.0, and 0.1 M EDTA. The solution was stirred under a stream of nitrogen for 1 hr at room temperature. To ensure the complete reduction of SH groups 1.5 ml of β-mercaptoethanol was then added and the solution was left under a nitrogen stream for 2 additional hours. IA, 3.5 g (approximately 110-fold excess over protein-SH groups), dissolved in water was then added, and the solution was stirred in the dark for 20 min. The reaction was stopped by lowering the pH to about 3 with 25% acetic acid. Excess reagents were removed by passing the solution over a 7 × 40 cm column of Sephadex G-10 equilibrated with 25% acetic acid. The column was maintained in darkness by covering it with aluminum foil. The optical density of the effluent was monitored at 280 nm and the fractions containing the protein peak were pooled and dried by rotary evaporation at 40°.

Cyanogen Bromide Cleavage. The dried, reduced, and β-carboxyamidomethylated subfragment-1 was dissolved in 70% formic acid to a concentration of 10–12 mg/ml and treated with a 500-fold excess (over methionine) of cyanogen bromide (CNBr) for 18 hr at 25°. Amino acid analysis indicated a 96–98% loss of methionine. The cyanogen bromide and formic acid were removed by evaporation under reduced pressure at 40°.

Tryptic Digestion of CNBr-Treated S-β-Carboxyamido-

TABLE I: Amino Acid Composition of Myosin and Subfragments of Heavy Meromyosin Expressed as Residues per 10^5 Gram.

Amino Acid	Myosin			HMM Subfragment-1					HMM Subfragment-2	
	Previous Reports		This	Previous Reports		This Work[c]			Previous	
	a	*b*	Work[c]	*d*	*b*	Column Purified	Ethanol Precipitated	Alkylated Ethanol Precipitated	Report *b*	This Work[e]
Lysine	85	82	93	71	83	85	85	80	121	127
Histidine	15	16	15	15	18	17	18	17	9	9
3-Methylhistidine			0.385			0.78	0.89	0.82		0
ϵ-*N*-Methyllysine			1.18			2.36	2.54			0
Arginine	41	43	45	32	34	34	34	35	37	35
Aspartic acid	85	85	84	91	85	85	88	91	87	86
Threonine[f]	41	44	41	50	49	49	49	49	43	43
Serine[f]	41	39	38	43	41	41	41	43	35	33
Glutamic acid	155	157	163	126	117	126	138	124	242	240
Proline	22	22	21	30	37	38	37	37	0	0
Glycine	39	40	40	55	61	57	55	57	19	14
Alanine	78	78	75	67	70	71	74	76	87	87
Valine	42	43	42	51	55	57	53	53	24	22
Methionine	22	23	21[g]	29	28	27[g]	28[g]	28	26	26
Isoleucine	42	42	40	52	53	48	48	48	34	35
Leucine	79	81	80	73	75	75	75	78	99	102
Tyrosine[f]	18	20	17	31	34	31	31	32	2.6	2
Phenylalanine	27	29	28	46	52	44	48	44	8.6	7
Cysteic acid[f]	8.6	8.8	9.0[g]	10	11	11[g]	11[g]		6.4	
Carboxymethyl-cysteine								11		

[a] Kominz *et al.* (1954). [b] Lowey *et al.* (1969). [c] Average of five determinations. [d] Jones and Perry (1966). [e] Purified on Sephadex G-100. [f] Corrections for destruction during hydrolysis: threonine, 0.95; serine, 0.90; cysteic acid, 0.90; tyrosine, 0.93. [g] Based upon analyses of three performic acid oxidized preparations.

methyl-Subfragment-1. The cyanogen bromide digest of subfragment-1 was suspended in 50 mM Tris, pH 8.5 to a concentration of 8–10 mg/ml. Trypsin, dissolved in 10^{-3} M HCl, was added to an enzyme–substrate ratio of 1:16 and the digestion was allowed to proceed for 16 hr at 30°. The reaction mixture was acidified by addition of glacial acetic acid and dried under reduced pressure at 40°. Pyridine was added then to a concentration of 0.2 M and the pH was adjusted to 3.1 by addition of appropriate amounts of glacial acetic acid. A small residue remained undissolved, and the soluble mixture of peptides was applied directly to the chromatographic columns. The undissolved material was shown by analysis to be devoid of 3-methylhistidine.

