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Myosin Heavy Chain Covalently Modified at Its Reactive Site Sulfhydryl Residues is Preferentially Degraded by Calpain

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N-ethylmaleimide at low concentrations is known to interact specifically with 2 sulfhydryl residues in myosin heavy chain. Calpain, a Ca^{2+}-dependent neutral protease isolated from chick skeletal muscle, was found to preferentially degrade the alkylated protein but much less significantly the unmodified protein. Exposure of myosin to $KMnO_4$, which is also known to interact with sulfhydryl groups, also caused the rapid degradation of the myosin heavy chain. Furthermore, treatment of each agent with increasing concentrations results in a greater loss of the myosin ATPase activity, indicating that the modification occurred at the reactive site sulfhydryl residues. These results suggest that the covalent modification at the reactive site sulfhydryl residues in the myosin heavy chain may mark the protein for degradation by intracellular proteases such as calpain.

KEY WORDS: Calpain, Myosin Heavy Chain, $KMnO_4$, N-ethylmaleimide

Oxidative modification has been suggested to be an important mechanism triggering the breakdown of critical cell proteins such as glutamine synthetase in *E. coli* (Stadtman and Oliver, 1991). Several mammalian proteolytic enzymes, including calpain and 20S proteasome, have been shown to preferentially degrade the glutamine synthetase that was irreversibly inactivated by a mixed function oxidant system (Rivett, 1985a, 1985b). Proteases Re and So in *E. coli* have also been demonstrated to degrade rapidly this damaged enzyme but not the native protein (Park *et al.*, 1988; Lee *et al.*, 1988). In addition, we have recently shown that sarcoplasmic reticulum Ca^{2+}/Mg^{2+}-ATPase, which is oxidatively modified at its reactive site sulfhydryl (SH) residues using $KMnO_4$, is preferentially degraded by calpain (Chung *et al.*, 1990). Various other types of covalent modification have also been implicated as marking steps that render proteins more susceptible to proteolytic attack (Rivett, 1986), and they include formation of mixed disulfides, oxidation of methionine residues or iron-sulfur centers, and phosphorylation (Horowitz and Bowman, 1987; Pontremoli *et al.*, 1987).

A myosin molecule contains over 40 SH residues, of which 12 or 13 reside on each of the two myosin heads (Lowey *et al.*, 1969). However, only a few of them are readily available for alkylation under mild conditions. Two SH residues, SH1 and SH2 located on myosin heavy chain,

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have been extensively studied, since their modifications dramatically affect the ATPase activity of myosin (Sekine and Kielly, 1964). Among the different SH reagents used for the modification, the reaction of N-ethylmaleimide (NEM) with myosin from skeletal muscle is particularly well documented and the peptide fragments containing the modified SH residues have been isolated and sequenced (Yamashita *et al.*, 1974). In addition, cross-linking of SH1 and SH2 groups with bifunctional SH reagents has been demonstrated that the SH residues involve in noncovalent trapping of adenine nucleotides, presumably at the active site (Wells and Yount, 1979). Therefore, we have examined if the covalent modification of the specific SH residues may render myosin molecules more susceptible to degradation by intracellular proteases.

Calpain has been implicated as an important protease involving in muscle protein turnover and degradation of proteins that were marked by covalent modifications, such as mixed function oxidation and phosphorylation (Rivett, 1986; Reddy *et al.*, 1975; Pontremoli *et al.*, 1987). Therefore, we chose this protease to test if myosin heavy chain, whose reactive site SH residues are modified by NEM or $KMnO_4$, is selectively degraded by the enzyme. The results of this study show that calpain from chick skeletal muscle specifically hydrolyzes the covalently modified myosin heavy chain.

Materials and Methods

Materials

Calpain was purified from adult chick skeletal muscle as described previously (Chung *et al.*, 1990). Purification of myosin from chick breast muscle was carried out by following the procedure of Margossian and Lowey (1973). All reagents used were of analytical grade and purchased from Sigma, unless otherwise indicated.

