Biochem. J. (1970) **119**, 293–298
Printed in Great Britain

Biological Activity and the 3-Methylhistidine Content of Actin and Myosin

By P. JOHNSON* and S. V. PERRY
Department of Biochemistry, University of Birmingham, Birmingham 15, *U.K.*

(*Received* 18 *May* 1970)

1. The 3-methylhistidine content of myosin varies according to muscle type. It is highest in myosin from white skeletal muscle and lower values are obtained from myosin of red skeletal and smooth muscle. 2. The 3-methylhistidine content of actin was similar in all of the types of muscle from which it was isolated. 3. The 3-methylhistidine of rabbit actin is localized in a single tryptic peptide that was readily modified during fractionation procedures. 4. Photo-oxidation studies indicated that the 3-methylhistidine residues are not essential for adeonsine triphosphatase and actin-combining activities of myosin. 5. During photo-oxidation G-actin lost completely the ability to polymerize to the F form before all the 3-methylhistidine was destroyed.

The analytical evidence currently available indicates that the occurrence of 3-methylhistidine in muscle proteins is restricted to actin and myosin (Asatoor & Armstrong, 1967; Johnson, Harris & Perry, 1967). Radiochemical experiments (Hardy & Perry, 1969), however, suggest that this amino acid may also be present in the sarcoplasmic protein fraction of rabbit skeletal muscle although the amount present is too small to be detected in the usual sample taken from amino acid analysis of the hydrolysate of this protein fraction. The methylation of histidine in actin is restricted to a single residue (Johnson *et al.* 1967) and presumably specific residues are also involved in myosin although direct evidence is not yet available in this case. Whereas 3-methylhistidine is present in actin isolated from skeletal muscle of the rabbit foetus, 3-methylhistidine does not appear in myosin from the same tissue until after birth. These studies also indicated that the 3-methylhistidine content of myosin from cardiac and red muscle is less than that of white skeletal muscle from the same species (Trayer, Harris & Perry, 1968).

The variable content of 3-methylhistidine in myosin from different muscle types suggests that methylation of the histidines may not be essential for the biological activity of the protein. Nevertheless within a given species the lower the specific ATPase† activity of the myosin in general the lower its 3-methylhistidine content, and there is evidence suggesting that histidine is involved in the active centres concerned with the biological activity of both myosin and actin. Photo-oxidation studies of disulphide-exchanged myosin (Stracher & Chan, 1964; Stracher, 1965) have shown that a single histidyl residue is involved at the ATPase active centre of the molecule, a finding in agreement with earlier kinetic studies (Hotta, 1961). In similar experiments on G-actin (Martonosi & Gouvea, 1961; Martonosi, 1968) it was concluded that the changes observed in the polymerizability of photo-oxidized actin were probably related to histidine-residue destruction and not thiol-group oxidation.

The present study is concerned with the distribution of 3-methylhistidine in myosin and actin from various species and muscle types. In addition, photo-oxidation studies suggest that the 3-methylhistidine residues are not directly involved in the biological activities of myosin and actin. Some aspects of the work have been briefly reported elsewhere (Johnson, Lobley & Perry, 1969).

METHODS

Preparation of proteins. Actin was prepared from muscle acetone-dried powders as described previously (Johnson *et al.* 1967). For the photo-oxidation studies myosin was prepared by the method of Perry (1955). To minimize actin contamination samples for analysis were prepared by a modification of this method (Trayer & Perry, 1966) from mixed longissimus dorsi and leg muscles of adult New Zealand White and Dutch rabbits, pigeon breast muscle, adult rabbit cardiac muscle, mixed skeletal muscle of white mice, smooth muscle of cow uterus, crab claw muscle and lobster tail muscle.

In addition, myosin was prepared from uterine smooth

* Present address: Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada.

