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Biochemistry

Proteolysis and structure of skeletal muscle actin

(limited proteolysis/organization of G-actin)

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ABSTRACT **Under standard conditions, G-actin has been submitted to nine proteases of varying specificity, and in each case the pattern of fragments produced has been studied by $NaDodSO_4$ gel electrophoresis. The results suggest that the actin monomer consists of a large region (*ca.* 33 kilodaltons) and a small, easily degraded region (*ca.* 9 kilodaltons). The COOH terminus is in the large region. Consideration of primary sequence homologies, medium resolution maps of actin crystals, and certain reactions of actin suggests that the NH_2 terminus is in the small region, as is the negative sequence to which a divalent metal cation is normally chelated, but that the nucleotide-binding site is on the large region near the junction between the regions. From analysis of these results, numerous properties of actin are understandable.**

Actin is one of the two major proteins comprising the contractile system of muscle. The structure of actin monomers and their assembly into F-actin filaments are being studied by various physical methods (1, 2). Here our methods and conclusions are chemical, but the latter seem to have structural implications. Guided by previous studies of actin proteolysis (3–7), we have submitted monomeric actin in solution to a battery of proteases in a test for the possible existence of domains within the monomer (see ref. 8 for a similar study of myosin S1). Analysis of the results shows that actin consists of two regions—a large, protease-resistant region of 33 kilodaltons (kDa) and a small, protease-vulnerable region of 9 kDa. The NH_2 terminus and the divalent metal cation (M^{2+})-binding regions are probably in the smaller region, whereas the COOH terminus is in the larger region, and the nucleotide-binding site is probably in the larger region near the junction with the smaller. From this knowledge and certain steric considerations, many characteristic properties of G- and F-actin can be rationalized.

MATERIALS AND METHODS

Preparation of Actin and Myosin. Rabbit skeletal muscle F-actin was prepared as described by Eisenberg and Kielley (9), and its concentration was estimated by using $A_{280}^{1\%} = 11.0$ (10).

G-actin was obtained by three successive dialyses of F-actin (3–6 mg/ml) with a 5-mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes) buffer (pH 7.6) containing 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.1 mM dithiothreitol, and 0.1 mM NaN_3 (buffer A). Its concentration was then estimated by using $A_{290}^{1\%} = 6.37$ (11).

For proteolysis, we always used G-actin solutions no older than 1 week, and we verified that these solutions were not denatured by adding 5 mM $MgCl_2$ and observing (viscometrically) F-actin polymerization as rapid as with fresh G-actin.

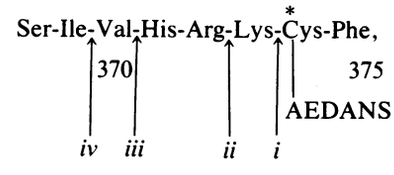
When labeled F-actin was used, the dye was *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS), known to react specifically with cysteine-374 (12). Then this fluorescent F-actin was dialyzed against dye-free solvent for 48 hr, like native actin, to obtain fluorescent G-actin.

Rabbit skeletal myosin was prepared according to Offer *et al.* (13); subfragment 1 (S1) was prepared by digestion of myosin filaments with α-chymotrypsin (14) and purified as in ref. 15. When Mg^{2+}-activated ATPase was followed, we used 50 μg of S1 in the presence of 20 times its weight of the actin fragment as is usually done in studying Mg^{2+} actin-activated ATPase (15).