Amino Acid Analyses. Analyses were performed on a Phoenix amino acid analyzer using a two-column system. The basic amino acids, including 3-methylhistidine and methylated lysines, were analyzed as described previously (Huszar and Elzinga, 1969a). In general, samples for analysis were hydrolyzed in 6 N HCl for 22 hr at 110° in sealed, evacuated tubes. Cysteine was measured as cysteic acid following performic acid oxidation (Moore, 1963), or as *S*-β-carboxymethylcysteine after alkylation of the SH groups. Asparagine and glutamine were determined by comparison of amino acid analyses of acid and aminopeptidase M (Henley and Co., New York, N. Y.) hydrolysates of peptides (Elzinga *et al.*, 1968). In some cases it was necessary to analyze simultaneously for glutamine and threonine; this was accomplished by using a lithium citrate buffer prepared as described by Benson *et al.* (1967), and a 0.9 × 25 cm column of Phoenix XX-860-0 resin. The column temperature was 50.3° and the flow rate was 100 ml/hr. In this system threonine was eluted at 36 min, and glutamine at 42 min.

Thermolysin Digestion. The 3-methylhistidine peptide was digested by thermolysin using the following reaction conditions. The peptide was dissolved in 0.1 M ammonium bicarbonate, pH 7.5. Thermolysin (Calbiochem, A grade containing 65% enzyme protein) was added in aqueous solution of 1 mg/ml in an enzyme to substrate ratio of approximately 500 μg per μmole of peptide. The solution was incubated for 6 hr at 40° and the digestion was stopped by lowering the pH to about 3 by addition of glacial acetic acid.

Carboxypeptidase A and B Digestion. Carboxypeptidase A (Worthington COADFP) was dissolved in 0.05 M ammonium bicarbonate containing 10% NaCl to a concentration of 2 mg/ml while carboxypeptidase B (Worthington, COBDFP) was dissolved in 0.05 M ammonium bicarbonate to a concentration of 1 mg/ml. Digestions were carried out in 0.05 M ammonium carbonate buffer. The amount of peptide used and incubation times are given in the Results.

Gel Filtration. Preliminary fractionation of the tryptic and cyanogen bromide digest of the alkylated subfragment-1 included two steps of gel filtration on Sephadex G-25 (fine)

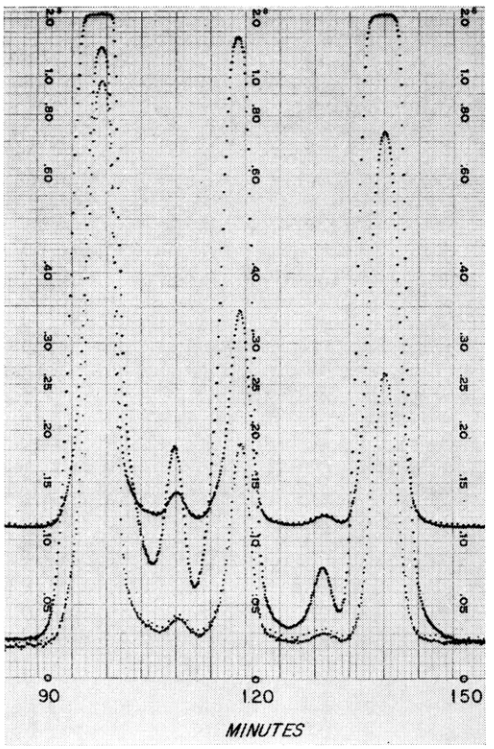

FIGURE 1: Separation of the basic amino acids of myosin subfragment-1 on a 0.9 × 40 cm column of Phoenix XX-860-0 resin. The temperature was 50.3°, the buffer was 0.35 M sodium citrate at pH 5.36, and the flow rate was 80 ml/hr. At 99 min, lysine; 110 min, ϵ-*N*-methyllysine (both mono- and trimethyl-); 119 min, histidine; 131 min, 3-methylhistidine; 140 min, ammonia.

and Bio-Gel P-6 columns. The column sizes and chromatography conditions are given in the legends.

