Cyanylation and Cleavage of Myosin Heavy Chains at Reactive Thiol Groups: Direct Localization of Thiol-1 and Thiol-2 Groups†

Jürg T. Walser, John G. Watterson, and Marcus C. Schaub*

ABSTRACT: The essential thiol-1 and thiol-2 groups of myosin and isolated heads prepared with chymotrypsin were specifically labeled in their native state with the cyano group with [^{14}C]nitrothiocyanobenzoate. The effects of the mono- and divalent cation dependent ATPase activities showed that the modification was similar to that obtained with *N*-ethylmaleimide or 5,5′-dithiobis(2-nitrobenzoic acid). Cleavage of the polypeptide chain, which can be induced at the resulting thiocyanoalanine residues, was more reproducible when the remaining free cysteine thiols were blocked by carboxamidomethylation under denaturing conditions before the splitting step. Molecular weight estimations by $NaDodSO_4$ gel electrophoresis showed that the intact myosin heavy chain becomes divided into a 70 000- and a 131 000-dalton fragment. Isolated myosin heads with a heavy chain of 90 000 daltons yielded also a 70 000 dalton, however, together with a 20 000-dalton fragment. In the latter case, the molecular weights of the cleaved products were confirmed by gel-filtration techniques. Since the 70 000-dalton fragment originated from both the intact myosin and isolated heads and the radioactivity was recovered in the 20 000-dalton fragment, the 70 000-dalton fragment must represent the NH_2 terminus of the myosin heavy chain. This alignment is supported by the recovery of the unique N^{τ}-methylhistidine in the 20 000-dalton fragment and COOH-terminal end group analyses on both fragments when compared with the sequence of a cyanogen bromide fragment containing thiol-1 and thiol-2 [Elzinga, M., & Collins, J. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4281–4284]. This places the essential thiols at a distance of around 580 amino acids from the NH_2 terminus of the myosin heavy chain and 170 amino acids from the chymotryptic cleavage point.

With the help of the electron microscope, a relatively clear picture of the structure of the contractile protein molecule myosin on a large scale has been able to be constructed. It contains three basic domains, the structural rodlike tail, the water-soluble rodlike heavy meromyosin subfragment-2, and the globular heavy meromyosin subfragment-1 or myosin head (Lowey et al., 1969). The three pieces are joined together at two hinges (Elliott & Offer, 1978), so allowing the molecular movement required for cross-bridge function (Huxley, 1969). On a small scale, the amino acid sequence of only a few thiol-containing peptides from the heavy chain (HC)[1] have been published to date. Among those, the second longest proteolytic peptide of 18 amino acid residues isolated by Weeds & Hartley (1968) overlaps by four residues with its COOH terminus the NH_2 terminus of a cyanogen bromide peptide of 92 residues isolated by Elzinga & Collins (1977). This latter peptide contains the reactive thiol-1 and thiol-2 groups as well as the unique N^{τ}-methylhistidine. Although the involvement of this posttranslational methylated histidine residue in binding Mg^{2+} remains speculative at present (Ramirez et al., 1979), it is since long known that in vitro modification of thiol-1 and thiol-2 affects the myosin ATPase activity in different ways, depending on conditions (Reisler et al., 1974a,b; Schaub et al., 1975; Kunz et al., 1980). As selective blockage of either of these reactive thiols invariably leads to full inactivation of the K^+-dependent ATPase activity and blocking of both types of thiol groups eliminates also the Ca^{2+}-dependent ATPase activity, they may be called essential on the basis of this criterion (Schaub et al., 1975; Kunz et al., 1980). Although there is no direct evidence that either thiol-1 or thiol-2 is involved in nucleotide binding, the above-mentioned arguments suggest that they are at or near the catalytic site of the myosin ATPase. Hence, the location of the peptide containing the essential thiols can be considered to be of biological importance. This peptide has been earlier shown to be contained entirely within the isolated globular head portion (Kunz et al., 1977), and theoretical speculations based on its primary structure indicate it could contain the active center itself (Elzinga & Collins, 1977; Ramirez et al., 1979). Indeed, it has recently been shown that binding of MgADP not only increase the reactivity of thiol-2 but also induces a conformational change that brings thiol-1 and thiol-2 into closer proximity (Burke & Reisler, 1977) so that the nucleotide remains trapped in the active site (Wells & Yount, 1979).

Preliminary attempts in localizing these reactive thiols have been done by following the time course of proteolytic digestion of native myosin or of heavy meromyosin after labeling them with radioactive IAA and MalNEt (Balint et al., 1978; Cardinaud, 1979). Alignment of the nascent peptide fragments indicates that thiol-1 and thiol-2 may be located in a peptide at least 85 000-dalton worth distant from the HC end of the globular head portion of the molecule. No end-group determinations have been reported, however, for corroborating the peptide alignment. We have therefore undertaken localization of the reactive thiol-1 and thiol-2 groups in the myosin HC by a direct approach in which these groups are first specifically modified in the native protein-producing thiocyanoalanine residues where selective cleavage of the peptide chain can then take place (Jacobson et al., 1973; Degani & Patchornik, 1974). The results indicate that they are located at a position in the myosin HC corresponding to

† From the Institute of Pharmacology, University of Zürich, CH-8006 Zürich, Switzerland. *Received June 9, 1980.* This work was financially supported by the Swiss National Science Foundation Grant No. 3.675.75 and 3.230.77.