Enzyme Cleavage Patterns. All enzyme cleavages were done in buffer A at 25°C in which exclusively G-actin exists; only in the case of plasmin and thrombin have we dialyzed the G-actin against calcium-free buffer A (with 1 mM EDTA). During the cutting, aliquots were taken at different times for $NaDodSO_4$/PAGE with slab gels [5–18% acrylamide gradient and a 50-mM Tris/100 mM boric acid buffer system (16)]. As protein markers, we used: (*i*) from S1 itself, its heavy chain of 95 kDa, its light chain 1 of 25 kDa, and its light chain 3 of 17 kDa; (*ii*) actin itself, 42 kDa; and (*iii*) the three fragments of tryptically split S1, 50 kDa, 27 kDa, and 20 kDa. From enzyme to enzyme we varied both the cutting time and the molar ratios of enzyme to actin as follows: chymotrypsin, 50 min at 1:5; trypsin, 60 min at 1:5; thermolysin, 30 min at 1:10; clostripain, 90 min at 1:5; nagarse, 90 min at 1:200; proteinase K, 90 min at 1:200; plasmin, 90 min at 1:5; thrombin, 360 min at 1:5; and *Staphylococcus aureus* V8, 90 min at 1:5. L-1-Tosylamido-2-phenylmethyl chloromethyl ketone (TPCK)-treated trypsin and α-chymotrypsin were from Worthington; porcine blood plasmin, *S. aureus* strain V8 (protease type XVII), clostripain (clostripeptidase B), nagarse (or subtilisin BPN', protease type VII), proteinase K (protease type XI), thermolysin (protease type X), and thrombin were from Sigma.

RESULTS

The fragmentation of G-actin by different enzymes was followed by PAGE in $NaDodSO_4$. Use of fluorescent G-actin [made so by attaching the 1,5-IAEDANS dye to cysteine-374 (12)] made it possible to discover which fragment contained the COOH terminus. The COOH-terminal sequence (17) is



and has four potential cutting sites: (*i*) for trypsin and plasmin, (*ii*) for trypsin and clostripain, (*iii*) for thermolysin, and (*iv*) for chymotrypsin and thermolysin. Therefore, the label

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Abbreviations: Subfragment 1, S1; kDa, kilodalton(s); 1,5-IAEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; M^{2+}, divalent metal cation.

(*) at cysteine-374 was always eventually lost, at various rates, depending on the enzyme used. However, the origin of the fragment (COOH- or NH_2-terminal) was generally identified before total loss of the label.

Trypsin. The first five wells of the gels in Fig. 1 *A* and *B* show the trypsin cutting pattern of G-actin. The fluorescence of intact actin disappeared completely after 10 min (Fig. 1*B*), leaving only a fluorescent area without any Coomassie counterpart in Fig. 1*A* (the Coomassie blue pattern of the same gel). A main product of 33 kDa without a 35-kDa precursor appeared. As demonstrated by Jacobson and Rosenbusch (4), this product is a COOH-terminal fragment containing the 69–372 sequence (having lost its labeled cysteine-374).

Chymotrypsin. The same 33-kDa fragment as also described in ref. 4 was obtained as a final product (Fig. 1 *A* and *B Middle*). With chymotrypsin, a fluorescent 35-kDa precursor appeared and then degraded to a fluorescent 33-kDa product. Finally, after 30 min of proteolysis, this 33-kDa product also lost the dye. Here too there appeared a fluorescent area not present in the native G-actin, having no counterpart in the Coomassie blue gel. The last 33-kDa fragment is the COOH-terminal fragment without the labeled cysteine-374.

Thermolysin. The thermolysin cutting pattern showed a rapid conversion of the 35-kDa fluorescent → 33-kDa fluorescent fragment, followed after 20 min by a complete loss of the dye (Fig. 1 *A* and *B Right*). Here, however, the dye migrated completely and massively to the bottom of the gel. After 30 min of proteolysis, a stable 33-kDa fragment, the COOH-terminal fragment, was obtained without a labeled cysteine-374.

Nagarse and Proteinase K. Fig. 2 *A* and *B Left* and *Center* compare the cutting patterns of these two enzymes. The most important fluorescent product was the 35-kDa fragment. Other cuts occurred and produced a fluorescent 16-kDa fragment, but no diffuse fluorescent area was detectable, thus showing that, with these enzymes, the 35-kDa fragment is a COOH-terminal fragment with its labeled cysteine-374 preserved.

