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IMMUNOCHEMICAL PROBING OF THE N-TERMINUS OF THE MYOSIN HEAVY CHAIN

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SUMMARY: The reactivity of myosin subfragment 1 (S-1) towards site specific polyclonal anti-N-terminus antibodies was examined in competitive ELISA titrations. Tryptic digestion of S-1 and specifically the cleavage at the 25/50K junction greatly increased the accessibility of the N-terminus region to the antibodies. The binding of actin to S-1 did not change significantly the reactivity of either tryptic or intact S-1 towards anti-N-terminus antibodies. These results suggest the interdependence of the N-terminus and 25/50K junction regions on S-1.

Many current studies on the role of myosin in muscle contraction focus on two important questions, the substructure of the myosin heads and the flexible motions of yet unidentified portions of S-1. The cleavage of S-1 by trypsin (1) and other proteolytic enzymes (2,3) into three major fragments, the 25, 50, and 20K units proved particularly helpful in generating new topographic information on specific sites on the myosin head. Such information is derived from electron microscopic observation of specific antibody binding sites on S-1 (4,5) as well as chemical modifications (7,8) and spectroscopic work (9,10).

Although the tryptic cleavage of S-1 doesn't affect its catalytic function or binding to actin, it appears to modify the coupling between the nucleotide and actin sites on myosin (11,12). Thus, dynamic aspects of myosin structure and function may be revealed by comparing the properties of intact and tryptic S-1. Recent observations indeed detected small changes in the α-helical content of S-1 upon its tryptic proteolysis (13) and suggested increased segmental motions in the cleaved protein (14,15).

In this study we have examined the effect of actin and that of tryptic cleavage of S-1 on the reactivity of this protein towards antibodies directed against the N-terminus of the myosin heavy chain (a generous gift

The abbreviations used are: SDS, sodium dodecyl sulphate; PBS, phosphate buffered saline containing 171 mM NaCl, 3.35 mM KCl, 10.11 mM Na_2HPO_4, 1.84 mM KH_2PO_4, pH 7.4; PTB, PBS with 0.05% Tween 20 and 0.1% BSA; IgG, immunoglobulin G, S-1, rabbit skeletal myosin subfragment 1.

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from Dr. K. Sutoh). We found that tryptic digestion of S-1 and mainly its cleavage at the 25/50K junction greately increased the reactivity of S-1 towards these antibodies. Actin, on the other hand, had only small if any effect on the reaction of S-1 with anti-N-terminus antibody.

MATERIALS AND METHODS

Materials. α-chymotrypsin, trypsin, soybean trypsin inhibitor, ATP, alkaline phosphatase conjugated goat anti-rabbit IgG antiserum and the substrate (5 mg tablets) were purchased from Sigma.

The antiserum against the N terminus of S-1 was a generous gift from Dr. K. Sutoh. The antiserum was raised in rabbits against a synthetic peptide with the sequence of acetyl-Ser-Ser-Asp-Ala-Asp-Met-Ala-Val-Lys (6) corresponding to the first eight N-terminal residues of the rabbit skeletal myosin heavy chain (16).

Rabbit skeletal myosin, myosin subfragment 1, and actin were prepared as described previously (17).

Tryptic digestions of S-1 were carried out at room temperature in 30 mM KCl, 10 mM imidazole, pH 7.0 and at a trypsin to S-1 ratio of 1 : 50 (w/w). Various tryptic species of S-1 were generated under the following conditions. The 25/50/20K S-1 was made by digestion of S-1 (3 mg/ml) for 30 minutes. The 25/70K S-1 was made by digestion of acto-S-1 complex (2 mg/ml each) for 45 minutes. After the digestion, actin and cleaved S-1 were separated by centrifugation in the presence of MgATP. The cleaved S-1 was freed from nucleotides by centrifugation through Sephadex columns (18). The 75/20K S-1 was obtained by tryptic cleavage (5 minutes) of S-1 in 0.6 M NaCl, 10 mM phosphate (pH 7.0) and in the presence of 10mM MgATP, a condition which inhibits tryptic cleavage at the 25/50K junction (19). The cleaved S-1 was freed from nucleotides as above. All digestions were quenched with soybean trypsin inhibitor.

Electrophoresis. All preparations of tryptically digested S-1 were denatured and examined on discontinuous SDS gels (10% upper, 15% lower) (20).