[1] Abbreviations used: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3); DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HC, heavy chain; IAA, iodoacetamide; MalNEt, *N*-ethylmaleimide; $NaDodSO_4$, sodium dodecyl sulfate; Nbs, 2-nitro-5-thiobenzoate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); NBzSCN, nitrothiocyanobenzoate; Tris, tris(hydroxymethyl)aminomethane.

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about a third of the overall length in the primary structure along from the NH_2 terminus.

Materials and Methods

N-Ethyl[1-^{14}C]maleimide, potassium [^{14}C]cyanide, and [1-^{14}C]iodoacetamide were obtained from New England Nuclear. All chemicals were of highest purity available. Bidistilled and deionized water was used throughout. Cold and radioactive [^{14}C]nitrothiocyanobenzoate were synthesized (Degani & Patchornik, 1971) and compared to commercially available cold NBzSCN by thin-layer chromatography, melting point, UV spectroscopy, and effects on the myosin ATPase activities. All three samples behaved identically, α-Chymotrypsin (EC 3.4.21.1) of bovine pancreas 3 times crystallized was obtained from Sigma Chemical Co. and carboxypeptidase-Y (EC 3.4.16.1) purified by affinity chromatography from baker's yeast from Pierce Chemical Co.

Protein Preparations. Myosin was prepared from fast-twitch skeletal rabbit muscles according to Trayer & Perry (1966), but after extraction, 5 mM EDTA was included during purification and storage throughout (Schaub et al., 1975). Heavy meromyosin subfragment-1, i.e., myosin heads, were obtained by chymotryptic digestion of myosin (Weeds & Taylor, 1975) and purified by ion-exchange chromatography on DEAE-Sephadex A-50 (Schaub et al., 1978) or by affinity chromatography on Sepharose 4B ribosyl-linked adipic hydrazide ATP (Kunz et al., 1980).

Thiol-Group Modification. Modification of thiols of intact myosin or isolated heads with NBzSCN, Nbs_2, and MalNEt (concentrations ranging from 50 μM up to 2 mM) in 50–100 mM imidazole–HCl buffer, pH 7.4 or 8.0, at 22–25 °C was carried out on 1–8 mg of protein/mL for various times in the presence of 20–50 mM KCl, 1–5 mM EDTA or 5 mM $MgCl_2$, and 5 mM ADP as specified in the text. Stock solutions of Nbs_2 and NBzSCN were made up in ethanol. Enzyme tests were performed at different experimental stages. For immediate tests, aliquots were withdrawn at different times, and the modification reaction was stopped by 20–50 times dilution with cold 20 mM Tris–maleate buffer, pH 7.1. Alternatively, after dilution, myosin was washed twice with the same buffer and resuspended in a small volume before enzyme tests. In some experiments, ATPase tests were performed after exhaustive dialysis against cold 10 mM Tris-maleate buffer, pH 7.1, containing 5 mM EDTA plus either 30 mM or 0.5 M KCl. For assessment of incorporated radioactivity when [^{14}C]-NBzSCN or [^{14}C]MalNEt was used, aliquots were removed and the modification reaction was stopped by addition of cold acetic acid to a final concentration of around 20% (v/v), lowering the pH to around 4 or less, followed by at least 4 times washing with cold acetone containing 3% HCl (v/v). Pellets were dissolved after centrifugation in 0.5 M NaOH, and the radioactivity was assessed by scintillation techniques. In the cases of Nbs_2 and NBzSCN upon reaction with the protein thiol groups, the liberated Nbs dianion could be assessed by absorption at 412 nm (Ellman, 1959) using a molar absorption coefficient of 14 150 (Riddles et al., 1979). With isolated heads in solution, the continuous production of the dianion Nbs was directly measured spectrophotometrically. With intact myosin in aliquots of the reaction mixture, the protein was precipitated with 5% trichloroacetic acid and filtered off, the pH readjusted to neutrality with NaOH in the filtrate, and absorption measured at 412 nm. In the case of Nbs_2, the values so obtained were compared with incorporated Nbs groups in the protein after precipitation and washing with acetone, as described above for the recovery of incorporated radioactivity. After dissolution of the protein in NaOH, the pH was readjusted to around 8, and 5 mM DTT was added for 15 min in the dark at 22 °C to liberate the bound Nbs groups before absorption measurements. In the case of [^{14}C]NBzSCN, the amount of liberated Nbs dianion was compared with that of incorporated radioactive cyano groups.