† Abbreviation: ATPase, adenosine triphosphatase.

muscle by the method of Needham & Williams (1963), and from lobster tail muscle by the method of Woods, Himmelfarb & Harrington (1963). The heavy alkaline component of myosin was prepared by the method of Gershman, Dreizen & Stracher (1966).

Photo-oxidation procedure. The photo-oxidation procedure used for myosin was essentially that of Stracher & Chan (1964). ATPase assays on the photo-oxidized myosin were also carried out by the method of Stracher & Chan (1964), and actin combination was studied by the method of Martonosi & Gouvea (1961) by altering the myosin/actin ratio from 5:1 to 1.6:1 by weight. Actin polymerizability was determined viscometrically by the method of the latter authors at 25°C by using a 3ml Ostwald viscometer with a 60s outflow time for water.

Actin was photo-oxidized in the absence of salyrgan by the method of Martonosi & Gouvea (1961). After photo-oxidation, mercaptoethanol (1:1 molar ratio with actin thiol groups) was added before the charcoal treatment to protect the actin-bound nucleotide (Barany, Nagy, Finkelman & Chrambach, 1961). The polymerizability of photo-oxidized actin was then studied by the method of Martonosi (1968).

Amino acid analyses. With the exception of the photo-oxidized proteins all protein samples were precipitated with 5% (w/v) trichloroacetic acid and analysed for 3-methylhistidine and histidine on the Beckman 120B amino acid analyser by the methods described previously (Johnson *et al.* 1967).

Before amino acid analysis of the photo-oxidized proteins, Methylene Blue was removed from solution by charcoal treatment (Martonosi & Gouvea, 1961) and the proteins were precipitated from the resulting colourless solutions by the addition of 3 vol. of ethanol. After being washed with ethanol, the precipitates were dried with acetone and ether and hydrolysed for 48h at 110°C *in vacuo* in 6M-HCl at a protein concentration of 5mg/ml.

Analyses of the lysine, arginine, histidine and 3-methylhistidine contents of photo-oxidized proteins were performed on a Technicon AutoAnalyzer in which the standard settings for amino acid analysis were used except for the chart recorder, which was set at half the normal speed. The column (130cm × 0.9cm) of grade A Technicon resin was pre-equilibrated and eluted with 0.35M-sodium citrate buffer, pH5.28, and the sample was applied in 1ml of 0.2M-sodium citrate buffer, pH2.2. Under these conditions, 3-methylhistidine was completely resolved from the histidine peak.

To obtain 3-methylhistidine peaks of reasonable size, it was necessary to use approx. 6mg of myosin and 4mg of actin per analysis. As lysine and arginine residues are not destroyed on photo-oxidation, it was found convenient to derive the numbers of histidine and 3-methylhistidine residues per mol of protein by comparison of their ratios to lysine or arginine. For these calculations, the molecular weight of myosin was taken as 500000 and its lysine and arginine contents as 419 and 199.6mol/mol respectively (Trayer, 1966), and for actin the molecular weight was taken as 44000 and its lysine and arginine contents as 19.2 and 16.9mol/mol respectively (Johnson & Perry, 1968).

Peptide studies. The tryptic fraction containing 3-methylhistidine was obtained after Dowex 1 chromatography of the tryptic digest of carboxymethylated actin (Johnson *et al.* 1967). Further purification of this fraction was made by electrophoresis on Whatman 3MM paper at pH6.5 and by descending chromatography on Whatman 3MM paper, by using the pyridine–3-methylbutan-1-ol–water solvent (Johnson *et al.* 1967).

Amino acid analyses of purified peptides were performed either on the Technicon or Beckman Unichrom amino acid analyser after hydrolysis of the peptides in 6M-HCl for 24h *in vacuo* at 110°C.

