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Biochemistry

Characterization of brevin, a serum protein that shortens actin filaments

(actin polymerization/actin-binding protein/plasma protein/cytoplasmic motility)

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ABSTRACT **We have purified a protein from rabbit serum with a molecular weight of 90,000 that inhibits the polymerization of actin measured viscometrically and that we have named "brevin" (from the Latin *breviare*, to shorten). From the extent of purification, we estimate that this inhibitor constitutes 0.3% of the total protein in plasma and serum. Brevin is also present in sera from humans and rats. Almost all of the activity in blood is extracellular; only 1% is present in platelets or other cellular elements. Several lines of evidence indicate that brevin is the same protein as the factor described by Fagraeus and Norberg [Fagraeus, A. & Norberg, R. (1978) *Curr. Top. Microbiol. Immunol.* 82, 1–13] as an actin-depolymerizing factor (ADF). If ADF and brevin are identical, then "ADF" is an inappropriate name because we find that the protein shortens actin filaments without depolymerizing them. Thus, brevin causes little change in the critical concentration of monomeric actin, even though the inhibitor binds to monomeric actin complexed to DNase I-agarose. Binding of brevin to filaments was demonstrated by sedimenting the inhibitor with F-actin. From the amounts of actin and brevin sedimented, and from the lengths of filaments measured by electron microscopy, we calculated that the stoichiometry of binding is one brevin molecule per filament over a wide range of inhibitor concentrations. This stoichiometry suggests that brevin inhibits polymerization by binding at the end of elongating actin filaments, a mechanism similar to that proposed for several intracellular actin-binding proteins and for the cytochalasins. Its abundance suggests that brevin plays an important physiological role in serum, but one not directly concerned with intracellular motility. Therefore its relationship to cytoplasmic actin-binding proteins remains to be determined.**

Fragraeus and Norberg (1, 2) described a factor in plasma that appeared to depolymerize actin filaments, which they called actin-depolymerizing factor (ADF). By microinjecting a partially purified preparation of ADF into the giant cerebral neuron of *Aplysia californica*, we obtained evidence that actin might be involved in fast axonal transport of serotonergic storage granules (3). The preparation injected was crude, however; we therefore decided to purify it, using as an assay its ability to inhibit the polymerization of actin (3). We describe here the isolation of the inhibitor from rabbit serum. Characterization of its mechanism of action indicates that the inhibitor does not depolymerize actin filaments, but rather its presence results in more numerous, shorter polymers. Thus ADF is not an appropriate name for the inhibitor, which we propose to call "brevin" (from the Latin *breviare*, to shorten).

MATERIALS AND METHODS

Blood was collected from New Zealand White rabbits and human volunteers; it either was treated with buffered sodium citrate as anticoagulant or was allowed to clot overnight at 4°C. Pooled normal rabbit serum was obtained from Pocono Rabbit Farms (Canadensis, PA). Platelets and other cellular elements were separated from plasma by differential centrifugation (4) and washed four times with buffered normal saline.

Protein was determined by the method of Bradford (5), using bovine gamma globulin as a standard, and G-actin was estimated spectrophotometrically, assuming an extinction coefficient at 290 nm of 617 ml/g·cm (6). Perfusates of isolated rat liver (7, 8) were kindly supplied by Leon L. Miller (University of Rochester School of Medicine and Dentistry).

Assay of Brevin. Samples were assayed in Ostwald viscometers (type 100, Cannon Instrument, State College, PA) by their effect on the salt-induced polymerization of actin purified (9) from rabbit skeletal muscle. Polymerization was initiated by adding 0.215 mg of G-actin to 0.1 M NaCl/5 mM ATP/5 mM $CaCl_2$/25 mM Tris·HCl (pH 8.0) in a total volume of 0.5 ml at 30°C. Readings were made every 2 min until the viscosity reached a plateau (usually within 5–15 min), and specific viscosity (η_{sp}) was calculated as the flow-time of the sample divided by the flow-time of the salt solution, minus 1. A control containing no brevin was assayed with each group of samples. Changing the composition of the assay mixture to 0.1 M KCl in buffer A (9) or taking readings at other time intervals had no effect on the final value of viscosity measured.

In the presence of brevin, actin polymerizes to a lower final viscosity than in its absence, and the decrease in viscosity is related to the amount of inhibitor added (see figure 1 of ref. 3). One milliunit is defined as the amount of brevin that reduces the final viscosity by 1% from that achieved without inhibitor. The assay was always performed with 20–50 milliunits because it is linear only within this range.

Purification of Brevin. One hundred milliliters of pooled normal rabbit serum was fractionated by gel filtration on a column (5 × 90 cm) of Sephadex G-200 as described by Norberg *et al.* (2). Protein emerged in three peaks containing IgM, IgG, and albumin (10). Brevin appeared between IgG and albumin. Active fractions were made 50% saturated with $(NH_4)_2SO_4$, and the redissolved precipitate was dialyzed overnight in 10 mM Tris·HCl (pH 8.0). The dialyzed protein was then chromatographed on a column (2.5 × 40 cm) of DEAE-cellulose (DE-52, Whatman) equilibrated at pH 8.0 with 10 mM Tris·HCl. After the column was washed with 0.05 M NaCl in the Tris buffer, brevin emerged at about 0.175 M NaCl during elution of the column with an 800-ml linear NaCl gradient (0.05–0.2 M in the Tris buffer). Fractions containing activity were concentrated by precipitation with $(NH_4)_2SO_4$ at 70% saturation and dialyzed overnight in 0.6 M NaCl and 0.01 M sodium phosphate (pH 6.8). Dialyzed fractions were purified further by chromatog-

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Abbreviations: ADF, actin-depolymerizing factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

raphy on a column (1.1 × 30 cm) of hydroxylapatite (Bio-Gel HTP, Bio-Rad) equilibrated with 0.01 M sodium phosphate in 0.6 M NaCl (pH 6.8). Brevin emerged at approximately 0.075 M phosphate when the column was developed with a 100-ml linear sodium phosphate gradient (0.01–0.15 M in 0.6 M NaCl, pH 6.8). Fractions containing activity were combined, dialyzed, and frozen at −70°C. Although activity in serum was unaffected by repeated freezing and thawing, the activity of the purified protein was quite labile. Addition of 1 mM $CaCl_2$ and bovine serum albumin at 1.5 mg/ml stabilizes the protein during storage at −70°C.