Plasmin. With plasmin (Fig. 2 *A* and *B Right*), the cutting process immediately caused a loss of the fluorescent dye, but when the diffuse fluorescent area was prominent, no counterpart in the Coomassie blue was obtained. Surprisingly, the proteolysis was very little, and only a small amount of a 30-kDa fragment was detectable. The plasmin (visible at

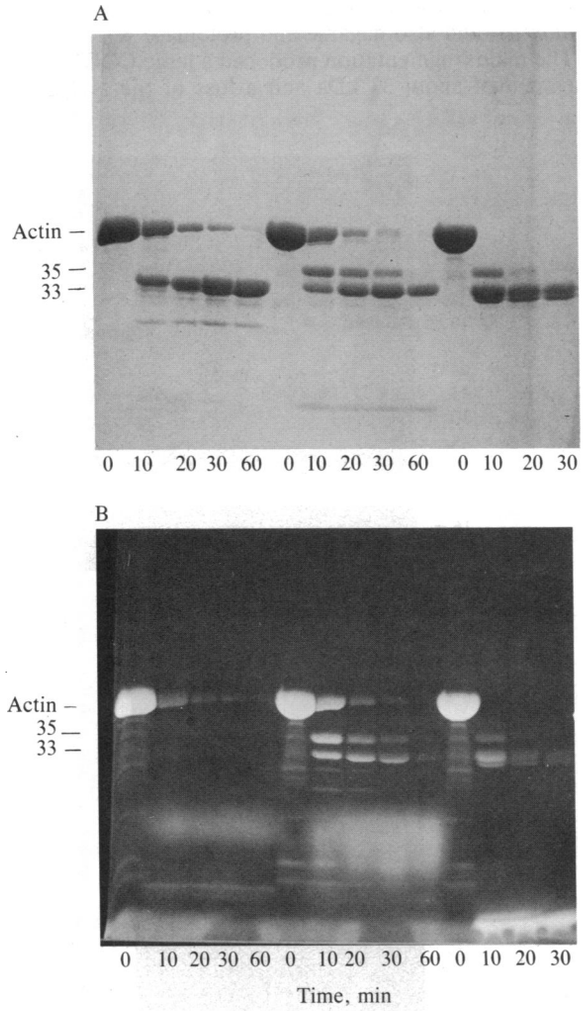

FIG. 1. Time-dependent fragmentation pattern resulting from the limited cleavage of G-actin with three different enzymes: trypsin at 0, 10, 20, 30, and 60 min (*Left*); chymotrypsin at 0, 10, 20, 30, and 60 min (*Center*); and thermolysin at 0, 10, 20, and 30 min (*Right*). At suitable time intervals, 50-μl aliquots of the proteolysis mixture were withdrawn and added to an equal volume of 5% $NaDodSO_4$/5% mercaptoethanol. After 3 min at 100°C, samples were subjected to $NaDodSO_4$/PAGE. The same gel is viewed under a UV lamp (*B*) and stained with Coomassie blue (*A*). Sizes are shown in kDa.

