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# INVOLVEMENT OF AN ARGININE RESIDUE OF ACTIN IN TROPOMYOSIN BINDING

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Summary: Using 1,2-cyclohexanedione, modification of three arginines per actin monomer in F-actin resulted in a loss of ability of the actin to interact with tropomyosin, although the F-actin polymer was not significantly depolymerized, the ability of the actin to activate the $Mg^{2+}$-ATPase of myosin was not affected, and the secondary structure of the actin monomers was not appreciably altered. Isolation of peptides from a digest of modified F-actin indicated that the modified residues were Arg-28, Arg-95 and Arg-147. When actin was combined with tropomyosin prior to the modification treatment, Arg-95 was not modified, and the actin retained its ability to bind tropomyosin. These results therefore indicate a direct involvement of Arg-95 in the tropomyosin binding function of F-actin.

Specific interactions between the proteins of the thin filaments of striated muscle are crucial events in the regulation of the contractile mechanism (1,2), and in particular it is now believed that the interaction between one tropomyosin and seven adjacent actin molecules can control the interaction between actin and myosin necessary for contraction (3-5). Although the interaction between actin and tropomyosin has long been recognized (6-8), specific details of the mutual interaction sites on these proteins have not been reported. Because of the fundamental importance of this interaction in contraction, it would be of value to identify the binding sites, so that when the tertiary structures of these molecules are established (9,10), more precise topological models of the thin filament can be developed.

We have therefore begun a study of the involvement of actin amino acid side chains in the interaction with tropomyosin, and this report identifies the involvement of arginine-95 of the actin molecule in tropomyosin binding.

Materials and Methods. Actin, myosin and tropomyosin were prepared from rabbit skeletal muscle as described elsewhere (11-13). The assays of actin-

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tropomyosin interaction and actin polymerizability were performed as reported previously (14) except that the combination buffer used was 80mM KCl, 5mM $MgCl_2$, 0.2mM ATP, 0.2mM DTT, 10mM borate, pH 8.0. Circular dichroism spectra of depolymerized actins (15) were recorded on a Jasco J-20 spectropolarimeter at 1mg/ml in 0.2mM ATP, 0.2mM DTT, 10mM borate, pH 8.0. Actin activation of $Mg^{2+}$-ATPase activity of myosin was determined by the method of Schaub and Perry (12).

For specific modification of arginine residues (16), F-actin (1mg/ml) in 80mM KCl, 5mM $MgCl_2$, 0.2mM ATP, 0.2mM DTT, 50mM borate, pH 9.0 was incubated at 37° for 2 h with various amounts of 1,2-cyclohexanedione (Aldrich), after which excess reagents were removed by dialysis against the buffer used in the combination studies. The extent of modification of the F-actin was determined according to the procedure of Patthy and Smith (16), with amino acid analysis performed on a Beckman 119 CL amino acid analyzer.

For isolation of peptides containing modified DHCH*-Arg residues, the modified protein was digested with 10% by weight pepsin in 5% formic acid for 18 h at 37°, after which the modified peptides were selectively purified from the digest by diagonal electrophoresis (17) or by ion-exchange chromatography and paper electrophoresis (18), using the $NH_2OH$-$NiCl_2$ stain for localization of DHCH-Arg-containing peptides (17). Purified peptides were hydrolyzed with and without prior $NH_2OH$ treatment (17) and the amino acid compositions obtained, in combination with observed peptide mobilities (19), enabled unambiguous assignment of the peptides in the actin primary structure (20) without further sequence analysis.

Results. When F-actin was modified with 1,2-cyclohexanedione at molar ratios of 1,2-cyclohexanedione: actin arginines from 0.5:1 to 4:1, up to three arginine residues were modified (Fig. 1). The circular dichroism spectra of the modified actins were identical and closely comparable to those previously obtained for G-actin (15,21). The biological properties of the modified F-actins indicated (Fig. 1) that activation of $Mg^{2+}$-ATPase of myosin and the polymeric state of the actin were essentially unaffected by modification, whereas the ability of the modified F-actins to interact with tropomyosin decreased as the extent of arginine modification approached three residues per mole of actin monomer.