RESULTS

3-Methylhistidine content of actin and myosin. All preparations of myosin from adult tissues analysed contained 3-methylhistidine. In the rabbit the amounts of 3-methylhistidine in cardiac and red skeletal muscle (Table 1) were significantly lower than that present in adult mixed skeletal muscle, which is mainly white. It is notable that 3-methylhistidine content of the mixed skeletal muscle of the mouse was also much lower than that of mixed skeletal muscle of the rabbit, probably reflecting the greater proportion of red fibres in the former tissue.

Extraction of crab claw muscle by the standard method gave a myosin preparation that had a low 3-methylhistidine/histidine ratio and was presumably contaminated with actomyosin. The direct extraction of myosin from invertebrate striated muscle tends to give preparations contaminated by actin and better preparations are reported to be obtained by dissociation of extracted actomyosin (de Villafranca, 1968). With lobster tail muscle, it was found, however, that the method of myosin preparation made little difference to the 3-methylhistidine/histidine ratio, for the heavy alkaline component prepared from myosin that had been purified by centrifugation for 30min at 30000*g* in 0.3M-potassium chloride to remove the actin as actomyosin (Perry, 1955) had a low 3-methylhistidine/histidine ratio, similar to that of the standard lobster myosin preparations.

Such results probably reflected the relatively high actin contamination in the preparations, for it has been stated that, in invertebrate muscle, actin and myosin combine very tightly in constant stoicheiometric amounts to produce a complex that is not readily dissociated (de Villafranca, 1968).

Limited studies on myosin obtained from cow uterus by the direct extraction procedure followed by ultracentrifugation of the dissociated system to remove actin suggested that the 3-methylhistidine content of myosin from cow uterus was lower than that of rabbit skeletal muscle. Myosin preparations obtained without the dissociation step had a relatively high 3-methylhistidine content (Table 1).

Whereas the 3-methylhistidine/histidine ratio varied between myosin preparations isolated from different muscle types, the ratio for actin was much

Table 1. *3-Methylhistidine/histidine ratios of actin and myosin prepared from different muscles*

The numbers in parentheses indicate the number of independent preparations used if greater than one, and the analytical values are the averages of duplicate estimations on each preparation. The 3-methylhistidine (3-MeHis) contents are calculated from the 3-MeHis/His ratios, by assuming that the molecular weights of actin and myosin are 45000 and 500000 respectively, and assuming by the same values for the histidine contents (myosin 14.4 residues/10^5g, actin 15.9 residue/10^5g) as in the rabbit skeletal proteins (Johnson *et al.* 1967; Trayer *et al.* 1968). HAC, Heavy alkaline component of myosin, see the Methods section.

Muscle		3-MeHis/His ratio				3-MeHis content (residues/mol of protein)	
Type	Source	Myosin	Actin	Actomyosin	HAC	Myosin	Actin
White striated	Adult rabbit mixed skeletal	1:44.2*	1:7.6*	1:26	1:44.7*	1.63*	0.95*
Red striated	Pigeon breast	1:79 (2)				0.91	
	Rabbit heart	1:178 (2)	1:10 (2)		1:119.9*	0.41	0.72
	Mouse mixed skeletal	1:93 (5)				0.78	
Smooth	Cow uterus	1:20.4‡	1:8.4 (2)			3.5	0.86
		1:68.3§				1.05	
Invertebrate striated	Crab claw	1:24.6‡				2.93	
	Lobster tail	1:25.7‡				2.80	
		1:28.2 (2)§	1:15.2	1.21	1:29.7	2.55	0.47

* From Trayer *et al.* (1968).
† From Johnson *et al.* (1967).
‡ Prepared by direct myosin extraction.
§ Prepared by dissociation of extracted actomyosin.

more constant. A slightly higher ratio than that obtained for rabbit skeletal muscle was given by actin from rabbit heart. In view of the difficulty of preparing pure actin, particularly from tissues other than rabbit skeletal muscle, it is more likely that the differences obtained reflect the presence of impurities in the preparations rather than real differences in the 3-methylhistidine content (Johnson *et al.* 1967). A similar explanation probably applied to the higher 3-methylhistidine/histidine ratio of lobster tail muscle actin.

