Conformational changes induced by Mg^{2+} on actin monomers

An immunologic attempt to localize the affected region

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The effect of Mg^{2+} ions on the conformation of G-actin and in particular on the accessibility of its antigenic regions has been tested. Experiments were performed with G-actin coupled to Sepharose 4B which was, therefore, maintained in the monomeric state. The results presented her show that the 2mM $MgCl_2$-perturbed antigenic site is located in a central region of the actin sequence.

Actin *Proteolysis* *Antigenic site* *Mg^{2+}*

1. INTRODUCTION

Studies of actin polymerisation in vitro show that a stable and monomeric conformer of actin, unable to polymerise in the absence of salts, undergoes, in the presence of 100 mM KCl [1–3] or 2 mM Mg^{2+} [4–6], structural transitions which promote filament formation when a critical concentration of actin [7] is reached. Thus, Frieden et al. [4], after 1,5-I-AEDANS labelling of actin, describe a fluorescence enhancement upon Mg^{2+} binding which correlates with a conformational change of the actin monomer. Furthermore, we have shown in a previous study [8], that actin monomers undergo a drastic decrease of antigenic reactivity in the presence of 2 mM Mg^{2+}.

Here, we attempt to localize the conformational changes which affect antigenic structures of actin and to distinguish between a global effect or a localized structural modification.

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Abbreviations: OxA, performic acid oxidized actin; ArA, trinitrophenylated actin; 1,5-I-AEDANS, *N*-iodoacetyl-*N*-(5-sulfo-1-naphthyl)ethylenediamine; IgG, immunoglobulin G

2. METHODS

Rabbit muscle actin was prepared as in [9]. Antisera directed towards performic acid oxidized, or trinitrophenylated unfolded actins, and trinitrophenylated F-actin were obtained as in [10,11]. The various antibodies were purified by affinity chromatography on immobilized actin [10]. Protein concentrations were determined spectrophotometrically [11,12]. Actin was cleaved into an actin core of 33.5 kDa by trypsin digestion [13]. Protein fragments were purified by preparative electrophoresis and by electrophoretic elution with an Isco sample concentration model 1750. C- and N-terminal sequences were determined by means of carboxypeptidase Y [14] and automatic Edman degradation [15]. Peptide transfer from SDS slab gel electrophoresis [16] to nitrocellulose sheets (0.2 μm) was performed as in [17]. F-actin was specifically labelled at Cys-374 using a 10 molar excess of 1,5-I-AEDANS [18]. G-actin in 2 mM Tris, 0.1 mM $CaCl_2$, 0.2 mM ATP buffer (pH 8.5) was

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linked to Sepharose 4B using cyanogen bromide activation [19]. Unsolubilized G-actin (2 mg actin/g gel) completely inhibits DNase I in a molar ratio interaction of about 2.

3. RESULTS

In a preliminary report [11], we have shown that the different fragments derived from a partial hydrolysis of actin by *S. aureus* V8 protease were diversely recognized by antibody populations derived from OxA and ArA antisera. Thus, we improved the proteolysis of actin under conditions in which the yield of cleavage products is enhanced. This allows selective purification of major fragments.

3.1. S. aureus *V_8 protease cleavage*

Selective digestion of actin at glutamyl residues [20] by *S. aureus* V_8 protease was carried out under unfolding conditions. As presented in fig.1, the presence of SDS in the reaction mixture increases the yield of actin cleavage products. High SDS concentrations (>2%) led to a rapid inactivation of the protease.

The standard procedure chosen was: 2 mg actin incubated in 1 ml of 1% SDS, 50 mM Tris, 2 mM $MgCl_2$ buffer (pH 7.8) with an enzyme/substrate ratio of 1/50 (w/w) at 25°C. The *S. aureus* V_8 protease first releases two major fragments of 26 and 16 kDa, respectively, which are further degraded (fig.2). Thus, another fragment of 19 kDa appears more slowly (fig.2).

The C-terminal fragment was identified after specific labelling at Cys-374 by a fluorogenic reagent (see section 2). The labelled protein was submitted to *S. aureus* V_8 protease action and the hydrolysate analyzed by SDS slab gel electrophoresis. As shown in fig.2, residual actin and