Identification of the arginine residues susceptible to modification was performed by isolation of DHCH-Arg containing peptides from a peptic digest of 120 mg F-actin which by analysis had 2.5 arginine residues modified per actin monomer. Three peptides were isolated and their amino acid compositions and electrophoretic mobilities are shown in Table 1. On the basis of these

*Abbreviations. CHD; 1,2-cyclohexanedione, DHCH- ; $N^7$,$N^8$-(1,2-dihydroxycyclohex-1,2-ylene)-

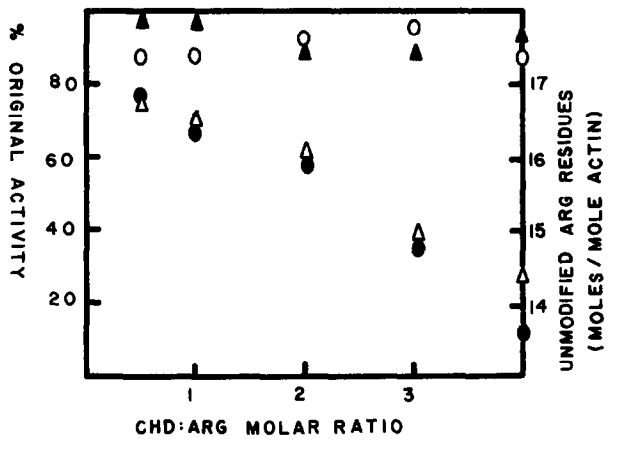


Figure 1: EFFECT OF ARGININE MODIFICATION OF F-ACTIN BY 1,2-CYCLOHEXANEDIONE Modification, amino acid analysis and biological activity assays are described in Materials and Methods. Polymeric state (▲); activation of $Mg^{2+}$-activated ATPase of myosin (O); tropomyosin combining ability (▲); number of unmodified arginine residues per mole of actin (●).

data, the known specificity of pepsin and the amino acid sequence of actin, it was possible to identify the arginine residues of peptides 1,2 and 3 as Arg-147, Arg-95 and Arg-28 respectively in the actin sequence.

The extent of the modification of each arginine residue at different modification ratios was estimated by peptic digestion of each modified actin followed by diagonal electrophoretic separation of the DHCH-Arg peptides using identical amounts of protein digests for each diagonal electrophoresis. The off-diagonal DHCH-Arg containing peptides were visualized by ninhydrin staining and the stained areas cut out and the color extracted into 1ml of ethanol and read at 504 nm. These studies indicated that essentially identical rates of modification of each of these 3 arginine residues occurred.

Identification of which of the three arginine residues is located at the tropomyosin binding site was investigated by studying the effect of prior combination of actin and tropomyosin on the modification pattern of actin. The actin-tropomyosin complexes (and appropriate controls of "unprotected" F-actin) were modified at various molar ratios of 1,2-cyclohexanedione : total