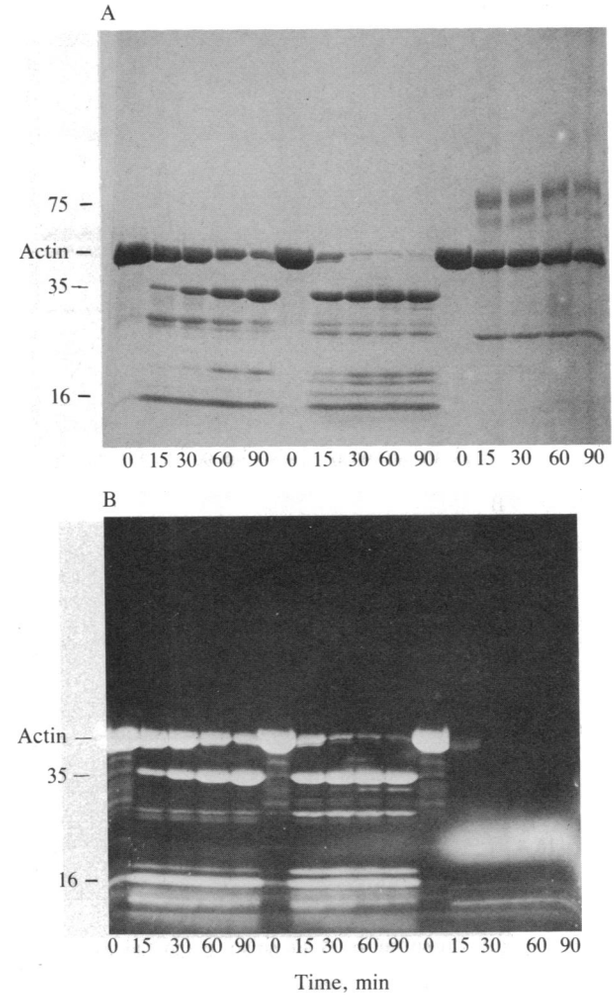

FIG. 2. Time course of the limited cleavage of G-actin with three different enzymes: nagarse at 0, 15, 30, 60, and 90 min (*Left*); proteinase K at 0, 15, 30, 60, and 90 min (*Center*); and plasmin at 0, 15, 30, 60, and 90 min (*Right*). The same gel is viewed under a UV lamp (*B*) and stained with Coomassie blue (*A*).

the top of the gel as a 75-kDa protein) was practically unable to cut G-actin under our conditions; G-actin was still intact except for the loss of cysteine-374.

***S. aureus* V8.** Fig. 3 *A* and *B Left* shows the pattern of V8 cutting (already described in ref. 3). Fig. 3*B* shows a 16-kDa fluorescent fragment and no loss of dye. But Fig. 3*A* shows a complementary fragment of about 30 kDa, which was identified as a 1–207 NH_2-terminal fragment (3); therefore, the 16-kDa fragment was a COOH-terminal fragment containing labeled cysteine-374.

Clostripain. With this enzyme a 33-kDa fragment rapidly appeared (Fig. 3*B Middle*), but after 30 min a diffuse fluorescent area appeared, and the 33-kDa product completely disappeared from the fluorescent picture. However (see Fig. 3*A*), a main 33-kDa fragment was obtained and identified as a COOH-terminal fragment without its labeled cysteine-374. An early fluorescent product of 37.5 kDa was also detectable.

Thrombin. The cutting pattern with thrombin (Fig. 3 *A* and *B Right*) shows the simultaneous appearance of a fluorescent 37.5-kDa fragment and a fluorescent 27-kDa fragment. No loss of the dye was observed; in accordance with ref. 6, we identified these two fragments as COOH-terminal fragments containing cysteine-374. In the Coomassie blue-stained gel, the two fragments, identified as "K" and "L" (6, 7), correspond to our 37.5-kDa and 27-kDa products, respectively.

We show in the same gel (Fig. 4*A*) the different proteolytic products of our battery of proteases. The first six enzymes, chymotrypsin, thermolysin, clostripain, nagarse, proteinase K, and trypsin gave a 35-kDa or 33-kDa fragment containing the fluorescent dye-labeled cysteine-374. (Because the label was sometimes lost at various rates depending on the enzyme used, we selected in these experiments an appropriate cutting time for each enzyme; only in the case of trypsin was the fluorescent dye too rapidly cut off.) Only among the *S. aureus* products (Fig. 4*B*, lane h), was the 30-kDa fragment NH_2-terminal; the 16-kDa fragment was the same fluorescent product obtained in the nagarse and proteinase K proteolysis. However, a minor fluorescent product, 27 kDa, migrating similarly to the "L" fluorescent product of the thrombin proteolysis (Fig. 3*B*) could be produced as a COOH-terminal segment.

Our results are summarized in Fig. 5 and take into account the fact that sometimes the COOH-terminal end of the molecule containing the labeled cysteine-374 was lost and also that sometimes an additional cutting process could occur at about 16 kDa from the COOH terminus (as was the case for *S. aureus*, and also nagarse and proteinase K).

