ISOLATION OF CYANOGEN BROMIDE AND TRYPTIC PEPTIDES CONTAINING THE ESSENTIAL THIOL GROUPS FROM ISOLATED MYOSIN HEADS

P. A. KUNZ, J. T. WALSER, J. G. WATTERSON and M. C. SCHAUB
Institute of Pharmacology, University of Zürich, Gloriastrasse 32, CH-8006 Zürich, Switzerland

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1. Introduction

The two classes of essential thiol groups of myosin have been defined according to the effect of their blockage on the enzymic properties of the protein [1]. According to this functional definition the so-called thiol-1 and thiol-2 groups are essential for the K^+-dependent ATPase. Blockage of thiol-1 has a variety of effects on the divalent cation-dependent ATPases depending on the conditions of the ATPase tests [2]. The second group, the thiol-2 group, can be differentiated since after blockage of thiol-1, it is essential for the functioning of the divalent cation-dependent ATPases. With the use of radioactive reagents it has been established that there is per heavy meromyosin subfragment-1, i.e., per myosin head, just one of each group and that both reside in the uncleaved head heavy chain of mol. wt 90 000 [3], obtained by chymotryptic digestion of myosin.

The results of experiments employing a thiol cross-linking reagent have shown that these two thiol groups are in close spatial proximity in the native myosin molecule [4], which raises the question of their positions relative to one another in the heavy chain. We prepared isolated myosin heads in which, in one case thiol-1, and in another thiol-1 and thiol-2, were specifically labelled according to their effect on the enzymic function. From these the labelled CNBr-peptides were isolated and the essential thiol groups identified after tryptic digestion. Since this work began we learned that Elzinga and Collins [5] have recently isolated and sequenced a CNBr-peptide of the intact myosin heavy chain. Its sequence indicates that this peptide contains the thiol-1 and thiol-2 from comparison of their immediate environment with published sequences of their tryptic peptides [6,7].

Abbreviations: ATPase, adenosine 5′-triphosphatase (EC 3.6.1.3); IAA, iodoacetamide; NEM, *N*-ethylmaleimide

Address correspondence to: Dr M. C. Schaub, Institute of Pharmacology, University of Zürich, Gloriastrasse 32, CH-8006 Zürich, Switzerland

2. Methods

Myosin heads were prepared by chymotryptic digestion [8] of myosin from fast skeletal rabbit muscles. For functional differentiation of the essential thiol groups of heads, progressive alkylation with radioactivity labelled [^{14}C]IAA and [^{14}C]NEM and subsequent analyses of incorporated radioactivity and K^+- as well as Ca^{2+}-dependent ATPase activities were performed as has been described in detail for myosin [9]. For identification of the essential thiol groups, in one head preparation thiol-1 was labelled with [^{14}C]IAA in the presence of Mg-pyrophosphate, at pH 8.5 and 22°C, incorporating around 5×10^6 dpm/μmol or 0.8–0.9 mol IAA. The same procedure was carried out on a second head preparation leading to an incorporation of around 1.1 mol IAA/head (i.e., approx. 6×10^6 dpm/μmol) which was followed by additional incorporation of 0.8 mol. [^{14}C]NEM/head corresponding to around 8×10^6 dpm/μmol, resulting from alkylation, at pH 7.6. After complete carboxymethylation with iodoacetic acid [10] in the presence of 6 M guanidine–HCl, the heavy chains of both preparations were separated from all smaller molec-

ular weight components by gel filtration on Sephadex G-100 (column dimensions: 2 × 150 cm) in 6 M guanidine–HCl [11]. The purified head heavy chains were then subjected to cyanogen bromide cleavage [12,13] and the radioactively labelled peptides isolated as described in the text.

Electrophoresis of CNBr-peptides was performed on 12% polyacrylamide gels in sodium dodecyl sulphate, at pH 7.0 [14] and at pH 4.5 in 4 M urea [15]. Tryptic digests were separated by 2-dimensional paper electrophoresis at high voltage in cooled tanks first at pH 6.5 [16] and secondly at pH 1.9 [17], and the radioactive peptides identified by autoradiography using X-ray films Kodirex from Kodak Co. (35 × 45 cm). For autoradiographic exposures at least 80 000 dpm were loaded and for preparative purposes for thiol group identification 50–100 nmol/peptide were loaded. Amino acid analyses were carried out according to standard procedures [18] on peptide hydrolysates. Following Edman degradation [19] amino acids were identified by thin-layer chromatography [20] as well as by analysis after regeneration of the phenylthiohydantoin derivatives [21]. Protein concentrations were estimated by the biuret reaction or according to Lowry et al. [22].