Ion-Exchange Chromatography. DOWEX-50 AND DOWEX-1. The resins used for ion-exchange chromatography (AG-50W-X-2, 200–400 mesh and AG-1-X-2 200–400 mesh, Bio-Rad, Richmond, Calif.) were prepared and poured as described by Schroeder (1967). Columns were developed at 40° at a flow rate of 25 ml/hr. Buffers, pH 3.1 and pH 5.0, for the Dowex-50 column were prepared as described by Schroeder (1967), and buffers with intermediate pH values were prepared by mixing appropriate amounts of these. For the preparation and elution of the Dowex-1 column the pH 9.4 α-picoline-*N*-ethylmorpholine–pyridine buffer was used as described by Schroeder (1967).

PHOSPHOCELLULOSE. Phosphocellulose (Cellex-P, Bio-Rad, Richmond, Calif.) was dry sieved through standard U.S. grade screens and the 100–200-mesh fraction was used. This was washed with 1 N NaOH and 1 N HCl as described by Bornstein and Piez (1966). The conditions for chromatography are given in the text. The ion-exchange columns were poured and equilibrated with the starting buffers.

Edman Degradation. Subtractive Edman degradation was carried out as described by Elzinga *et al.* (1968).

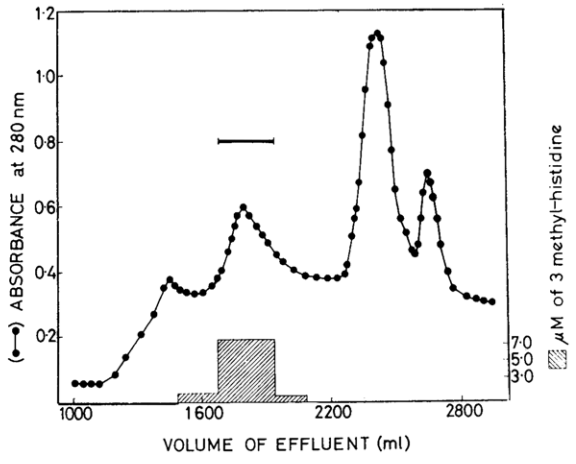

FIGURE 2: Preliminary fractionation of 10 μmoles of the subfragment-1 peptide mixture. The column (5 × 180 cm) was packed with Sephadex G-25 fine, and was eluted with 0.2 N pyridine–acetic acid buffer. The flow rate was 80 ml/hr. Fractions (15 ml) were collected. The 3-methylhistidine-containing tubes that were pooled for rechromatography are indicated by the solid bar. The cross-hatched area shows the distribution of the 3-methylhistidine.

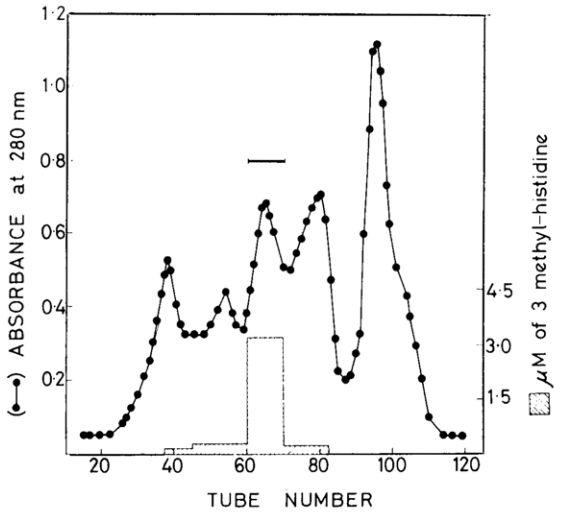

FIGURE 3: Rechromatography of the 3-methylhistidine-containing fraction from the Sephadex G-25 column on a 2.5 × 200 cm Bio-Gel P-6 column. The eluent was 25% acetic acid and the column was run at a flow rate of 11 ml/hr. The fraction size was 4.8 ml. Tubes were pooled for ion-exchange chromatography as indicated by the solid bar. The cross-hatched area shows the distribution of 3-methylhistidine.

Results

Amino Acid Composition of Myosin, HMM Subfragment-1, and HMM Subfragment-2. The amino acid composition of the myosin used in this study is shown in Table I and is compared with two sets of literature values obtained by others. There is good agreement within experimental error for all amino acids.