The main fragmentation produced a large COOH-terminal fragment of about 33 kDa and a loss of the NH_2-terminal

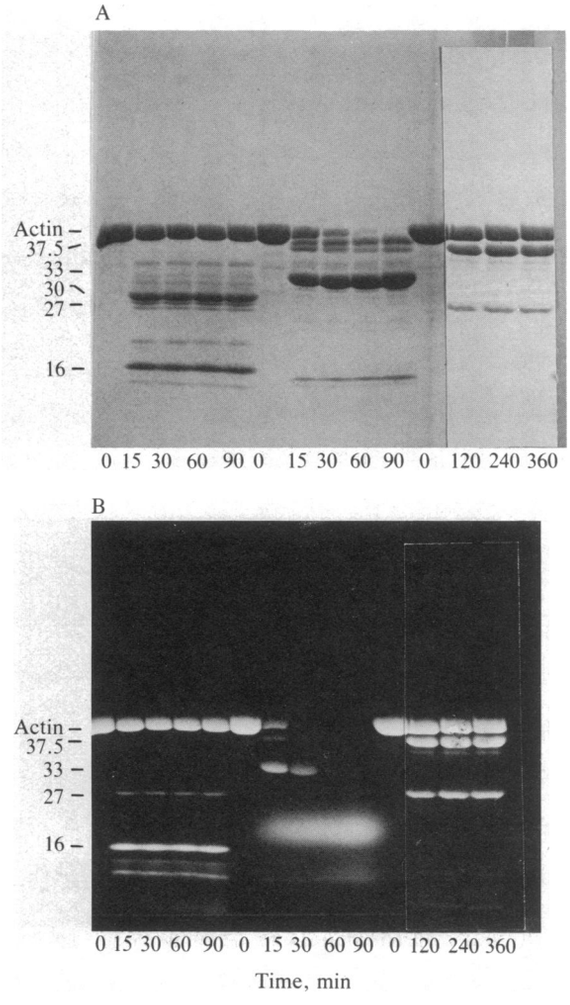

FIG. 3. Time course of the limited cleavage of G-actin with three different enzymes: *S. aureus* V8 at 0, 15, 30, 60, and 90 min (*Left*); clostripain at 0, 15, 30, 60, and 90 min (*Center*); and thrombin at 120, 240, and 360 min (*Right*). The same gel is viewed under a UV lamp (*B*) and stained with Coomassie blue (*A*).

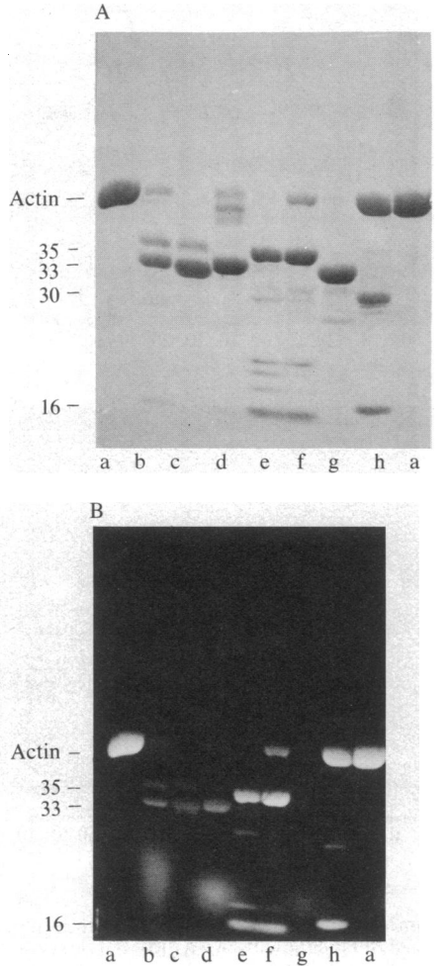

FIG. 4. Comparative migration of limited cleavage of G-actin. The same gel is viewed under a UV lamp (*B*) and stained with Coomassie blue (*A*). Lane a shows native G-actin. Other lanes show a selective cutting time where the final product is still fluorescent with different enzymes: b, chymotrypsin, 30 min; c, thermolysin, 10 min; d, clostripain, 30 min; e, nagarse, 90 min; f, proteinase K, 90 min; g, trypsin, 60 min; h, *S. aureus* V8, 90 min.