3. Results and discussion

3.1. *Functional differentiation of the 2 essential thiol groups in isolated myosin heads*

Isolated heads prepared in various ways have been reported to contain around 12 thiol groups/mol [23–26]. However, of these only one reacts readily with [^{14}C]IAA in the heads used in this work which contained an uncleaved heavy chain of 90 000 daltons and one so-called alkali light chain/mol [3]. Even carrying out the alkylation for 1 h at 25°C with a 100-times molar excess of IAA, at pH 9.5, or with addition of 1 M urea, at pH 8.0, did not increase the amount incorporated. The group blocked by this reagent is invariably the thiol-1 group as the Ca^{2+}-dependent ATPase is activated and the K^+-dependent ATPase simultaneously destroyed (fig.1). Alkylation with [^{14}C]NEM, also under a wide range of conditions, shows that just 2 thiol groups become blocked. In the presence of Mg-pyrophosphate, or without ligands, the order of reaction is thiol-1 followed by thiol-2 as indicated by the course of the Ca^{2+}-dependent ATPase activity (fig.1). Because of the selectivity of IAA for the thiol-1 group it is possible to perform sequential alkylation steps whereby thiol-1 is first blocked with IAA and then thiol-2 with NEM. Hence, identification and localization of the 2 essential thiol groups could be achieved by comparison of the radioactive peptides originating from 2 head preparations, one with thiol-1–[^{14}C]IAA, and the other with thiol-1–[^{14}C]IAA as well as thiol-2–[^{14}C]NEM labelled.

3.2. *Isolation of radioactively labelled CNBr-peptides*

After cyanogen bromide cleavage the radioactive CNBr-peptides of both preparations were subjected to the same purification procedure. They were passed over Sephadex G-50 fine in 5% acetic acid (column dimensions: 1.5 × 200 cm). The major part of the radioactivity appeared in a single peptide fraction of low absorbance at 280 nm well separated from most of the non-radioactive peptide material. It was further subjected to ion exchange chromatography on SE-Sephadex C-50 in 50 mM Tris–Cl, pH 8.5. In this medium the material remained adsorbed on the ion exchange resin even after a salt gradient of 0–1 M KCl and was eluted with 6 M urea. After desalting on Sephadex G-25 in 10% formic acid, it was adsorbed on CM–Sephadex C-25 and further non-radioactive peptide material eluted. A medium containing 6 M urea had to be again applied to recover the radioactive

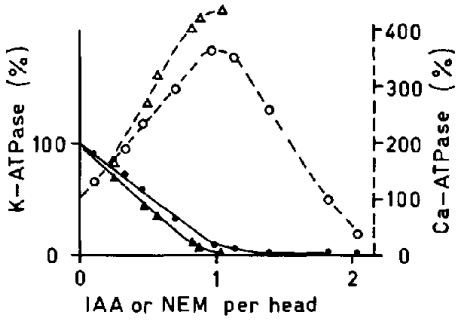

Fig.1. Effect of incorporation of radioactive [^{14}C]IAA and [^{14}C]NEM on ATPase activities of chymotryptic myosin heads, mol. wt 120 000. Prior to ATPase tests alkylation was performed on 8.2 μM heads in the presence of 5 mM $MgCl_2$ plus 2.5 mM pyrophosphate for 30 min at pH 8.0, 0°C and different concentrations of IAA or NEM. Closed symbols, K-ATPase, open symbols, Ca-ATPase. (▲ △) IAA; (● ○) NEM.

material. After 2 further passages over Sephadex G-25 in 10% acetic acid a single radioactive peak was obtained in the case of both preparations. This peptide peak was examined for purity by checking the N-terminal amino acids. Two successive Edman steps [19] yielded the sequence Glu–His– with both preparations. Electrophoresis on polyacrylamide gels, in the presence of 4 M urea and pH 4.5, gave a single peptide band in each case which migrated at the same position. Gel electrophoresis in the presence of sodium dodecyl sulphate also revealed one band from each preparation as well as their mixture, migrating in the 9000–11 000 mol. wt region as judged from comparison of electrophoretic mobilities with those of the 3 myosin light chains, ribonuclease and cytochrome *c*. It can be assumed that the radioactivity present in this band equals that originally in the corresponding heavy chains on a molar basis, because it is known that both thiol-1 and thiol-2 groups are located in the heavy chain [3]. Thus it was also possible to estimate the molecular weight of the peptide from its specific radioactivity based on dry weight and the amount incorporated in its parent heavy chain. As this procedure gave values in the range 9000–10 000, a mol. wt of 10 000 was used for the evaluation of the amino acid composition (table 1). The facts that both peptides contain almost identical sets of amino acids and bear the same N-terminal ends, lead directly to the conclusion that both essential thiol groups are located in the same CNBr-peptide. For identification of the labelled thiol groups within these peptides the latter will be referred to as CNBr-P–thiol-1 stemming from the head preparation whose thiol-1 group was blocked, and CNBr-P–thiol-1+2 stemming from those heads whose thiol-1 as well as thiol-2 groups were blocked.