Chemical Modifications

The purified myosin (1.4 mg/ml) was alkylated by incubating with NEM for 50 min at 30°C in 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) buffer (pH 7) containing 100 mM KCl, 5 mM $MgCl_2$ and 50 μM $CaCl_2$ as described (Kawakita *et al.*, 1980). Myosin was also oxidatively modified by treating $KMnO_4$ at 22°C for 50 min in 30 mM 3-(N-morpholino)propane sulfonic acid (MOPS) buffer (pH 6.9) containing 100 mM KCl and 125 mM sucrose as described (Ariki and Shamoo, 1983). After the incubations, the reactions were terminated by adding 2-mercaptoethanol to a final concentration of 5 mM. The protein was also oxidized by a metal-catalyzed mixed oxidation system (i.e., using Fe^{3+}/O_2/ascorbate) as described previously (Lee *et al.*, 1988).

Assays

The ATPase activity of myosin was assayed by incubation at 30°C for 1 h with 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM ATP, 5 mM $CaCl_2$ and 0.5 M KCl. After the incubation, the reaction was stopped by adding 2% (w/v) sodium dodecyl sulfate (SDS) and the release of inorganic phosphates was assayed colorimetrically as described previously (Hwang *et al.*, 1988).

Proteolysis

The modified myosin was incubated at 30°C with 1 μg of the purified calpain in 50 mM Tris-HCl buffer (pH 8) containing 5 mM $CaCl_2$ and 15 mM 2-mercaptoethanol. After the incubation for appropriate periods, the reaction was terminated by adding 2% SDS and the resulting samples were electrophoresed on 8% (w/v) polyacrylamide slab gels containing SDS (Laemmli, 1970). Protein bands were visualized by staining with Coomassie R-250.

Results and Discussion

To examine if calpain preferentially hydrolyzes the covalently modified form of myosin, the protease was incubated with the purified myosin that had been alkylated with NEM. As shown in Fig. 1, calpain hydrolyzed to greater extents the myosin heavy chain that had been treated with higher amounts of NEM. Under the same condition, however, calpain showed insignificant

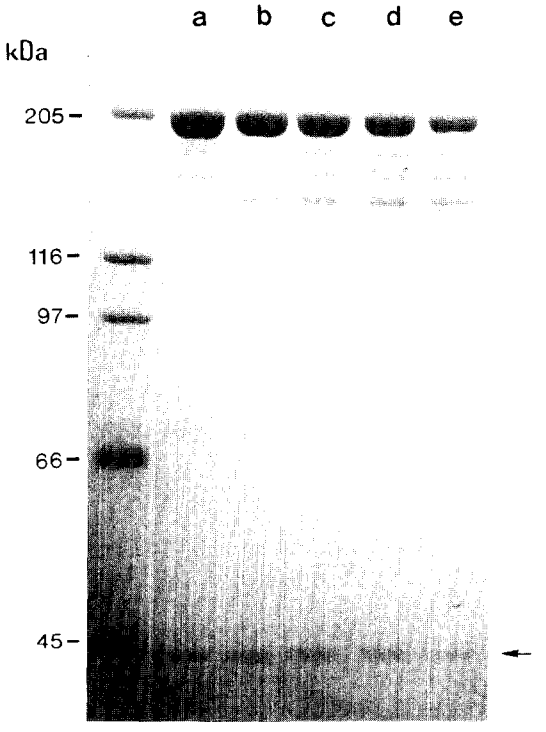

Fig. 1. Hydrolysis of the NEM-modified myosin heavy chain by calpain. The purified myosin preparation (20 μg each), that had been treated with 0 (lane b), 0.1 (c), 0.2 (d) or 0.3 mM NEM (e), was hydrolyzed by incubation with 1 μg of calpain for 1 h at 30°C as described in Materials and Methods. The untreated myosin was also incubated without calpain as a control (lane a). The samples were then electrophoresed in the presence of SDS and stained with Coomassie R-250.

activity against the myosin light chain (data not shown) or actin molecules (indicated by the arrow), which were copurified with the myosin preparation. These results clearly suggest that the covalent modification by NEM appears to occur specifically to the myosin heavy chain and results in preferential degradation of the alkylated protein by calpain. Of particular interest is the finding that gel electrophoretic pattern of the cleavage products of the alkylated myosin heavy chain (lanes b-e) is very similar to that of the untreated protein (lane a), although the extent of proteolysis was much less in the latter case. Thus, it appears that the alkylation of myosin heavy chain may increase its susceptibility to the target protease.