Chemical Cleavage at Thiocyanoalanine Residues. For chemical cleavage, specific cyanylation on thiol-1 and thiol-2 groups was carried out by incubating 5–10 mg of native protein/mL in 100 mM Tris-HCl buffer, pH 7.8–8.0, with either 200–500 μM unlabeled NBzSCN or [^{14}C]NBzSCN at 22 °C in the presence of 5 mM EDTA and 2.5 mM ADP so that 1–2 cyano groups were incorporated per myosin head. If necessary, NaOH was added to keep the pH between 7.8 and 8.0. Acetic acid was added to 20% (v/v) to stop the reaction, and the precipitated protein was washed several times with cold acetone containing 3% HCl (v/v) and lyophilized for storage. For initiation of cleavage (Jacobson et al., 1973) Degani & Patchornik, 1974) at the thiocyanoalanine residues, the protein (3–8 mg/mL) was dissolved in 100 mM Tris-HCl buffer, pH 9.0, containing 0.5–1.0% $NaDodSO_4$ (w/v) and incubated for 15 h at 37 °C. Such treated samples were then diluted appropriately and could immediately be subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. For preparative purposes, about 10 mg of protein/mL was dissolved in 50 mM Tris-HCl buffer, pH 7.8, containing 6 M guanidine hydrochloride, 5 mM EDTA, and IAA in 10–20% molar excess over total thiol groups in the protein and incubated for 5 min at 22 °C for carboxamidomethylation of the nonmodified thiols before the cleavage reaction was started by raising the pH and temperature. In several experiments, a first pulse of 200 μM radioactively labeled IAA was given for 3 min before the cold IAA was added for full carboxamidomethylation. In other experiments, only radioactive NBzSCN was used. Alternatively, the carboxamidomethylation step was applied immediately following the modification reaction with NBzSCN in the same medium by addition of the guanidine hydrochloride and IAA before the pH and temperature were raised again to induce cleavage. After chemical cleavage in the guanidine hydrochloride medium, samples were exhaustively dialyzed against water and lyophilized for storage. Separation of cleavage fragments was achieved by chromatography on agarose (Bio-Gel A-5M; column dimensions 190 × 2 cm) in 6 M guanidine hydrochloride, pH 7.8, at 22 °C by monitoring the eluate for absorbance at 280 nm or for radioactivity. After dialysis against water and analysis in $NaDodSO_4$ gel electrophoresis, fractions were pooled. For molecular weight determination of the fragments, the following proteins were used after full reduction with DTT and carboxymethylation with iodoacetic acid when appropriate for calibration of the agarose column: phosphorylase *b*, 94 000; myosin head HC, 90 000 (Weeds & Pope, 1977; Schaub et al., 1978); bovine serum albumin, 67 000; actin, 42 000; tropomyosin, 34 000; soybean trypsin inhibitor, 20 100; ribonuclease, 13 700 daltons.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis in $NaDodSO_4$ of myosin, myosin heads, and their fragments on 7.5% and 10% polyacrylamide gels was carried out according to Dunker & Rueckert (1969) in a Tris–borate buffer system, pH 7.5 (Cummins & Perry, 1973), by using the standardized fixation and staining procedure of Sobieszek & Bremel (1975). Stoichiometry of subunits and fragments was determined densitometrically as described in detail earlier (Schaub et al., 1978). For estimation of apparent molecular weights, the same marker proteins were used for calibration as for column chromatography. For assessment of radioactivity

specifically incorporated with the cyano groups, gels were sliced and processed for counting (Gray & Steffensen, 1968) exactly as described earlier (Schaub et al., 1978).

Amino Acid and End-Group Determinations. Amino acid determinations on hydrolysates of isolated peptides were done according to standard procedures (Moore & Stein, 1963) on a Labotron Model Liquimat-2 amino acid analyzer (Kontron, Switzerland). Thr and Ser were extrapolated to zero time of hydrolysis. Val, Ile, and Leu were determined after 72 h of hydrolysis. N^{τ}-Methylhistidine was eluted between His and Arg by using a citrate buffer of pH 4.0 at 47 °C from Durrum resin 4-A. The amino acids at the COOH termnini of head HC and the fragments were determined on the amino acid analyzer after digestion with carboxypeptidase-Y (Hayashi, 1977). Incubations were carried out on 1–3 mg of peptides/mL with around 20 μg of enzyme/mL for various periods of time at 25 °C in 50 mM ammonium acetate buffer, pH 6.0, in the presence of 4 M urea and 5 mM EDTA. The reaction was stopped either by addition of trichloroacetic acid or by boiling, and amino acids were determined in the supernatant after centrifugation.