3.3. *Identification of the essential thiol-1 and thiol-2 groups*

The purified CNBr-peptides were subjected to tryptic digestion with a peptide : enzyme ratio of 100:4 (w/w) at pH 8.5. Passage over Sephadex G-25 in 5% acetic acid gave one radioactive peak in the case of CNBr-P–thiol-1 and complete separation of 2 peaks in the case of CNBr-P–thiol-1+2. The second peak of CNBr-P–thiol-1+2 eluted at the same volume as that of CNBr-P–thiol-1 (fig.2). Complete isolation of the radioactive tryptic peptides from each peak was achieved by 2-dimensional paper electrophoresis.

Table 1
Amino acid composition of radioactively labelled CNBr-peptides (residues/10 000 daltons)

Amino acid	CNBr-P–thiol-1	CNBr-P–thiol-1+2
Lys	9.1	8.8
His	2.9	3.5
Arg	3.6	4.7
Asp	8.0	7.1
Thr	4.3	2.9
Ser	5.2	5.8
Glu	11.8	12.5
Pro	2.8	2.1
Gly	7.1	7.7
Ala	7.0	6.2
Val	4.6	5.3
Met	1.2	0.9
Ile	4.6	5.3
Leu	11.3	10.8
Tyr	3.2	3.0
Phe	4.6	4.8
Trp	0.05	0.02
Cys	1.8	1.7

Thr and Ser were extrapolated to zero time of hydrolysis. Val, Ile and Leu were determined after 72 h hydrolysis. Met was determined as homoserine plus homoserine lactone. Trp was determined after hydrolysis with *p*-toluenesulfonic acid [27]. Cys was determined as CM–Cys, Cys–COOH and succinyl–Cys

Autoradiography revealed one radioactive spot from the material in the peak common to both CNBr-P–thiol-1 and CNBr-P–thiol-1+2, which migrated towards the cathode in the first dimension at pH 6.5 and which

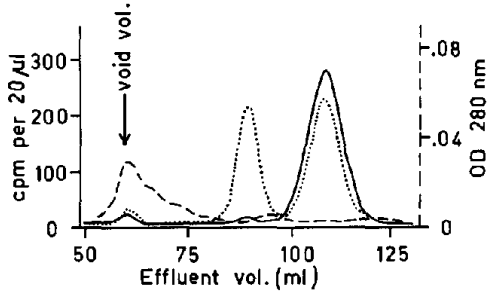

Fig.2. Elution profiles of tryptic digests of CNBr-P–thiol-1 (solid line) and CNBr-P–thiol-1+2 (dotted line) peptides from the Sephadex G-25 column in 5% acetic acid by following radioactivity. Also shown in $A_{280\,nm}$ (dashed line) which was approximately the same for both preparations. Column dimensions, 1.7 × 90 cm; flow rate, 6.4 ml/h.

remained as a single spot when samples from the peaks were mixed before electrophoresis. The peptides were eluted from the paper with 50% acetic acid and subjected to amino acid analysis. In both cases it was found to contain the 3 amino acids Arg, Ile and Cys, which agrees with the published sequence for the tryptic peptide of the thiol-1group, Ile–Cys–Arg [6].

Material from the first peak of CNBr-P–thiol-1+2, corresponding to peptides of larger molecular weight, yielded 2 radioactive spots after the same procedure. But in this case both migrated with negative charge towards the anode at pH 6.5. Amino acid analyses demonstrated that both peptides contained Arg, Asp, Glu, Gly, Val, Ile, Leu and Cys, and that both therefore represented the tryptic peptide of thiol-2 [7]. Since this group was labelled with NEM, the splitting of the electric charge carried by the peptide can be attributed to a modified form of NEM known to occur [28], which is ionized at pH 6.5, and not to a complication arising from incomplete tryptic digestion.

4. Conclusion

The essential thiol-1 and thiol-2 groups, labelled according to the effect of their blockage on the enzymic function, have been identified and could also be located in a single CNBr-peptide of the head heavy chain. Comparison of its amino acid composition and N-terminal end with those of the CNBr-peptide isolated from myosin heavy chains [5] indicates that they are identical. Thus this peptide resides entirely within the mol. wt 90 000 fragment of the heavy chain forming the myosin head.

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