3-Methylhistidine peptide fraction of actin. Although the analytical results clearly indicate one 3-methylhistidine residue per molecule of actin (mol. wt. 44000) the earlier fractionation studies on tryptic digests indicated that two 3-methylhistidine-containing peptides might be present (Johnson *et al.* 1967). To clarify this point the 3-methylhistidine-containing peptide fraction obtained by chromatography on Dowex 1 (fraction XV; Johnson & Perry, 1968) was re-examined.

Electrophoresis at pH 9.0 of 3-methylhistidine separated the fraction into two peptide bands, one of which contained tyrosine and the other tryptophan. The slower-moving tryosine-containing peptide band (peptide A) was found to be homogeneous on examination by electrophoresis at pH 3.5 and pH 6.5 and by paper chromatography. On amino acid analysis, peptide A was found not to contain 3-methylhistidine or histidine (Table 2).

Table 2. *Amino acid composition of peptides in 3-methylhistidine-containing peptide fraction isolated from tryptic digests of actin*

Ratios are based on Arginine = 1.0 for peptide A and Lys = 1.0 for peptides B and C. Results are averages of analyses of two independent preparations. Peptide A was hydrolysed for 24 h and 72 h. Tryptophan was determined in single preparations after alkaline hydrolysis for 68 h (Noltmann, Mahowald & Kuby, 1962).

	Peptide A	Peptide B	Peptide C
Asp	2.3	3.0	3.6
Thr	1.0	0.8	1.0
Ser	1.0	Nil	0.9
Glu	3.7	1.9	2.1
Pro	1.5	1.1	1.5
Gly	2.4	1.0	1.1
Val	1.0	Nil	Nil
Ile	2.2	1.9	2.7
Leu	1.1	Nil	Nil
Tyr	0.8	0.1	0.4
3-MeHis	Nil	0.9	0.4
His	Nil	0.02	Nil
Trp	Nil	0.8	0.8
Arg	1.0	Nil	Nil
Lys	Nil	1.0	1.0

Although the faster-moving 3-methylhistidine-containing peptide band was found to be homogeneous on electrophoresis at pH 1.5, 3.5 and 6.5, this band was resolved into two peptide bands by

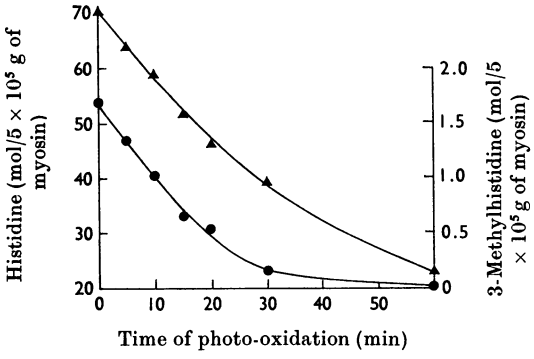

Fig. 1. Effect of photo-oxidation on the histidine and 3-methylhistidine content of myosin. The results plotted are the averages from two independent photo-oxidation experiments. ▲, Histidine; ●, 3-methylhistidine.

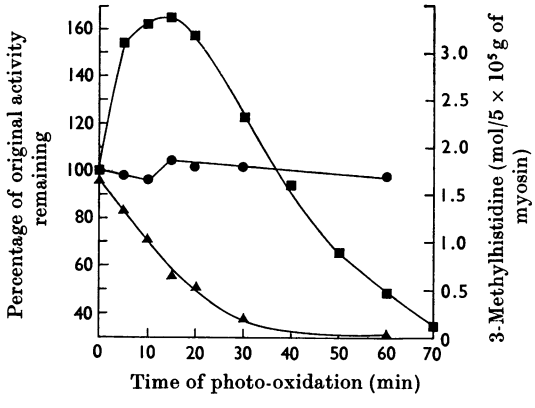

Fig. 2. Effect of photo-oxidation on the Ca^{2+}-activated ATPase, the actin-combining activity and the 3-methylhistidine content of myosin. The results for actin combination and 3-methylhistidine content are the averages of two independent experiments; those for ATPase activity are averaged from four independent experiments. ■, Ca^{2+}-activated ATPase; ●, actin combination; ▲, 3-methylhistidine content.