Calpain was also found to selectively hydrolyze the myosin heavy chain that had been oxidatively damaged by treatment with $KMnO_4$ (Fig. 2). It is also noteworthy that the oxidative modification of the myosin light chain (data not shown) or the copurified actin does not affect its susceptibility to calpain (shown by the arrow). Since NEM and $KMnO_4$ are known to interact with SH residues (Ariki and Shamoo, 1983; Kawakita *et al.*, 1980), the chemical modification (i.e., alkylation or oxidation) of SH residues may be responsible for the recognition and degradation by calpain. However, the $KMnO_4$-oxidized form of myosin heavy chain was degraded more extensively by calpain than the NEM-modified protein. Thus, it appears that $KMnO_4$ and NEM have different accessibility to the SH residues in the myosin heavy chain molecules.

To define more clearly the selectivity of the covalently modified form of the myosin heavy

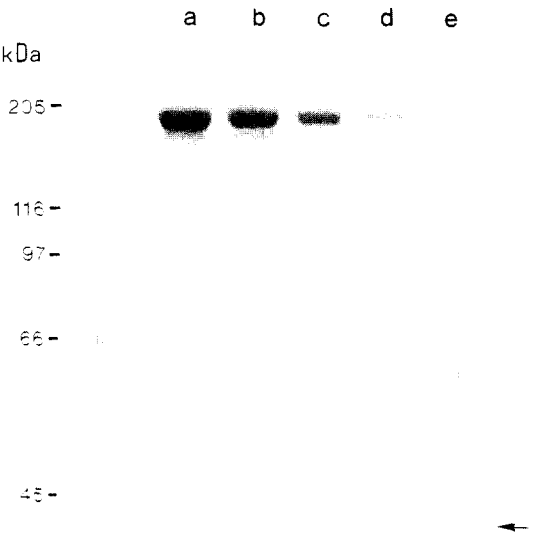

Fig. 2. Hydrolysis of the $KMnO_4$-oxidized myosin heavy chain by calpain. The purified myosin preparation (20 μg each), that had been treated with 0 (lane b), 50 (c), 100 (d) or 200 μM $KMnO_4$ (e), was hydrolyzed by incubation with 1 μg of calpain for 1 h at 30°C. The untreated myosin was also incubated without calpain as a control (lane a). The samples were then electrophoresed and stained as in Fig. 1.

chain over the unmodified polypeptide in proteolysis, calpain was incubated with these proteins for varying periods. As shown in Fig. 3, both the NEM-modified and $KMnO_4$-oxidized forms are degraded at much higher rates than the untreated myosin heavy chain. Thus, it is at least clear that calpain preferentially degrades the myosin heavy chain that were chemically modified at its SH residues.

To determine whether the chemical modifications occur at the SH residues near or at the reactive site or other structural locus of the myosin heavy chain, the ATPase activity of myosin was measured in the presence of increasing concentrations of NEM and $KMnO_4$. Fig. 4 shows that treatments of higher amounts of the agents cause greater reductions in the ATP hydrolysis. On the other hand, the myosin preparation that had been exposed to a mixed oxidant system (i.e., Fe^{3+}/O_2/ascorbate) was neither inactivated nor degraded by calpain (data not shown). The mixed oxidant system has been reported to alter a single histidine residue but not SH groups in glutamine synthetase (Levine, 1983). These results suggest that the irreversible inactivation by the covalent modification correlates well with susceptibility to proteolytic attack and that the modifications by NEM and $KMnO_4$ occur specifically at the reactive site SH residues of the myosin heavy chain.