Solid Phase Immunoassays. The wells of Dynatech Immulon plates were coated with S-1 or tryptic (25/50/20) S-1 (5 ug per well) at 4°C overnight. The plates were blocked with the buffer solution containing 5% BSA in PTB. The anti-N-terminus antiserum was then added to the wells in triplicate at 10^2 to 10^5 dilutions. The antibodies were allowed to bind at 4° C overnight. The plates were washed and then incubated for 2 hours at room temperature with alkaline phosphatase conjugated goat anti-rabbit IgG antiserum (1:1000 dilution in PTB). After washing, the alkaline phosphatase substrate (1 mg/ml in 1M diethanolamine) was added and the developed color was measured at 410 nm in a Dynatech MR600 microplate reader.

Competitive Immunoassays. The procedures employed in the competitive immunoassays were basically the same as those in solid phase assays. The only modification of that assay involved the addition of competing antigen (i.e. intact S-1 and various tryptic species of S-1) together with the anti-N-terminus antiserum ($2x10^4$ dilution) to the coated wells as opposed to additions of antiserum alone in the standard solid phase assays. The competition step was carried out a 4°C overnight or at 37°C for 3 hours with no apparent difference in results.

RESULTS AND DISCUSSION

Proteolytically Induced Changes in S-1. The goal of this work was to explore the properties of the myosin's heavy chain N-terminus. The interest in this part of the protein is related to its mapping to the middle portion

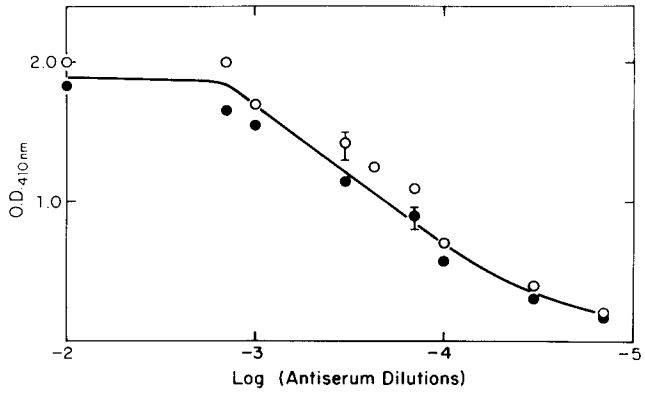

Fig. 1. Reactivity of the anti N-terminus antiserum towards S-1 (○) or tryptically cleaved (25/50/20) S-1 (●) immobilized in the microtiter wells. The solid phase immunotitration was carried out as described in Materials and Methods. Each point represents the average of three triplicate assays.

of S-1 (4,5) and the availability of N-terminus specific antibodies. The antibodies can be used to probe the accessibility and the flexible motions of the N-terminus region.

As previously observed (6), the antiserum against the N-terminus segment of S-1 showed high reactivity towards S-1 absorbed to microtiter plates (Fig. 1). In addition, ELISA titration curves for intact and the (25/50/20) tryptically split S-1 (i.e. S-1 cleaved at both 25/50K and 50/20K linker peptides) were virtually identical over a wide range of antiserum dilutions. Thus, when tested by solid phase immunoassays, the environment of the myosin's N-terminus appears unchanged by tryptic cleavage of S-1.

This situation was drastically changed when the antigen-antibody interactions were probed in solution i.e. in competitive ELISA experiments. In agreement with Sutoh, et al., (6), we note much lower reactivity of anti-N-terminus antibody towards S-1 in solution than in the solid phase. This is evidenced from the fact that large excess of competing S-1 (up to 200 ug) results in only partial inhibition of antibody binding to the immobilized S-1 (Fig. 2). In contrast to intact myosin heads, the tryptically cleaved 25/50/20K S-1 competes rather efficiently against the immobilized S-1 for the anti N-terminus antibodies. This difference in the relative solution reactivities of intact and tryptic S-1 towards the antibody was observed irrespective of whether the immobilized S-1 was intact or tryptically cleaved. The trypsin-induced increase in the reactivity of S-1 towards anti-N-terminus antibodies is consistent with the recent electric birefringence observations on the increased flexibility or segmental motions in S-1 following its proteolysis (15).