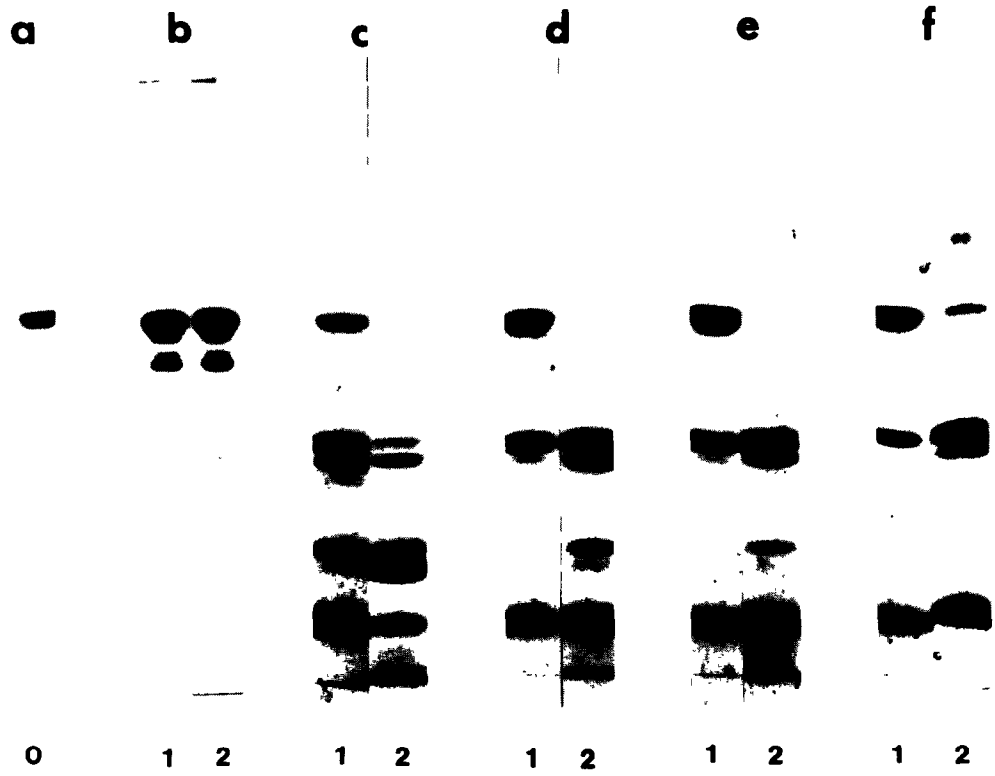

Fig.1. Cleavage of actin by *S. aureus* V_8 protease. Effect of SDS concentration. Actin (2 mg/ml) (a) was incubated in 50 mM Tris, 2 mM $MgCl_2$ buffer (pH 7.8) in the presence of SDS (b, 0%; c, 0.1%; d, 0.5%; e, 1%; f, 2%) at 25°C for 1 min (1), and 6 min (2) with protease (0.04 mg/ml).