The separation of the basic amino acids including 3-methylhistidine and the methylated lysines is illustrated in Figure 1; even in the presence of large amounts of lysine and histidine, the 3-methylhistidine and methyllysine peaks are well resolved. The amino acid composition of subfragment-1 and subfragment-2 is shown in Table I. The overall composition of subfragment-1, and the presence of 1 mole of 3-methylhistidine and about 3 moles of methyllysine per mole (115,000 g) of protein are taken as evidence that, although the subfragment-1 is prepared in a denatured form, it is chemically very similar to enzymatically active subfragment-1 prepared by gel filtration procedures.

Gel Filtration of the Peptide Mixture Containing 3-Methyl-

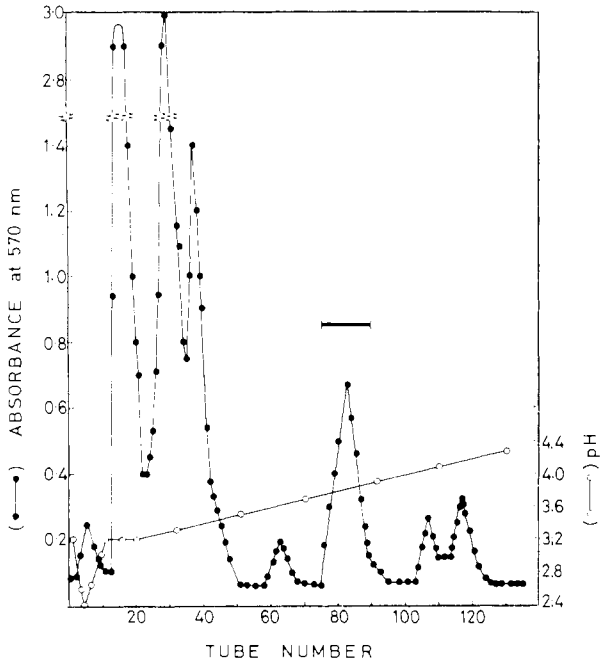

FIGURE 4: Ion-exchange chromatography of the 3-methylhistidine containing peak from the P-6 column. A 0.6 × 60 cm Dowex-50 column was developed using a linear gradient of 350 ml of pH 3.25, and 350 ml of pH 4.4 pyridine–acetic acid buffer at 40°, at a flow rate of 25 ml/hr. Fraction size is 3.4 ml. The points represent the color obtained by analyzing 3% of the fractions by the manual ninhydrin method. The solid bar indicates the peak at pH 3.8 which contains the 3-methylhistidine peptide.

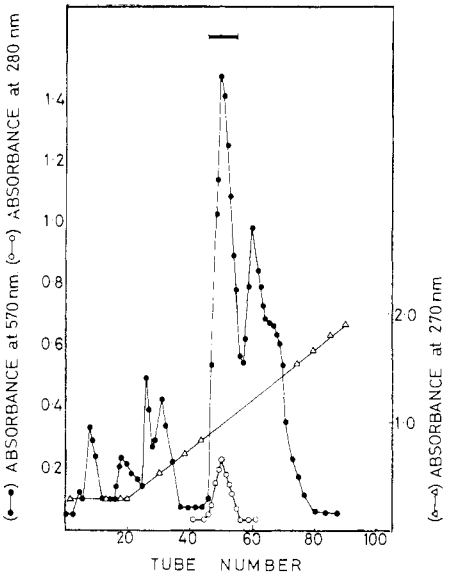

FIGURE 5: Elution pattern of the phosphocellulose column used in the final purification step. The column was 0.6 × 40 cm and was developed using a linear gradient of 28% acetic acid and pH 3.2 pyridine–acetic acid buffer at 40° with a flow rate of 25 ml/hr. The fraction size was 3.3 ml and peaks were detected by the manual ninhydrin method. The solid bar indicates the 3-methylhistidine peptide. Since this is the only peptide in this mixture that contains tyrosine or tryptophan it can be located by reading the tubes at 280 nm. The 270-nm readings represent the contribution of pyridine.

histidine. The combined tryptic and cyanogen bromide digest of alkylated subfragment-1 should contain about 160–170 peptides, assuming hydrolysis at the 96 lysines, 38 arginines, and 33 methionines in subfragment-1. This digest was the starting material for the isolation of the peptide that contained 3-methylhistidine.