In order to determine which junction on S-1 is linked to the observed

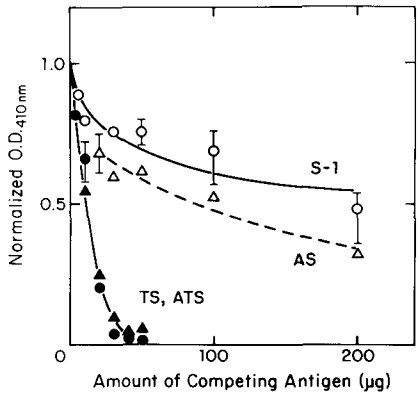

Fig. 2. Inhibition of anti-N-terminus antiserum binding to immobilized S-1, by S-1 (AS), tryptically split (25/50/20) S-1 (TS), and their respective complexes with actin, AS and ATS. The competitive immunoassays were carried out as described in Materials and Methods. ○, intact S-1; △, acto-S-1 (AS); ●, (25/50/20) tryptically cleaved S-1 (TS); ▲, acto-(25/50/20) tryptically cleaved S-1 (ATS). Every point represents the average of three sets of assays each one performed in triplicate. All data are normalized to the amount of antiserum binding in the absence of competing antigens.

changes in the accessibility of myosin's N-terminus to antibodies, two additional tryptic S-1 preparations were tested in competitive immunoassays. The cleavage of S-1 at the 25/50K junction (25/70K S-1 in Fig. 3a) resulted in a strong increase in the reactivity of S-1 towards the N-terminus antibody (Fig. 3b) similar to changes observed for myosin heads cut at both linker peptides. On the other hand a single cut of S-1 at the 50/20K junction (75/20K S-1 Figs. 3a and 3b) produced only partial changes in the accessibility of S-1 to the anti N-terminus antibody. It is very likely that even this small change in reactivity is due in some extent to the imperfect nature of the 75/20K S-1 preparations in which we were unable to completely block the tryptic cleavage of S-1 at the 25/50K junction (Fig. 3a).

Taken together the results of competitive immunoassays shown in Figs. 2 and 3 suggest the cleavage of S-1 at its 25/50K junction is responsible for the increased reactivity of tryptic (25/50/20) S-1 towards N-terminus antibodies. If the accessibility to antibodies can be indeed linked to the mobility of the antigenic epitope (21,22), then a correlation can be established between the state or events on the 25/50K linker peptide and the flexible motions of myosin's N-terminus. The interdependence between the N- and C-terminus of the 25K fragment may reflect their steric proximity in the middle region of S-1 and could have important functional implications.