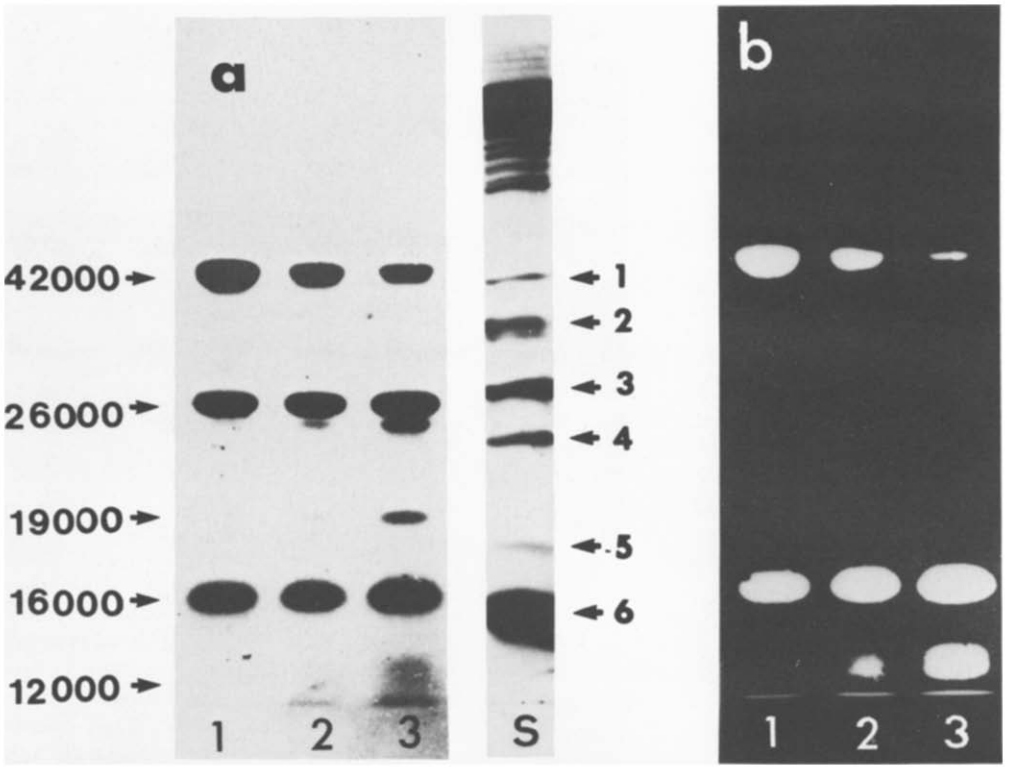

Fig.2. Cleavage of 1,5-I-AEDANS labelled actin in its Cys 374. SDS – 12.5% polyacrylamide slab gel, (1–3) 1, 2, 4 min incubation time, respectively; (S) marker proteins (kDa): thrombin digest of actin, 1 (37), 3 (27); tryptic core of actin, 2 (33.5); myosin light chains, 4 (25), 5 (18) and lysozyme, 6 (14.4). (a) Coomassie blue coloration, (b) detection of fluorescent fragments.

the 16-kDa fragment were only detected under UV light. This fragment, then, can be identified as the C-part of the protein.

The N-terminal fragments were identified by an immunological approach. The anti-trinitrophenylated F-actin antiserum presents the specificity shown in fig.3a and b. As presented in this figure, antibodies react with actin but not with the tryptic actin core lacking the end terminal sequence 1–62 of actin [13]. These antibodies, directed towards the N-terminal part of actin present a reaction with both 26- and 19-kDa fragments. This result shows that these two fragments would include at least a part of the 1–62 sequence. Furthermore, when the modification of cysteinyl residues by 1,5-I-AEDANS was conducted under more drastic conditions (fig.3c) than those described above, the 19-kDa fragment, as well as the 26-kDa fragment, also became fluorescent. As Cys-10 is the only thiol group accessible in the N-terminal part of native actin [21], it is obvious that the two peptides (26- and 19-kDa fragments) include this residue.

3.2. *Sites of cleavage*

The major site of cleavage in the primary structure was determined after fragment isolation. The 16-kDa fragment containing Cys-374 was submitted to automatic Edman degradation. Its N-terminal amino acid sequence is Met-Ala-Thr-Ala. This sequence corresponds to about 95% of the analyzed material. Another sequence (2% of the material) is Asn-Glu-Met-Ala which corresponds to an overlap of the major component. We can conclude that the main cleavage occurs at Glu-226. The digestion of the second and complementary fragment (26-kDa fragment) by carboxypeptidase Y releases Glu, Asn and some Phe, which confirms the postulated cleavage site. A secondary and minor cleavage site could be tentatively located at Glu-167 according to the size and the N-terminal

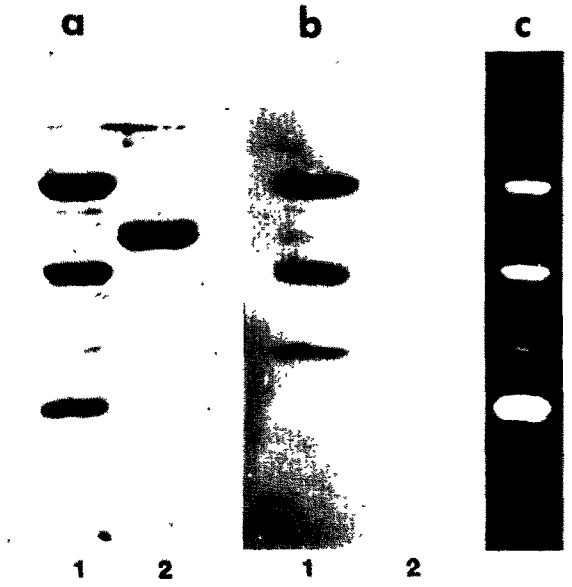

Fig.3. Identification of N-terminal fragments in *S. aureus* V_8 protease digest of actin. (a) SDS – 15% polyacrylamide slab gel (Coomassie blue coloration). (b) Electrophoretic replicates reaction with the anti-trinitrophenylated F-actin antiserum. (1) *S. aureus* V_8 protease digest, (2) tryptic digest. (c) Cleavage of 1,5-I-AEDANS labelled actin by *S. aureus* V_8 protease. Actin was incubated for 2 h with a 50 molar excess of fluorogenic reagent. SDS–15% polyacrylamide slab gel was observed upon UV light.