TABLE I
Properties of DHCH-ARG Containing Peptides

| Peptide | | Composition | Relative Mobility (and calculated Net Charge) at pH 6.5 in 0.1M Borate[b] | Relative Mobility (and calculated Net Charge) at pH 6.5 no Borate Present | Peptide and ARG Residue Identification from Actin Primary Structure[c] |
|---|---|---|---|---|---|
| 1 | $NoNH_2OH$ treatment[a] | $Thr_{1.7}Ser_{1.3}Gly_{2.0}Ala_{1.0}Tyr_{0.9}Arg_{0}$ | 0 (0) | +0.28 (+1) | 143-150 (Arg-147) |
| | $NH_2OH$ Treatment[a] | $Thr_{1.6}Ser_{1.1}Gly_{2.1}Ala_{1.0}Tyr_{0.7}Arg_{0.7}$ | | | |
| 2 | $NoNH_2OH$ treatment[a] | $Thr_{1.0}Glu_{2.0}Pro_{2.2}Ala_{1.0}Val_{0.9}Leu_{1.0}His_{0.9}Arg_{0}$ | -0.24(-1) | 0 (0) | 94-103 (Arg-95) |
| | $NH_2OH$ treatment[a] | $Thr_{0.9}Glu_{2.0}Pro_{1.9}Ala_{1.0}Val_{0.9}Leu_{1.1}His_{0.9}Arg_{1.0}$ | | | |
| 3 | $NoNH_2OH$ treatment[a] | $Asp_{2.0}Pro_{1.1}Gly_{1.2}Ala_{3.1}Val_{1.0}Phe_{0.9}Arg_{0}$ | -0.45(-2) | -0.25(-1) | 21-30 (Arg-23) |
| | $NH_2OH$ treatment[a] | $Asp_{1.7}Pro_{1.1}Gly_{1.0}Ala_{2.8}Val_{1.2}Phe_{1.4}Arg_{1.1}$ | | | |

[a]Hydrolyses performed according to ref. (17).
[b]Mobility calculations based on ref. (18).
[c]Residue numbers of actin from ref. (20).

arginine residues in the sample, and after modification, the F-actin was selectively sedimented after increasing the KCl concentration in the solution to 0.6M (11). The tropomyosin-free actin so obtained was then used for amino acid analysis and tropomyosin combination assay using fresh unmodified tropomyosin. Significant differences existed for extent of modification and tropomyosin binding ability between "protected" and "unprotected" actins at the modification ratios used. At a modification ratio of 3:1, in unprotected actin, 3.84 arginine residues per mole were modified, and the modified actin retained 30% of its tropomyosin binding ability. In contrast, at the same modification ratio, in "protected" actin 2.45 residues were modified, and the modified actin retained 87% of the original tropomyosin binding ability. These results indicate that prior combination with tropomyosin protects a single arginine residue in actin from modification by 1,2-cyclohexanedione, and allows the modified actin to retain its tropomyosin binding ability.

Identification of the protected arginine residue was performed by comparison of peptic digests of unprotected and protected actins, using the borate pH 6.5 diagonal method (17). In these studies, it was observed that peptide 2 was absent from the diagonal map of protected modified actin, indicating that Arg-95 is the residue which can be protected from modification in an actin-tropomyosin complex.

Discussion: This study of the role of actin arginines in tropomyosin combination was prompted by previous suggestions (22,23) that the actin-tropomyosin interaction probably involves ionic interactions between charged groups of the two proteins. The reagent 1,2-cyclohexanedione was used for this work as it has the advantages of specificity, quantification of modification and reversibility (17). That the extent of arginine modification was restricted to approximately three out of eighteen residues per monomer is presumably a reflection of residue availability in F-actin, with many of the residues being protected from modification by their involvement in the F-actin double-helix polymer.

The initial results of this study clearly indicate the involvement of one or more of arginines 28, 95 and 147 in tropomyosin combination whereas modification of these residues did not significantly alter other properties of the actin polymer. That Arg-28 and Arg-147 were not themselves directly located at the tropomyosin binding site was demonstrated experimentally by the protection of only Arg-95 from modification in actin-tropomyosin complexes.

These studies therefore indicate that Arg-95 is located directly at the tropomyosin binding site of actin, and on the basis of the actin molecule containing alternate tropomyosin binding sites (5,23,24), this residue is part of the "activated" state binding site. Further studies are currently in progress to further define the actin residues involved in the "activated" state tropomyosin binding site, and to investigate the existence of an alternate ("relaxed" state) binding site. These results will be of considerable interest when the tertiary structure of actin is established (9), as

identification of surface regions of the actin molecule involved in tropomyosin binding will be possible.

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