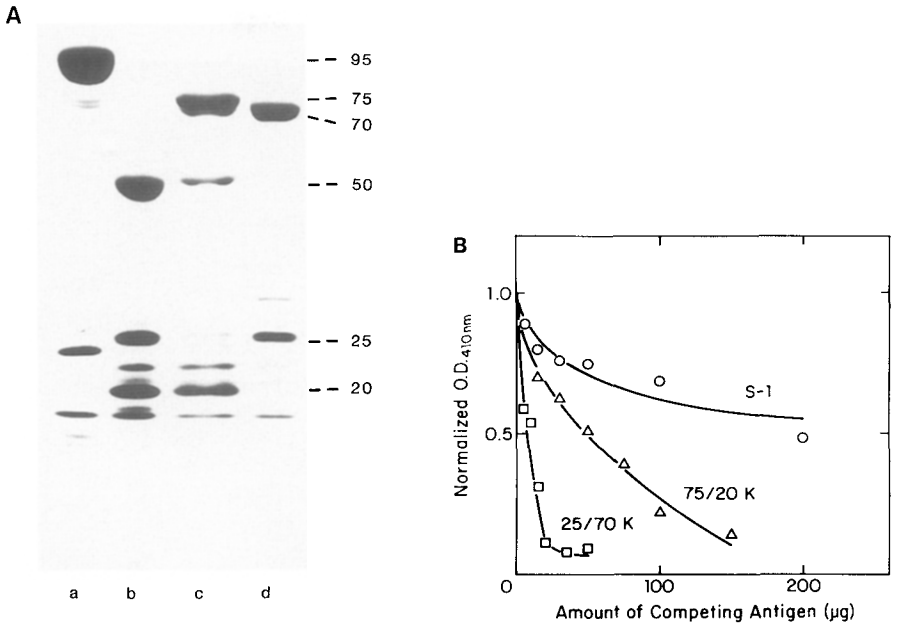

Fig. 3.(A). SDS-polyacrylamide gel electrophoresis showing various S-1 preparations. a, chymotryptic S-1 with the 95K heavy chain; b, tryptically cleaved (25/50/20) S-1; c, S-1 tryptically cleaved in 0.6 M NaCl and 10 mM MgATP to give mostly 50/20K cut, i.e. (75/20) S-1, (Note, however, the formation of 50K product indicating tryptic cut at the 25/50K junction); d, S-1 tryptically cleaved in the presence of actin to give mostly 25/50K cut, i.e. (25/70) S-1. The conditions for S-1 preparations are described in Materials and Methods. The S-1 species shown here were used as competing antigens in competitive ELISAs presented in (B).

(B). Inhibition of antiserum binding to immobilized S-1 by S-1 cleaved at either 50/20K or 25/50K junctions. S-1 (○), (75/20)S-1 (△), and (25/70) S-1 (□) (as shown in A) were used as competing antigens (see Materials and Methods). Each point represents the average of three triplicate assays.

Myosin's N-Terminus and the Binding of Actin to S-1. Competitive ELISA titrations presented in Fig. 2 reveal that the binding of actin to S-1 has very little if any effect on accessibility of the myosin's N-terminus to the antibody. This is particularly striking for tryptic S-1 preparations which show high and unimpeded by actin reactivity towards antibodies. It should be noted that separate turbiodometric experiments verified that N-terminus antibodies did not dissociate the acto-S-1 complex. Our results indicate that the binding of actin to S-1 does not change significantly the disposition or mobility of myosin's N-terminus.

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REFERENCES

1. Balint, M., Wolf, L., Tarcsfalvi, A., Gergely, J., Sreter, F.A. (1978) Arch. Biochem. Biophys. 190, 793-799.
2. Applegate, D., and Reisler, E . (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 7109-7112.
3. Mornet, D., Ue, K., and Morales, M.F. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 736-739.
4. Sutoh, K., Yamamoto, K., and Wakabayashi, T. (1984) J. Mol. Biol. 178, 323-329.
5. Sutoh, K, Yamamoto, K., and Wakabayashi, T. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 212-216.
6. Sutoh, K. Tokunaga, M., and Wakabayashi, T. (1987) J. Mol. Biol. 195, 953-956.
7. Lu, R.C., Moo, L., and Wong, A.G. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6392-6396.
8. Mornet, D., Ue, K., and Morales, M.F. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 1658-1662.
9. Botts, J., Takashi, R., Torgerson, P., Hozumi, T., Muhlrad, A., Mornet, D., and Morales, M.F. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 2060-2064.
10. dos Remedios, C.G., Mini, M., and Barden, J.A. (1987) J. Musc. Res. and Cell. Motil. 8, 97-117.
11. Mornet, D., Bertrand, R., Pantel, P., Audemard, E., and Kassab, R. (1981) Biochemistry 20, 2110-2120.
12. Botts, J., Muhlrad, A., Takashi, R., and Morales, M. (1982) Biochemistry 21, 6903-6905.
13. Chaussepied, P., Mornet, D., Barman, T.E., Travers, F., and Kassab, R. (1986) Biochemistry 35, 1141-1149.
14. Goodearl, A.J., Levine, B.A., and Trayer, I.P. (1985) J. Musc. Res. and Cell Motil. 8, 97-117.
15. Highsmith, S., and Eden, D. (1987) Biochemistry 26, 2747-2750.
16. Tong, S.W., and Elzinga, M. (1983) J. Mol. Biol. 258, 13100-13110.
17. Reisler, E. (1980) J. Mol. Biol. 138, 93-107.
18. Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891-2899.
19. Mathern, B.E., and Burke, M. (1986) Biochemistry 25, 884-889.
20. Laemmli, U.K. (1970) Nature (London) 277, 680-685.
21. Geysen, H.M., Tainer, J.A., Rodda, S.J., Mason, T.J., Alexander, H., Getzoff, E.D., and Lerner, R.A. (1987) Science 235, 1184-1190.
22. Berzofsky, J.A. (1985) Science 229, 932-940.