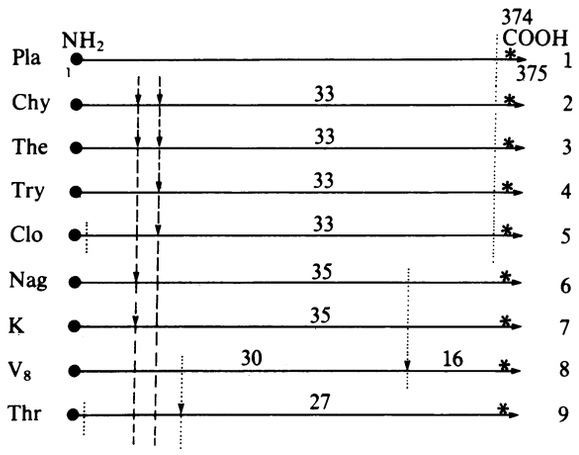

FIG. 5. Schematic representation of the proteolytic fragments of actin. Positions of the amino acid residues are referred to the primary structure of vertebrate skeletal actin (17, 18). The locations (dark arrowheads) of the main proteolytic cleavages that are obtained on actin by various enzymes: plasmin (Pla), chymotrypsin (Chy), thermolysin (The), trypsin (Try), clostripain (Clo), nagarse (Nag), proteinase K (K), *S. aureus* V8 (V8) and thrombin (Thr). Vertical dashed lines emphasize that each of the two cleavages made by an enzyme lies in a rather narrow range. Vertical dotted lines show additional cutting locations that occur with particular enzymes. Sizes are shown in kDa. The asterisks refer to fluorescent cysteine-374.

segment. In this main fragment [which in the chymotrypsin and trypsin cutting process was thought to retain the ATP-binding site (4)], we searched for an adenine-binding sequence homology.

The amino acid sequence of skeletal actin contains 375 amino acids (18) and a long, mostly nonpolar, sequence (120–153) containing a Gly-Arg-Thr tripeptide surrounded by hydrophobic residues. From comparison with the adenine nucleotide-binding sequence homology (19) substituting an arginine for a lysine, we believe that this region corresponds to the hydrophobic sequence:

X-X-X-X-Gly-Lys-Thr-X-X-X-X-X-,

where the X are hydrophobic residues and are described as the "A" sequence. This consideration suggests that the nucleotide binding site is toward the NH_2 terminus of the 33-kDa fragment.

The actin molecule as normally isolated contains 1 mol of bound ADP (20) and also 1 mol of Ca^{2+} bound to a specific M^{2+}-binding site (21). A proteolytic removal of residues 1–68 eliminates the ability of actin to bind M^{2+} (4, 22). The calcium-binding protein from carp, the "CBP" (23), has been investigated, and there exist homology studies (24) that have compared the amino acid sequences involved in various calcium-binding sites. The calcium-ligating positions are the vertices of an octahedron; they are contained in a 12-residue peptide and are codified as (X, Y, Z, -Y, -X, and -Z) (25). If we look for similarities between amino acids of the calcium-binding sites and the beginning of the sequence of the actin, we can find three acidic residues within the six coordinating positions and also three additional conservative replacements. Thus, there is present a good 50% of the homology. The correlation of this homology with actual Ca^{2+}-binding, however, is not straightforward. For example, although an even better homology exists in one of the "alkali" light chains of myosin, this chain is unable to bind Ca^{2+}. Troponin C and calmodulin contain the sequence homology and do bind Ca^{2+} for known physiological reasons. The Ca^{2+} affinity of actin is considerable but seems to be less than that of Mg^{2+} *in vivo* (26). Despite these equivocations, we believe that the first 12 NH_2-terminal residues contain a partial sequence homology and are likely to constitute the M^{2+}-binding site. Barden and co-workers drew the same general conclusion by different reasoning. Assuming that Tb^{3+} has the highest affinity for the M^{2+}-binding site, they sought perturbations of aromatic residues using NMR (27) and UV spectroscopy (28); on finding none they reasoned that this portion of the primary sequence (the first 20 residues) was uniquely distant from aromatic residues. On also failing to find effects of bound Tb^{3+} on the ring protons of bound nucleotide, they concluded that the two sites were more than 1.6 nm distant (22); this observation too fitted the idea that the M^{2+}-site was in the NH_2-terminal residues. It should be noted that Brauer and Sykes (29) found the phosphorus nuclei of bound nucleotide to be near the M^{2+}-binding site, but the finding may not be contradictory in as much as intranucleotide distances can be considerable.