paper chromatography on Whatman 3MM paper by using either pyridine–3-methylbutan-1-ol–water (Johnson *et al.* 1967) or butan-1-ol–acetic acid–water–butyl acetate (65:15:25:3, by vol.). In both solvents, the two peptides had very similar R_F values; for example in the former solvent the values were 0.18 for peptide B and 0.22 for peptide C.

Separation of peptides B and C was attempted by preparative chromatography with the pyridine–3-methylbutan-1-ol–water solvent. However, after each separation it was found that the faster peptide

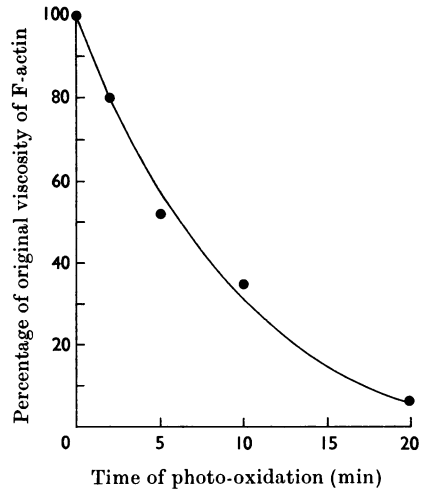

Fig. 3. Effect of photo-oxidation in the polymerizability of G-actin. The results plotted are the averaged results of three independent photo-oxidation experiments.

C appeared to be converted into peptide B, and consequently after three cycles of chromatography only a very small yield of peptide C was obtained whereas the amount of peptide B had increased.

Amino acid analyses of peptides B and C indicated that their compositions were very similar (Table 2) and the only notable differences were that peptide C possessed serine and possibly an extra aspartate residue. Neither peptide possessed significant amounts of histidine, and the small amounts of tyrosine, which were not present in all preparations, were ignored. It was therefore concluded that both peptides C and B were derived from the same unique sequence about the single 3-methylhistidine residue in actin and that peptide B was a modified form of peptide C.

Photo-oxidation of myosin. Measurement of the histidine and 3-methylhistidine content after different periods of photo-oxidation indicated that both residues were destroyed progressively with increasing time (Fig. 1). The two amino acid residues were destroyed at a roughly similar rate, although there were indications that the 3-methylhistidine remaining after 30min was rather more resistant than that destroyed in the earlier stages of photo-oxidation.

As photo-oxidation progressed, the Ca^{2+}-activated ATPase increased, probably due to oxidation of thiol groups (Kielley & Bradley, 1956), reaching a maximum after about 20min and then decreased sharply until it was almost completely destroyed after 70min (Fig. 2). Similar effects of photo-oxidation have been reported by Stracher &

Table 3. *Effect of photo-oxidation on the 3-methylhistidine and histidine contents of G-actin*

Values in parentheses indicate the number of independent experiments used for analysis.

Actin	Amino acid content (mol/44000g of actin) Histidine	3-Methylhistidine
Control	7.35 (2)	0.95 (2)
Photo-oxidized for 20min	4.95 ± 0.3 (4)	0.5 ± 0.05 (4)

Chan (1964). Some ATPase activity remained even after photo-oxidation of myosin to the stage at which 3-methylhistidine could no longer be detected.

In contrast with the ATPase activity, the actin-combining activity was virtually unchanged after 60min photo-oxidation (Fig. 2). In this period about 65% of the histidine and all of the 3-methylhistidine had been destroyed.