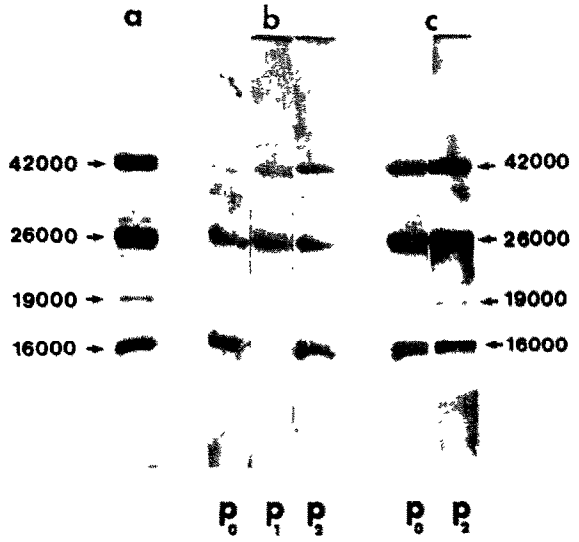

Fig.4. Antigenic reactivity of antibody populations eluted from Sepharose-G-actin column. P_0, initial population; P_1, 2 mM $MgCl_2$ eluted population; P_2, 4 M $MgCl_2$ eluted population. *S. aureus* V_8 protease digest. (a) Coomassie blue coloration, (b) electrophoretic replicates revealed with OxA antibodies, (c) electrophoretic replicates revealed with ArA antibodies.

position of the produced fragment (19 kDa) and the specificity of the *S. aureus* V_8 protease [20].

3.3. *Structural changes induced by Mg^{2+} binding*

To follow the Mg^{2+} effect on actin monomers, it was necessary to link G-actin to Sepharose 4B. Under these conditions the nucleation and elongation phases do not take place and the conformational changes observed can be related to the actin monomeric structure.

Two lots of antibodies were used in these experiments (see section 2). OxA antibodies were bound to Sepharose 4B-G-actin at low ionic strength. After addition of 2 mM $MgCl_2$, about 30% antibodies were released. The remaining antibodies were released by 4 M $MgCl_2$, a reagent used to break down antigen–antibody interactions. In contrast, 2 mM $MgCl_2$ was unable to release any antibody in the case of ArA antibodies.

The different fractions eluted were then tested using nitrocellulose replicates of electrophoretic patterns from *S. aureus* V_8 protease digest of actin. It appears (fig.4) that the two lots of antibodies eluted by 4 M $MgCl_2$ (P_2) present reactions analogous to that of the initial populations (P_0). In contrast, a specific reaction of the 2 mM $MgCl_2$-eluted OxA antibodies (P_1) is observed against the 26-kDa peptide only. Finally, the observation (fig.4) that the initial OxA antibody population (P_0) is unable to interact in any appreciable amount with the 19-kDa fragment as compared with ArA antibodies, restrains the region able to interact with the P_1 population to the sequence 168–226.

4. DISCUSSION

A previous study [22] has revealed that *S. aureus* V_8 protease is able to cleave actin and to yield an N-terminal peptide of 25 kDa. This fragment conserves a large capacity to interact with antiactin antibodies and is able to complex DNase I [23]. The ability of this protease to cleave actin into large domains which retain antigenic activity was used in our study to map the antigenic structure of actin.

In the presence of 1% SDS which allows a complete cleavage of actin in 5 min, three fragments were identified with relative molecular masses of 26, 19 and 16 kDa.

That SDS enhances the yield of cleavage by *S. aureus* V_8 protease is also demonstrated in the case of *Dictyostelium* actin in a paper just published after completion of our manuscript (Simpson, P.A., Spudich, J.A. and Parham, P., J. Cell. Biol., 1984, 99, 287–295). However, in the case of this particular actin, the major site of cleavage seems to be at Glu-167.

Structural changes which affect the antigenic reactivity of actin [8] can be located from the present results. We show that when G-actin binds Mg^{2+} in conditions where the polymerisation process is prevented (G-actin linked to Sepharose-4B), a part of OxA antibodies is excluded from actin.

These results seem to infer that the effect of magnesium on antigenic reactivity of actin monomers could be linked to a modification of accessibility or conformation of antigenic determinants concerned in OxA antibody specificity. These antigenic determinants are located in a limited sequence between Glu-167 and Glu-226 in the central part of the sequence of actin. A general reorganisation of tertiary structure which would affect the whole antigenic structure of actin monomer could be excluded.

The located conformational change evidenced here could be an important step preceding nuclei formation as established in the mechanism described in [5]. Thus, as shown by Frieden, the first step in actin polymerisation is the replacement of Ca^{2+} by Mg^{2+} followed by a 'time-dependent conformational change'. Furthermore, the third step is also concerned with the binding of Mg^{2+} to weak binding sites, but the author does not observe any conformational change. The fact that the central part of the polypeptide chain of actin may be implicated in the polymerisation process is strengthened by the observation [11] that Fab fragments obtained from OxA antibodies are able to decrease the polymerisation rate which is not the case with ArA antibodies.

In conclusion, we can suggest that the binding of Mg^{2+} by actin monomer at least induces a limited conformational change which affects the exposure of residues in the central part of actin where an antigenic region is located.

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