Although the undamaged 33-kDa COOH-terminal fragment could be obtained readily (using thermolysin or clostripain) free of intact G-actin, it could not polymerize, even on adding S1; these observations confirm Jacobson and Rosenbusch (4).

DISCUSSION

Our experimental results show that, aside from minor complications (three of the enzymes also create 16-kDa COOH-terminal fragments, and most enzymes eventually destroy the very end of the COOH terminus), a battery of nine proteases with different specificities leaves undamaged a 33- to 35-kDa COOH-terminal region of actin but cuts off and degrades the 9-kDa NH_2-terminal region. Without at this point drawing any structural inferences, we feel justified in regarding the actin monomer as consisting of a "large" and a "small" region and in thinking of the latter as less compact and joined to the former by a protease-vulnerable connection. Stimulated by the foregoing, we additionally have sought and found good indications (sequence homologies) that in the negative NH_2-terminal part (first 12 residues) of the small region there is a M^{2+}-binding site and that on the large region adjoining the connection there is the adenine portion of a nucleotide-binding site.

When the results above are considered together with those of others, some very interesting suggestions emerge. There are activities associated with the small region (or with a part of the large region adjoining the small region)—(*i*) the binding of antibody (30–32), (*ii*) attack by proteases (3–7, 33), (*iii*) binding of DNase I (1, 33, 34), (*iv*) exchange of M^{2+} (35), and (*v*) exchange of nucleotide (36)—that are well expressed in G-actin but poorly or not at all expressed in F-actin, at least at room temperature. A rather obvious interpretation is to think that actin monomers are assembled into F-actin filaments in such a way that their small regions become sterically shielded from solvent. Properties *i–iii* have not been studied as a function of temperature, but Asai and Tawada (36) did study activities *iv* and *v* in F-actin and found that near 50°C these activities become manifest without extensive depolymerization. Since that time there has accumulated much evidence that actin monomers move relative to one another while remaining members of F-actin filaments (37). Therefore, it is plausible to think that at higher temperatures thermally activated motions compromise the steric shielding of the small regions or that there is a temperature-dependent equilibrium between monomers in an "F-like" state and those in a "G-like" state.

These ideas ramify to other actin phenomena. Cysteine-374 (which our work shows to be on the large region) is very reactive, even in F-actin, and, therefore, must be very accessible to solvent. The energy-transfer measurements of Taylor *et al.* (38) assign to it a considerable radial distance from

the filament axis, so its location on the large region may be rather "outward" in the F-actin assembly. On the other hand, the low reactivity of cysteine-10, even in G-actin, even to positively charged reagents (39), has been perplexing, but now one can think that, because cysteine-10 is among the residues that form the M^{2+}-site, it may be prevented from expressing its normal character. Finally, G-actins in the absence of M^{2+} would bear negative charges in their small regions, making polymerization impossible; therefore, M^{2+} would strongly promote polymerization, which is certainly the case.

The most interesting implications concern the fundamental contractile interaction of F-actin with S1 moieties. Sutoh (40) has shown that in the "rigor complex," the 20-kDa domain of S1 can be readily cross-linked to the first 12 NH_2-terminal residues of the small region. At first sight, this seems to contradict our proposals, since we suggest that in F-actin the small region is shielded from macromolecules even smaller than S1. There are, however, clues on how to resolve this difficulty. First, it has been reported (41) that activities *iv* and *v*—and so, presumably *i–iii* (i.e., access of the small regions to macromolecules)—are expressed during the contractile interaction. Second, directional probes (fluorescent nucleotides) inserted into actin detect unique rotational motion of the monomers during the contractile interaction (42, 43). So it is possible that, unlike other macromolecules, S1s actively evert small regions, perhaps by means of their secondary 50-kDa contacts (44) with the actins.

We are aware of exciting studies on the bilobar structure of the actin monomer (1) and on how such monomers fit into the F-actin helix (45, 46). Warned that the mass ratios of the lobes are different from our 9 kDa/33 kDa ratio, we have refrained from identifying "regions" with "lobes." It is, however, obvious that the several chemical and functional implications discussed above must eventually be accommodated by proposed actin structures.

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