Enzyme Tests. ATPase activities were carried out routinely (Watterson et al., 1975) at 25 °C in 25 mM imidazole–HCl buffer, pH 7.4, or 25 mM Tris-HCl buffer, pH 7.6, and 2.5 mM ATP on the following: 0.02–0.15 mg of protein/mL in the presence of 10 mM EDTA and 1 M KCl and 0.2–0.8 mg/mL in the presence of 10 mM $CaCl_2$ and 0.5 M KCl. The K^+-dependent ATPase activity was in the range 11–16 s^{-1} for myosin and 5.3–8.7 s^{-1} for isolated heads and the Ca^{2+}-dependent one, 1.5–2.0 s^{-1} and 0.8–1.0 s^{-1}, respectively.

Protein Concentrations. These were determined by the biuret reaction or according to the Lowry method standardized by ultramicro-Kjeldahl estimation of nitrogen (Strauch, 1965). For calculations, molecular weights of 470 000 (Dreizen et al., 1967) and 120 000 (Weeds & Taylor, 1975) were taken for myosin and isolated heads, respectively.

Results

Specificity of Cyanylation with NBzSCN. Incorporation studies of the cyano group into intact myosin and isolated myosin heads using NBzSCN were carried out prior to cleavage studies in order to determine the specificity of this reagent toward the essential thiol groups. When radioactively labeled NBzSCN was used, a pattern of ATPase behavior with increasing degrees of incorporation into myosin similar to that found with MalNEt was observed (Figure 1). We therefore call on the same interpretation as in the case of MalNEt, i.e., that the first two essential thiols (thiol-1) become blocked, activating the Ca^{2+}-dependent ATPase and inactivating the K^+-dependent ATPase, followed by two more essential thiols (thiol-2), inactivating the Ca^{2+}-dependent ATPase subsequently (Reisler et al., 1974a,b; Schaub et al., 1975). Figure 1 also illustrates the parallel behavior of the enzyme produced by incorporation of Nbs, although, in this case, the reagent Nbs_2 is less specific since two to three groups in addition to the four essential thiols are also modified before loss of all enzymatic activity. With both reagents, it was found that the maximal activation of the Ca^{2+}-dependent ATPase was higher if the modification was carried out in the presence of ADP rather than in its absence, indicating an increase in the ordering of the thiol-1 followed by thiol-2 groups in the reaction sequence. These results were not affected when $MgCl_2$ was present during the reaction with either NBzSCN or Nbs_2.

When samples were, however, dialyzed at high or low ionic strength at pH 7 and 2 °C after modification with either NBzSCN or Nbs_2, the activation of the Ca^{2+}-dependent ATPase gradually disappeared and followed the inactivation curve of the K^+-dependent activity in a parallel way. This loss of activation was accompanied by a decrease in the number of incorporated Nbs or cyano groups per mole of myosin to about one-third its value before dialysis. This behavior is reminiscent of that observed after cross-linking the thiol-1 and thiol-2 groups in myosin (Reisler et al., 1974b; Burke & Reisler, 1977) with the bifunctional reagent *N,N'-p*-phenylenedimaleimide. Thus, our observations indicate the possible formation of a disulfide cross-link between thiol-1 and thiol-2. The formation of such a cross-link induced by Nbs_2 has recently been reported for isolated myosin heads (Wells & Yount, 1980).

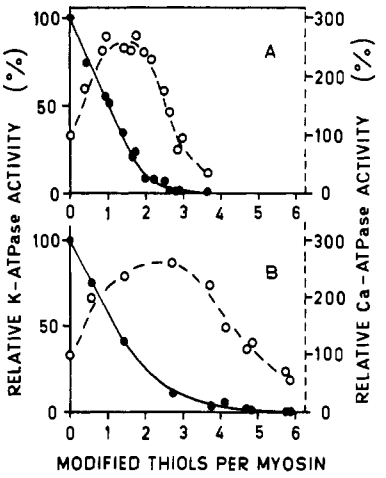

FIGURE 1: Effect of incorporation of cyano and Nbs groups on ATPase activities of myosin. Prior to ATPase tests, modification on 16.5 μM myosin was performed for 0–40 min at 22 °C and pH 7.4 in the presence of 5 mM EDTA and 5 mM ADP. The incorporated groups were assessed on sample aliquots after washing with acetone as described under Materials and Methods. (A) Modification with 266 μM radioactive NBzSCN; (B) modification with 245 μM Nbs_2. (●) K^+-dependent ATPase; (○) Ca^{2+}-dependent ATPase.

As it is known that both essential thiols reside in the 90 000-dalton head HC (Kunz et al., 1977), this subfragment offers a more direct approach to the localization of the essential thiols than the larger myosin molecule. Table I presents the data showing that cyanylation of isolated heads with NBzSCN, as in intact myosin, modifies the essential thiols. The time course followed by the K^+-dependent and Ca^{2+}-dependent ATPase activities indicates that the order of blockage is thiol-1 followed by thiol-2. The degree of incorporation of the cyano group, measured with radioactivity and by following the release of the Nbs dianion spectrophotometrically, indicates further that the reaction carried out under these mild conditions involves almost exclusively just these two essential thiols. Subsequent reaction of such modified samples with radioactive MalNEt, which is known to react exclusively with the two essential groups (Schaub et al., 1978), supports the conclusion that cyanylation only blocks these two thiols, since, as the incorporation of cyano groups increases, that of *N*-ethylsuccinimido groups reciprocally decreases (Table I). On the other hand, when head preparations were first reacted with radioactive MalNEt so that around 2 mol of *N*-ethylsuccinimido groups was found incorporated per head, subsequent treatment with NBzSCN under comparable conditions led to negligible further incorporation of cyano groups.