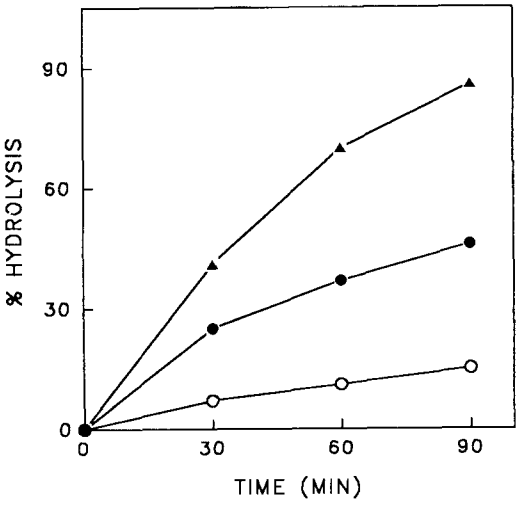

Fig. 3. Time-dependent hydrolysis of the NEM-modified and $KMnO_4$-oxidized myosin heavy chain by calpain. The purified myosin preparation (20 μg each), that had been treated with 200 μM NEM (●) or 50 μM $KMnO_4$ (▲), was incubated with 1 μg of calpain at 30°C for various periods. The protein was also incubated without calpain (○). The samples were then electrophoresed and stained as in Fig. 1. The bands corresponding to the myosin heavy chain were scanned using a densitometer, and the intensity disappeared by the incubation with calpain was expressed as the percent of that shown by the incubation in its absence.

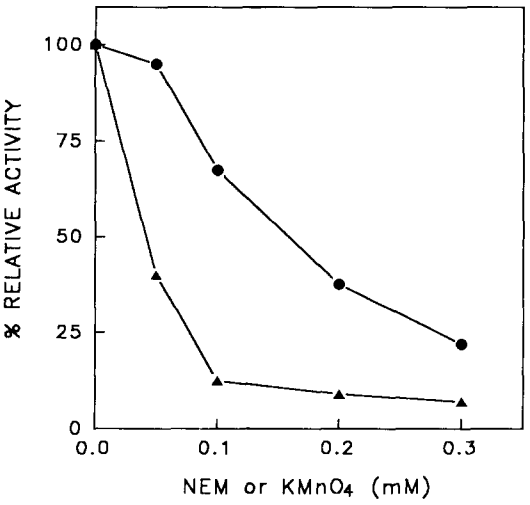

Fig. 4. Dose-response effects of NEM (●) and $KMnO_4$ (▲) on the ATPase activity of myosin. The purified myosin preparation (1.4 μg) was assayed for its ATPase activity in the presence of increasing amounts of each agent as described in Materials and Methods. ATP hydrolysis shown in the absence of the reagents was expressed as 100% activity.

Thus, it appears possible that calpain may involve within cells in the selective hydrolysis of the oxidized or covalently modified proteins such as myosin. Nevertheless, the physiological significance of this process is perplexing, because the majority of calpain molecules in chick skeletal muscle require millimolar concentration of Ca^{2+} (Kwak *et al.*, 1993). Neither is clear about the mechanism how the modified proteins are recognized by calpain, although specific, undefined alteration(s) in the structure of the proteins can be an easy explanation for it.

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활성화 부위의 황화기가 화학적으로 변형된 Myosin Heavy Chain의 Calpain에 의한 선택적 분해

곽규봉, 정성수, 이창호*, 하두봉, 정진하(서울대학교 자연과학대학 분자생물학과 및 세포분화 연구센터; *강릉대학교 생물학과)

N-ethylmaleimide는 낮은 농도에서 Myosin heavy chain의 활성화 부위에 존재하는 2개의 황화기에 선택적으로 결합하는 것으로 알려져 있다. 계 근조직에서 분리된 Ca^{2+}-의존성 단백질 분해효소, Calpain은 이와같이 알킬화된 Myosin heavy chain을 알킬화 되지 않은 것에 비하여 우선적으로 분해하는 것으로 나타났다. 또한, 황화기를 특이하게 산화시키는 $KMnO_4$가 처리된 Myosin heavy chain도 산화되지 않은 것에 비하여 훨씬 빠른 속도로 분해됨을 관찰하였다. 뿐만아니라, N-ethylmaleimide나 $KMnO_4$의 처리는 농도-의존적으로 myosin에 의한 ATP 분해를 불활성화 시키었다. 이러한 결과는 활성화 부위에 존재하는 황화기의 화학적 변형은 Myosin heavy chain이 Calapin과 같은 세포내 단백질 분해효소에 의하여 인식되는 기구임을 시사한다.