SEPHADEX G-25. About 1.15 g (10 μmoles) of digested subfragment-1 was dissolved in 40 ml of 0.2 N pyridine–acetic acid buffer, pH 3.1, and subjected to gel filtration on G-25. The elution pattern obtained from this column is shown in Figure 2. Small aliquots taken from each 10–15 tubes were collected and analyzed for 3-methylhistidine. Tubes that contained most of the 3-methylhistidine were pooled (see Figure 2) and dried by rotatory evaporation.

BIO-GEL P-6. The dried material from the G-25 column was dissolved in 15–25 ml of 25% acetic acid and chromatographed on a column packed with Bio-Gel P-6. Figure 3 shows the elution pattern of a peptide mixture with about 4 μmoles of 3-methylhistidine. The fractions containing the 3-methylhistidine were pooled and dried, and subjected to ion-exchange chromatography. The position at which the 3-methylhistidine peptide was eluted from the Bio-Gel P-6 suggested that its molecular weight was about 2000.

Isolation of the 3-Methylhistidine Peptide by Ion-Exchange Chromatography. DOWEX 50-X2. The peptide mixture from the P-6 column that contained most of the 3-methylhistidine was dissolved in 25% acetic acid and applied to a 0.6 × 60 cm Dowex-50 column equilibrated with pH 3.25 pyridine–acetic acid buffer. Elution was accomplished with a linear gradient of pH 3.25 and pH 4.4 buffer using a total of 700-ml eluent. The tubes were monitored with the use of the ninhydrin reaction after alkaline hydrolysis (Hirs, 1967). The fractions containing the 3-methylhistidine peptide were located by amino acid analysis and were pooled as indicated (Figure 4). The recovery of the 3-methylhistidine peptide from the Dowex-50 column was about 70%.

PHOSPHOCELLULOSE. The final purification step was carried out on a phosphocellulose column (0.6 × 40 cm) developed with a very shallow linear gradient obtained by mixing 200 ml of 28% acetic acid and 200 ml of pH 3.2 pyridine–acetic acid buffer. The sample was applied to the column in 28% acetic acid. The fractions were monitored at 280 nm (in the first part of the gradient the absorption due to pyridine is very low) and by ninhydrin reaction after alkaline hydrolysis. Figure 5 shows the elution pattern of the 3-methylhistidine-containing peptide mixture; the peak that contained the 3-methylhistidine was pooled as indicated by the solid bar and this peak represented the purified 3-methylhistidine peptide from myosin. The phosphocellulose column gave a recovery of 3-methylhistidine of about 80%.

The overall uncorrected yield of the 3-methylhistidine peptide was about 40% based upon the amount of myosin used. It gave a single spot after high-voltage electrophoresis; the amino acid analysis indicated that it contained 13 amino acids present in stoichiometric amounts, and after one step of Edman degradation only one residue (leucine) was lost.

Determination of the Sequence of the Peptide That Contains 3-Methylhistidine. The amino acid composition of the pure peptide is shown in Table II. It is apparent from analysis of the amino peptidase digest (300 μg of enzyme–0.1 μmole of peptide–0.07 M phosphate buffer, pH 7.5, 24 hr, 22°) of the peptide that it contains two residues of aspartic acid, but the glutamic acid actually exists in the peptide as glutamine. Two steps of Edman degradation were performed on the intact peptide as follows. Step 1: Lys, 1.1; 3-methylhistidine, 1.06; Asx, 1.96; Thr, 0.93; Ser, 1.01; Glx, 1.35;

TABLE II: Amino Acid Composition of the 3-Methylhistidine Peptide of Myosin and Its Thermolysin Peptides.

Amino Acid	3-MeHis Peptide	Th-1	Th-2	Th-3
Aspartic acid	2.04 (2)			2.0
Threonine	0.96 (1)			0.95
Serine	0.97 (1)		0.89	
Glutamine	1.19 (1)			1.36
Glycine	1.0 (1)		1.01	
Alanine	0.24			
Valine	0.91 (1)			1.02
Isoleucine	1.0 (1)			1.1
Leucine	2.02 (2)		2.18	
Tyrosine	0.93 (1)	0.83		
Phenylalanine	0.17			
Lysine	1.1 (1)	1.0		
Histidine	0.046			
3-Methylhistidine	0.93 (1)			0.94

TABLE III: The Release of Amino Acid Residues from the 3-Methylhistidine Peptide by Carboxypeptidases A and B.[a]

Amino Acid	Hours of Digestion 4	8	24	36
Lysine	1.0	1.0	1.0	1.0
Tyrosine	1.02	1.0	0.97	0.94
Threonine	1.04	0.87	1.0	1.1
Glutamine	0.19	0.28	0.46	0.53

[a] Peptide, 0.1 μmole, was incubated with 200 μg of carboxypeptidase A and 300 μg of carboxypeptidase B for the times shown.