Cleavage of Heavy Chains at the Thiocyanoalanine Residues. For the cleavage experiments, samples of both intact myosin and isolated myosin heads were cyanylated under mild conditions, chosen so that one to two cyano groups per head were incorporated. The degree of incorporation was estimated either from radioactivity measurements or from the mono–

Table I: Modification of Isolated Myosin Heads by Radioactive NBzSCN and Its Effects on ATPase Activities[a]

time of cyanylation reaction (min)	relative K^+-ATPase activity (%)	relative Ca^{2+}-ATPase activity (%)	incorporated radioactive cyano groups per head	liberated Nbs groups per head	subsequent additional incorporation of radioactive MalNEt per head
0	100	100	0	0	1.80
2	82	123	0.35	0.28	1.58
5	54	139	0.51	0.42	1.50
10	28	169	0.68	0.84	1.21
20	13	168	0.94	1.15	0.88
30	4	115	1.45	1.35	0.26
60	2	55	1.89	1.69	0.07

[a] Cyanylation with 1.05 mM [^{14}C]NBzSCN was performed on heads at 22 °C and pH 7.4 in the presence of 5 mM EDTA and 5 mM ADP. ATPase tests were carried out immediately after 20 times dilution on sample aliquots. Incorporation of radioactive cyano groups was assessed on sample aliquots after washing with acetone. The amount of incorporated Nbs groups was also assessed spectrophotometrically on acetone-washed sample aliquots after liberation with DTT. Subsequent incorporation of radioactive *N*-ethylsuccinimido groups was carried out in a second modification step on sample aliquots incubated for further 5 min at 0 °C and pH 7.4 with 2.50 mM [^{14}C]MalNEt in the original medium and assessed for additional incorporated radioactivity again after washing the protein with acetone. For procedural details see Materials and Methods.

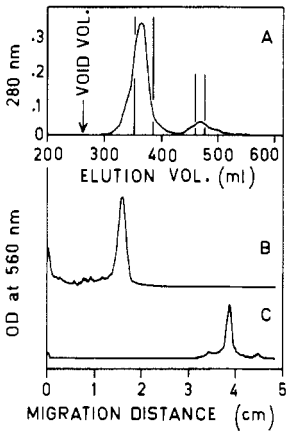

FIGURE 2: (A) Gel filtration of cleavage products obtained after cyanylation of isolated myosin heads on Bio-Gel A-5M in 6 M guanidine hydrochloride (column dimensions = 2 × 190 cm; flow rate = 8.2 mL/h): 2 mL containing a total of 53 mg of protein was loaded, the elution was monitored at 280 nm, and fractions of 2 mL were collected. Peak 1 corresponds to apparent molecular weight 71 000 and peak 2 to 21 000. The arrow indicates void volume, and fractions betwen the bars were pooled. Underneath are shown densitometric tracings of pooled fractions on $NaDodSO_4$ electrophoresis in 7.5% polyacrylamide gels as described under Materials and Methods. (B) Peak 1 and (C) peak 2.

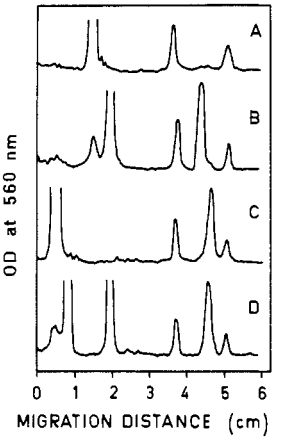

FIGURE 3: Electrophoretograms of myosin and isolated heads before and after cleavage at the modified thiocyanoalanine residues as described in the text. $NaDodSO_4$ electrophoresis of 20–30 μg of protein on 7.5% polyacrylamide gels. Staining and densitometry were performed as described under Materials and Methods. Protein bands (apparent molecular weights) are designated with increasing migration distance as follows: (A) head HC (90 000), light chain-1 (24 800), and light chain-3 (14 700); (B) residual head HC, cleavage fragment (68 000), light chain-1, cleavage fragment (20 000), and light chain-3; (C) myosin HC (200 000), light chain-1 (24 800), light chain-2 (17 500), and light chain-3 (14 700); (D) residual myosin HC, cleavage fragment (131 000), cleavage fragment (68 000) followed by the unchanged light chain pattern.