Photo-oxidation of actin. Photo-oxidation rapidly destroyed the ability of G-actin to polymerize to the F form (Fig. 3). After 20min exposure, the actin–actin interaction necessary for the viscosity increase was practically entirely abolished. Over this period, approximately half the actin molecules still possessed intact 3-methylhistidine residues. The histidine content had decreased by about 35% over the same period (Table 3).

DISCUSSION

The fact that 3-methylhistidine is present in actin and myosin isolated from seven species of vertebrates and invertebrates strongly suggests that it is a normal component of these proteins in all species. The 3-methylhistidine content of actin, however, appears to be much more constant than is the case with myosin and probably corresponds in all species to one residue per molecule, as is the case with rabbit actin. Although in some cases the analytical results suggested rather less than this, it is more likely that the lower values reflect the presence of protein impurities that do not contain 3-methylhistidine, e.g. tropomyosin. Methylation occurs at a specific histidine residue in actin, although the tryptic peptide in which the 3-methylhistidine is localized readily undergoes partial conversion into a modified form during separation, giving rise to a mixture of two 3-methylhistidine-containing peptides.

The values for the 3-methylhistidine content of myosin were much more variable than those of actin and in all cases the 3-methylhistidine/histidine ratio is higher. Nevertheless because of its high molecular weight adult white muscle myosin contains more 3-methylhistidine residues per molecule than does actin. Higher 3-methylhistidine contents than that obtained with rabbit white muscle myosin are of doubtful significance for the primary structure, as they probably merely reflect contamination of the myosin preparations with actin. It is well known that myosin, particularly from smooth and invertebrate muscle, is difficult to prepare free of actin. Significantly, high-speed centrifugation of cow uterus myosin under conditions in which actomyosin is dissociated and actin is sedimented increased the 3-methylhistidine/histidine ratio to a higher value than that of myosin from rabbit white muscle.

Similar high ratios obtained with certain myosin preparations implying lower 3-methylhistidine content are significant and confirm earlier findings (Trayer, Harris & Perry, 1968) with foetal and cardiac muscle. Thus the 3-methylhistidine content of myosins from red muscle lies between those isolated from white muscle and the foetal form of skeletal muscle, which for the rabbit is zero. Since cardiac and foetal muscles are slower than the highly specialized fast white muscle and their myosins have lower ATPase specific activities (Trayer & Perry, 1966; Bailey, 1942) it may be concluded that the extent of methylation of histidine in myosin increases with the speed of the muscle, as does the ATPase activity.

Although this correlation is apparent its significance has yet to be determined, for 3-methylhistidine is obviously not essential for enzyme activity, as foetal rabbit myosin contains none and photo-oxidation destroys this residue without destruction of ATPase activity. Further, the ability of myosin to combine with actin appears to be independent of the methylation of the histidine. The fact that the ATPase activity of myosin is more sensitive to photo-oxidation than is the ability to interact with actin is further evidence that separate centres on the myosin molecule are associated with these activities (Perry & Cotterill, 1965).

So far as the myosin is concerned, it appears that its biological activities do not depend on the methylation of histidine and the presence of this residue probably reflects the availability of histidine residues to the methylating-enzyme system of the muscle. The difference in extent of methylation of histidine would not appear to be due to the absence of the appropriate enzyme system, for the histidine residue in actin is methylated in both foetal and adult muscle. In this respect it is notable that certain lysine residues in myosin can be methylated to give ϵ-*N*-monomethyl- and trimethyl-lysines, which are almost as abundant in foetal as in adult myosin (Hardy, Harris, Perry & Stone, 1970).

We thank Dr P. T. Grant of the National Environmental Research Council Fisheries Biochemical Research Unit, University of Aberdeen, for a supply of crabs and lobsters. This work was in part supported by grants from the Muscular Dystrophy Associations of America Inc. and the Medical Research Council.

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