Gly, 1.15; Val, 0.91; Ile, 0.98; **Leu, 1.18;** Tyr, 0.72. Step 2: Lys, 1.1; 3-methylhistidine, 1.06; Asx, 2.07; Thr, 0.97; Ser, 0.99; Glx, 1.35; Gly, 1.12; Val, 0.94; Ile, 1.03; **Leu, 0.40;** Tyr, 0.92. These results indicate that the amino-terminal sequence is Leu-Leu.

The peptide was then treated with carboxypeptidase A and B, and the results are summarized in Table III. These results, together with the data on peptide Th-1 (see below), suggested that the sequence from the carboxyl end was -Gln-Thr-Tyr-Lys.

In order to determine the rest of the sequence it was necessary to digest the peptide further, and the presence of a valine and an isoleucine in the interior of the peptide suggested the use of thermolysin. This enzyme preferentially hydrolyzes peptide bonds in which the amino group is contributed by an amino acid having a hydrophobic side chain (Matsubara *et al.*, 1966; Ambler and Meadway, 1968; Bradshaw, 1969). The peptide was treated with thermolysin and the digest was chromatographed on a Dowex-1-X2 column (0.6 × 20 cm) as shown in Figure 6. Three peptides (Th-1, Th-2, TH-3) were obtained and their compositions are given in Table II.

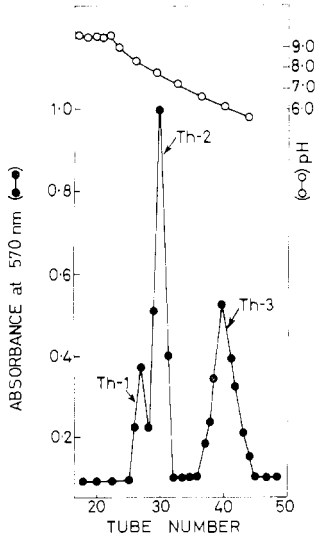

FIGURE 6: Separation of the thermolysin peptides of the 3-methylhistidine peptide on Dowex-1-X-2. A column, 0.6 × 15 cm, was developed using a linear gradient of 200 ml of α-picoline–*N*-ethylmorpholine–pyridine buffer, pH 9.4, and 200 ml of 1 N acetic acid at 40°. The flow rate was 25 ml/hr and the fraction size was 4 ml. Peaks were detected by the manual ninhydrin method.

Th-1 clearly represents the carboxyl terminus of the intact peptide; this assignment is based on the specificity of trypsin, which usually releases peptides that have a basic residue at the carboxyl end, and the results of carboxypeptidase digestion of the intact peptide, which place both lysine and tyrosine near the carboxyl end.

Th-2 contains both leucine residues; since the leucines had been placed at the amino terminus by Edman degradation of the intact peptide, Th-2 is placed at the amino end of the peptide.

The sequence of the tetrapeptide Th-2 was established to be Leu-Leu-Gly-Ser by three steps of Edman degradation. Step 1: Ser, 0.89; Gly, 1.01; **Leu, 1.00.** Step 2: Ser, 0.88; Gly, 1.00; **Leu, 0.20.** Step 3: Ser, 0.88, **Gly, 0.37;** Leu, 0.00.

Peptide Th-3 was subjected to four steps of Edman degradation with the following results. Step 1: Asx, 2.10; Thr, 0.95; Glx, 1.36; Val, 1.02; **Ile, 0.00;** 3-methylhistidine, 0.98. Step 2: **Asx, 1.38;** Thr, 0.95; Glx, 1.47; Val, 1.02; Ile, —; 3-methylhistidine, 1.0. Step 3: Asp, 1.40; Thr, 0.99; Gln, 1.44; **Val, 0.00;** Ile, —; 3-methylhistidine, 1.02. Step 4: **Asx, 0.71;** Thr, 0.96; Glx, 1.35; Val, —; Ile, —; 3-methylhistidine, 1.0. The results established the amino-terminal sequence of Th-3 to be Ile-Asp-Val-Asp.