and divalent cation dependent ATPase activity levels when cold NBzSCN was employed. Splitting of the modified chains was carried out immediately after cyanylation without prior dialysis, or, alternatively, the cyanylation reaction was stopped by addition of acetic acid, and the modified protein was subsequently washed with acetone and lyophilized for storage before cleavage as described under Materials and Methods. Splitting was induced in either $NaDodSO_4$ or guanidine hydrochloride (Stark, 1977). Analysis of the products by $NaDodSO_4$ gel electrophoresis revealed that cleavage performed in the presence of $NaDodSO_4$ was more reproducible, yielding fewer protein bands than in guanidine hydrochloride. In the latter denaturing medium, the cyano radical appears to be rather labile with respect to other thiol groups, producing multiple bands when subsequently analyzed in $NaDodSO_4$ gel electrophoresis. However, it was found that this complication could be prevented by carboxamidomethylation in the guanidine hydrochloride medium before raising the pH to 9 and the temperature to 37 °C necessary for the cleavage reaction. Isolated myosin heads first purified by ion-exchange chromatography or affinity chromatography were treated in this way, and the material was filtered on agarose in 6 M guanidine hydrochloride to separate the cleavage products. Fractions were pooled containing the fragments corresponding to 70 000 and 20 000 daltons, as judged by $NaDodSO_4$ gel electrophoresis (Figure 2).

The electrophoretic protein band pattern of cleaved heads before separation by column chromatography showed a faint residual HC of 90 000-dalton apparent molecular weight (Schaub et al., 1978), while most of the material appeared in new bands at positions corresponding to 68 000 ± 4000 and 20 000 ± 1500 daltons, besides the bands of ligh chain-1 and light chain-3 at 24 800 and 14 700 apparent molecular weight, respectively (Figure 3). In chymotryptic head preparations, the light chain-2 is absent (Weeds & Pope, 1977). Independent molecular weight estimations of two cleaved head preparations from the column chromatography in 6 M guanidine hydrochloride yielded, on average, values of 71 000 and 21 000 daltons for the two fragments. In the case of myosin, again some residual 200 000-dalton material was present in $NaDodSO_4$ gel electrophoresis, while from five experiments after cleavage, the new bands containing most

of the heavy material appeared on average at the 131 000- and 69 000-dalton positions, and the light-chain pattern appeared unchanged as in the parent myosins. In the case of both isolated heads and myosin, there are therefore only two cleavage products whose molecular weights add up to about the corresponding parent heavy-chain species.

Characterization of the 70 000- and 20 000-Dalton Cleavage Products. Three approaches were employed to characterize the 70 000- and 20 000-dalton fragments obtained from isolated myosin heads after cyanylation.

(i) Using NBzSCN bearing a [^{14}C]-labeled thiocyano group for cyanylation allowed us to follow the radioactive distribution over the fragments after cleavage. During the splitting reaction, the radioactive carbon of the thiocyanolalanine becomes locked into the five-membered ring forming the iminothiazolidine derivative. In this form, the radioactive carbon is stable at the now modified NH_2 terminus of the nascent peptide. In several cleavage experiments starting from a myosin head preparation containing 1.6 mol of cyano groups per head, the distribution of radioactivity over the stained protein bands in $NaDodSO_4$ gel electrophoresis corresponding to the apparent molecular weights of 70 000 and 20 000 daltons was assessed. Around 80–90% of the recoverable radioactivity was associated with the 20 000- and only around 10% with the 70 000-dalton protein bands. Quantitative densitometric evaluation of the protein mass in these bands reveals that the radioactivity found corresponds to almost 1 mol of label incorporated in the 20 000-dalton fragment and only 0.1–0.2 mol in the 70 000-dalton fragment.

Since the two fragments obviously originate from the 90 000-dalton head HC, the incorporated label would be expected to add up to the originally incorporated 1.6 mol/HC. However, the results show that around 0.4 mol of label is lost. One likely explanation is that after incorporation of between one and two cyano groups per myosin head, those 90 000-dalton head HC which happen to be cyanylated on both thiol-1 and thiol-2 become split twice. The decapeptide originating from in between the two essential thiols and carrying one label in its modified NH_2 terminus was later lost during the experimental procedures such as dialysis and electrophoresis. On the other hand, the little radioactivity recovered in the 70 000-dalton fragment stems possibly also from double-labeled chains followed by incomplete cleavage at the thiol-1 position only, so that the modified thiol-2 remained in the 70 000-dalton fragment.

(ii) The position of the unusual amino acid N^{τ}-methylhistidine relative to the thiol-1 and thiol-2 groups is known since it is also contained in the sequenced cyanogen bromide peptide referred to above (Elzinga & Collins, 1977). As it lies 48 amino acid residues toward the COOH-terminal side of thiol-1, it should be located in the 20 000 molecular weight fragment resulting from cleavage of the head HC at either thiol-1 or thiol-2. Two independent estimations yielded 0.91 and 1.21 mol of N^{τ}-methylhistidine per mol of this fragment. Negligible amounts were obtained for the remaining 70 000-dalton fragment, indicating that it is absent from this piece of the HC (Table II). When the values for amino acid content are expressed on the basis per mole of fragment, there is a good correspondence between the sum of the values for the 20 000- and 70 000-dalton fragments and the 90 000-dalton HC of the isolated heads (Table II).