Carboxypeptidase A + B released from the intact 13 member peptide Lys, Tyr, Thr, and Gln sequentially (Table III); this indicates that the carboxyl-terminal sequence of Th-3 is -Gln-Thr. (Lysine and tyrosine are known to arise from Th-1.) The 3-methylhistidine is placed between aspartic acid and glutamine by difference, and the sequence of Th-3 is thus Ile-Asp-Val-Asp-3-methylhistidine-Gln-Thr. The sequence data are summarized in Figure 7.

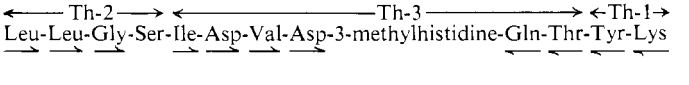

FIGURE 7: Sequence of the 3-methylhistidine peptide of myosin. The half-arrow pointing to the right indicates that the residue was placed by Edman degradation; a half-arrow pointing to the left indicates that the residue was placed by carboxypeptidase digestion.

Myosin: Leu-Leu-Gly-Ser-Ile-Asp-Val-Asp-3-methylhistidine-Gln-Thr-Tyr-Lys
Actin: Leu-Thr-Leu-Lys-Tyr-Pro-Ile -Glu-3-methylhistidine-Trp-Gly-Ile-Ile

FIGURE 8: Amino acid sequences around the single 3-methylhistidine in actin and in myosin.

Discussion

In attempting to isolate a specific peptide from myosin that contained the 3-methylhistidine residues it seemed logical to begin with subfragment-1; it was known that all of the 3-methylhistidine in myosin was located in the globular subfragment-1, and prior removal of light meromyosin and subfragment-2 was expected to reduce the complexity of the peptide mixture that would be obtained after enzymatic and chemical digestion. Enzymatically active subfragment-1 had previously been prepared from myosin or heavy meromyosin by limited proteolysis and purified by gel filtration (Lowey *et al.*, 1969; Young, 1969). This method probably produces a more homogeneous subfragment-1 than the method described here, but the use of a column for purification somewhat limits the amount of material that can be obtained at one time. In the studies described here it was not necessary that the subfragment-1 from which the 3-methylhistidine peptide was isolated be active, and this fact permitted development of an alternative procedure by which relatively large amounts of "denatured" subfragment-1 could be prepared. This procedure (see Methods) involves ethanol precipitation of a limited tryptic digest of heavy meromyosin; both subfragment-1 and subfragment-2 are precipitated by ethanol, while a small amount of peptide material remains in solution. The precipitate is then extracted with dilute salt at neutral pH; under these conditions the subfragment-2 is solubilized and removed while the subfragment-1 remains as a precipitate. While there may, in fact, be some differences in chemical composition, the subfragment-1 prepared in this way is very similar in amino acid composition to subfragment-1 prepared by gel filtration on Sephadex G-200, and essentially all of the 3-methylhistidine was found in the subfragment-1.

Isolation of the 1 peptide out of about 160 from the combined cyanogen bromide and tryptic digest of myosin subfragment-1 was facilitated by the ability to analyze regions of column effluents directly for 3-methylhistidine. In all of the gel filtration and ion-exchange columns, the 3-methylhistidine was found in single discrete peaks, and the overall recovery of the 3-methylhistidine peptide from myosin was about 40%. These results suggest that, as in actin, the 3-methylhistidine in myosin represents a fully methylated histidine at a single position in the main chain of myosin.

Determination of the amino acid sequence of this peptide was facilitated by the use of the enzyme thermolysin. This enzyme usually hydrolyzes peptide bonds in which the amide bond is contributed by an amino acid with a hydrophobic side chain, and in this peptide it hydrolyzes a Gly–Ile bond and a Thr–Tyr bond. Experiments in which the peptide was hydrolyzed for times less than 6 hr indicated that the Gly–Ile was hydrolyzed faster than Thr–Tyr. It should be noted that thermolysin did not split the Asp–Val bond. Both aminopeptidase M and carboxypeptidase A seemed to release 3-methylhistidine at a slower rate than the other amino acids.