(iii) As the COOH termini of the fragments produced by cleavage assumed to occur at thiol-1 and thiol-2 are known from the cyanogen bromide peptide sequenced by Elzinga & Collins (1977), we subjected both fragments to end-group analysis by digestion with carboxypeptidase Y. Within the first hour of incubation, 0.4–0.6 mol of Ile was released from the 70 000-dalton fragment. Longer incubations up to 5 h led to release of around 1 mol of Arg together with smaller amounts of Ile, Gly, Leu, and Gln. In the case of the 20 000-dalton fragment as well as of the parent 90 000-dalton HC, short incubations up to 20 min released a mixture of Phe, Gly, Glu, and Ile in near-stoichiometric amounts. Of these, Phe as the COOH-terminal amino acid would be compatible with the specificity of chymotrypsin.

Table II: Amino Acid Composition of Head Heavy Chain and Cleavage Fragments after Cyanylation (Residues per Mole of Protein; Averages from Two Preparations)

amino acid	cleavage fragment 20 000	cleavage fragment 70 000	sum of both cleavage fragments	head heavy chain 90 000
Lys	18.8	52.8	72	72
His	3.3	11.4	15	15
Arg	8.4	21.7	30	32
Asp	15.1	55.7	71	74
Thr	7.7	35.4	43	48
Ser	8.0	30.0	38	40
Glu	26.8	90.8	118	106
Pro	8.7	23.9	33	32
Gly	11.9	38.5	50	51
Ala	13.3	46.8	60	63
Val	8.7	34.8	44	46
Met	4.6	17.0	22	23
Ile	6.9	31.7	39	41
Leu	16.0	47.5	64	64
Tyr	3.9	25.5	29	31
Phe	9.5	34.6	44	45
3MHis[a]	1.1	0.2	1.3	0.9

[a] 3MHis stands for N^{τ}-methylhistidine.

Discussion

Balint and co-workers (1978) have placed the essential thiol-1 and thiol-2 groups at a distance corresponding to 85 0000–95 000-daltons worth in the primary structure from the NH_2 terminus of the HC in the head portion of the myosin molecule. They employed different digestion procedures on heavy meromyosin, and so their method relies on molecular weight estimations of a number of proteolytic fragments in a $NaDodSO_4$ gel electrophoretic system. In the work presented here, the molecular weight of only one fragment needs, in principle, to be estimated, and alignment of this fragment relative to the second one generated by the chemical cleavage step is sufficient for location of the essential thiols within the myosin HC. Two facts lead to this situation: First, thiol-1 and thiol-2 lie only a few amino acids apart in the primary structure (Elzinga & Collins, 1977); hence chemical cleavage at either residue produces fragments of virtually identical molecular weights. Second, experimental conditions were chosen so that only these two thiols became specifically modified by cyanylation and allowed chemical cleavage there. The cyanylation reaction is strictly specific for thiol groups and has been used for modification of reactive thiols in a number of undenatured enzymes (Stark, 1977).

It has recently been claimed that modification of the essential thiols in myosin by attaching groups of small size such as the cyano (Wiedner et al., 1978) or thiomethyl group (Botts et al., 1979) does not have the characteristic effects on the enzymatic properties as shown in Figure 1. They even dismissed the essentiality of thiol-1 and thiol-2. However, close examination of their results reveals that nonspecific reaction on groups other than thiol-1 and thiol-2 must have occurred.

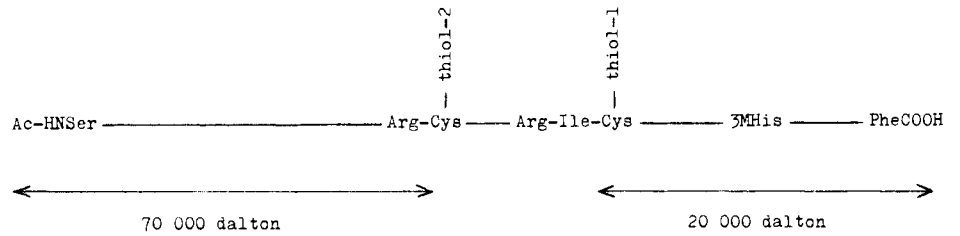

FIGURE 4: Schematic representation of the head heavy chain showing the alignment of the 70000- and 20000-dalton fragments. 3MHis stands for N^{τ}-methylhistidine. For further explanation see Discussion.