Establishment of the amino acid sequences around 3-methylhistidine in both myosin and actin (Elzinga, 1971) permits a comparison of these sequences in the two proteins. Our interest in this comparison is based on a number of considerations. First, actin and myosin are the only myofibrillar proteins that have so far been shown to contain this amino acid. In fact, the only other proteins which have been reported to contain this residue are amoeba actin (Weihing and Korn, 1969; D. E. Woolley, 1970, personal communication) and a histone that has been partially isolated from the immature duck erythrocyte (Gershey *et al.*, 1969). Secondly, the possibility that the myosin molecule has evolved from smaller proteins must be considered; of the myofibrillar proteins, both the light meromyosin part of the myosin and tropomyosin exist in rod-like highly α-helical configurations, while the subfragment-1 part of myosin and actin are globular. Also, both actin and subfragment-1 contain sites of interaction with nucleotides and metal, although actin apparently does not have true ATPase activity, while myosin subfragment-1 does. The molecular weights of actin and subfragment-1 are, however, quite different, actin being about one-half the size of myosin subfragment-1. If actin and subfragment-1 have, in fact, evolved from a common precursor, it seemed to us that homology in sequence might be apparent from a comparison of the sequences around 3-methylhistidine in these two proteins. The corresponding regions around 3-methylhistidine in myosin and actin are compared in Figure 8, and the sequences do not appear to be homologous. This finding does not, of course, prove that homology between these two proteins does not exist, and more sequence information from subfragment-1 is needed before this question can be adequately evaluated. One feature common to both peptides is the acidic residue adjacent to 3-methylhistidine; acidic side chains often lie at the surface of a protein and if the acid side chains in these peptides are at the surfaces the side chains of 3-methylhistidine would also be at or near the surfaces of actin and myosin.

It is clear from the experiments of Asatoor and Armstrong (1967) with actin and Hardy and Perry (1969) with myosin, that 3-methylhistidine is incorporated into the polypeptide as histidine, and that the methyl group is transferred enzymatically from *S*-adenosylmethionine. Since there are many histidine residues in these proteins, and only one is methylated, there must be some aspect of the structure that serves as a recognition site for the methylating enzyme. This problem is somewhat analogous to the problem of the biosynthesis of glycoproteins, where carbohydrate moieties are attached at specific amino acid side chains in proteins. Here the sequences around the points of attachment seem to fall into two classes: Asn(CHO)-polar-(Ser or Thr) for those sites at which long polysaccharide side chains are attached, and Asn(CHO)-apolar-(Ser or Thr) where smaller sugar groups are bound (Jackson and Hirs, 1970; Eylar, 1965). Thus it appears that a short specific segment in the amino acid sequence may serve as a part of the recognition site for an enzyme that attaches the sugar moiety.

By analogy, one might expect that the enzymes that methylate a specific histidine would recognize a short segment of primary structure in actin and myosin. If this is the case, the dissimilarity of these two peptides suggests that the enzymes which methylate a histidine in actin and myosin

are different. It is not clear at this time whether the methylation of histidine occurs before or after the polypeptide chains have been folded into their final tertiary structure; if the synthesis occurs after folding, the site that is recognized by the methylating enzyme may, in fact, be unrelated to the primary structure around 3-methylhistidine. Red, cardiac, and fetal muscle myosins are devoid of 3-methylhistidine and this absence could be due to (1) a lack of enzyme system for the synthesis of 3-methylhistidine in these tissues, or (2) differences in the sequence or folding of the proteins such that this histidine is not recognized by the methylating enzyme. The possibility that it is due to differences in sequence could be experimentally tested by obtaining from red, cardiac, or fetal myosin the peptide that corresponds to the region represented by the 3-methylhistidine peptide. If the sequence were identical except for the methyl groups on histidine, this would suggest that the histidine-methylating system is absent from muscle other than white skeletal muscle.

The function of 3-methylhistidine in myosin, as well as that of the other methylated amino acids in myosin and other proteins, has not yet been elucidated. There is a correlation between the content of 3-methylhistidine and the ATPase activity of myosin; both red and cardiac muscle myosins, which lack 3-methylhistidine, typically have lower ATPase activity than white muscle myosin, while fetal rabbit myosin, which lacks 3-methylhistidine, has a lower ATPase activity than adult white muscle myosin. However, this correlation should be interpreted with caution, and additional information is necessary before the function of 3-methylhistidine can be defined.

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