In addition, the introduced cyano group is labile under certain experimental conditions, and side reactions may therefore have subsequently occurred. In the present work, these complications were avoided by taking the precaution of rapid blocking of the remaining free thiols. The nonspecific labeling cited may have been also due to the vehicle moiety of the molecule which introduces the adduct. For cyanylation, Wiedner et al. (1978) employed an active site directed ribofuranosylpurine derivative. Wagner & Yount (1975), who also used an active site directed purine disulfide analogue of ATP for introducing cyano groups, found them indeed bound to thiols other than thiol-1 and thiol-2. In our experiments, the cyano groups were introduced via the vehicle moiety Nbs, not at all chemically related to the substrate ATP. Modification with Nbs_2, where Nbs again serves as the vehicle, this time introduces an Nbs group which is large in comparison to the cyano group but, nevertheless, produces the same effects on the enzymatic activities (Figure 1). Furthermore, this effect parallels exactly the pattern obtained by modification with MalNEt under specified conditions in intact myosin as well as in isolated heads (Schaub et al., 1978). Finally, the fact that addition of the three distinct groups, namely *N*-ethylsuccinimido, cyano, and Nbs with their widely differing sizes, produces identical effects on the enzymatic properties demonstrates the essentiality of thiol-1 and thiol-2 and the specificity of the cyanylation reaction, using NBzSCN for these groups. In the case of modification with radioactively labeled MalNEt, we have isolated and identified the thiol-1, containing tryptic tripeptide, and the thiol-2, containing tryptic nonapeptide, from both proteins (Kunz et al., 1977, 1980).

We now turn to the alignment of the fragments obtained after chemical cleavage at the essential thiols. The fact that a fragment of the same apparent molecular weight of 70 000 daltons is produced from both intact myosin and isolated heads (Figure 3) suggests immediately that it originates from the NH_2 terminus of the HC, which is itself located in the head portion. This terminal amino group is blocked by acetylation (Starr & Offer, 1973), and remains blocked in chymotryptic head preparations (Schaub et al., 1978). The new NH_2 terminus produced by the chemical cleavage employed here is also blocked as it is built into the iminothiazolidine ring. Therefore, only the COOH termini are available for end-group analysis. In the sequence of the cyanogen bromide peptide, Ile-Arg-Ile-Gly follows thiol-1 in the NH_2-terminal direction and Arg-Leu-Gln follows thiol-2 in the same direction (Elzinga & Collins, 1977). Our finding of less than stoichiometric amounts of Ile followed by 1 mol of Arg indicates that the chemical cleavage produced two types of 70000-dalton fragments. This interpretation is compatible with splitting at thiol-1 only or at both thiol-1 and thiol-2, which are only ten amino acid residues apart, and so giving rise to fragments with and without this decapeptide (Figure 4). This supports the placing of the 70000-dalton fragment at the NH_2 terminus of the HC. Complementary evidence is given by the similar results of end-group analysis between the 20000-dalton fragment and the parent 90000-dalton head HC. As Phe was among the initially released amino acids, the only one typical for the specificity of chymotrypsin, it probably represents the COOH terminus.

Additional strong support for our alignment is given by the presence of the unique amino acid N^{τ}-methylhistidine as well as the recovery of 1 mol of label introduced with the bound cyano group, both in the 20000-dalton fragment (Figure 4). Taken together, the overall loss of radioactive label in the decapeptide originating when splitting occurred at both thiol-1 and thiol-2 and the little radioactivity sometimes recovered in the 70000-dalton fragment indicate that cleavage at thiol-2 was not as efficient as at thiol-1. Therefore this fragment represents a mixture of species with and without the decapeptide at its COOH terminus, corroborating the end-group analysis.

In conclusion, our alignment of the fragments obtained together with their molecular weight estimations by two independent methods places the essential thiol-1 and thiol-2 groups at around 580 amino acid residues from the NH_2 terminus in the head portion of the myosin molecule. In isolated heads prepared by chymotrypsin, they are around 170 residues from the position where proteolysis takes place. This stretch contains the COOH-terminal 72 amino acids, including the unique N^{τ}-methylhistidine of the cyanogen bromide peptide sequenced by Elzinga & Collins (1977). As these 72 residues contain two Pro, there are seven more Pro within the remainder of the 20000-dalton fragment of around 108 residues, lying toward its COOH terminus (Table II). In other words, this small fragment at the end of the 90000-dalton head HC has a Pro content of 65 per 1000 residues, which is higher than that of any myosin subunit or other fragment isolated so far. This base of the myosin head rich in Pro seems to be the region where not only chymotrypsin but also other proteinases preferentially attack the myosin HC (Lu, 1980). The location of the essential thiols at 170 amino acid residues from this point is somewhat further along the head HC than that suggested by Balint et al. (1978) and does not allow any conclusion to be drawn about their spatial position within the globular head, as this distance could well span the entire head. It is, nevertheless, noteworthy that their microenvironment is affected by such diverse events as binding of actin (Barany et al., 1969; Schaub & Watterson, 1972), nucleotides (Yamaguchi & Sekine, 1966; Schaub et al., 1975), and divalent cations to the light chain-2 (Watterson et al., 1979).

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