DNase-agarose, prepared from CNBr-activated Sepharose (Pharmacia) and DNase I (type I, Sigma), was treated with 0.25 mM phenylmethylsulfonyl fluoride (Sigma) and was then saturated with rabbit G-actin. Affinity chromatography was carried out using conditions of elution described (11).

Electron Microscopy. Actin filaments, diluted to a concentration of 0.02–0.08 mg/ml, were applied to Formvar/carbon-coated grids and negatively stained with 1% uranyl acetate. Montages containing 70–180 filaments were constructed from overlapping grid areas photographed at ×19,000 or ×34,000 in a Philips 301 electron microscope. All filaments in a montage were measured. Many montages could be constructed from a single grid, but the average filament length measured in different areas of the same grid had a coefficient of variation of less than 10%.

RESULTS

Brevin is present in sera of rabbits (0.10 ± 0.005 [SEM] units/mg of protein, $n = 7$), humans (0.04 ± 0.005, $n = 4$), and rats (0.06 ± 0.002, $n = 3$). Fractionation of both rabbit and human anticoagulant-treated fresh blood revealed that serum contains 99% of the activity in the blood. A small amount is in the platelet fraction, which has a specific activity of 0.4 unit/mg. No activity was detected in other cellular elements. In addition, we found that serum has the same activity as plasma, indicating that the presence of brevin is not influenced by the clotting process.

Purification of Brevin from Rabbit Serum. We purified brevin 88- to 242-fold with an overall recovery of 1–3% in six independent preparations. A summary of one purification is presented in Table 1, with polyacrylamide gel electrophoretic analyses in Fig. 1A. The predominant protein remaining after hydroxylapatite chromatography has a molecular weight of 90,000 and constitutes 81% of the total protein at this stage. Three minor proteins having molecular weights of 73,000, 50,000, and 45,000 are also present, but densitometry demonstrates that brevin activity closely correlates with the amount of the 90,000 molecular weight protein and shows no correlation with the amounts of the minor constituents. We suspect that the 45,000 molecular weight protein, usually the major contaminant, is actin. Actin binds to brevin (see below); in addition, the 45,000 molecular weight protein migrates with brevin during electrophoresis on a nondenaturing polyacrylamide gel. It is unlikely that the purified 90,000 molecular weight protein accounts for only a minor fraction of the total brevin activity originally present in serum, because recovery of activity of each stage of the purification was substantial, ranging between 27% and 73%. On the basis of the degree of purification, we calculate that brevin is quite abundant in blood, representing 0.3% of the total protein in serum or plasma.

Table 1. Purification of brevin from rabbit serum

Purification step	Volume, ml	Activity, units	Protein, mg	Specific activity, units/mg	Purification, fold
Serum	100	1073	10,400	0.10	1
Sephadex G-200	89	482	685	0.70	7
50% $(NH_4)_2SO_4$	7.2	315	101	3.1	31
DEAE-cellulose	82	157	13.1	12.0	120
70% $(NH_4)_2SO_4$	1.3	115	10.3	11.2	112
Hydroxylapatite	3.3	31.4	1.3	24.2	242

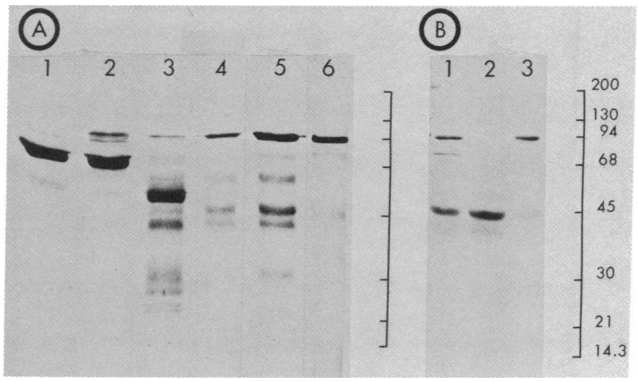

FIG. 1. Electrophoretic analyses of protein fractions on 10% polyacrylamide slab gels in $NaDodSO_4$ (12). Gels were stained with Coomassie blue. Myosin (200,000), β-galactosidase (130,000), phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300) were used as molecular weight markers (scale on right, molecular weight × 10^{-3}). (*A*) Purification of brevin (see Table 1). Lane 1, rabbit serum (15 μg of protein was applied); lane 2, active fractions from Sephadex G-200 (15 μg); lane 3, precipitate from 50% $(NH_4)_2SO_4$ fractionation (15 μg); lane 4, active fractions from DEAE-cellulose (8 μg); lane 5, precipitate from 70% $(NH_4)_2SO_4$ fractionation (15 μg); lane 6, active fractions from hydroxylapatite (5 μg). The 90,000 molecular weight protein that appears in lanes 2 and 3 is not all brevin, because the amount estimated densitometrically does not correlate with the brevin activity of these samples. (*B*) Affinity chromatography of brevin on actin-DNase I-agarose. Lane 1, active hydroxylapatite fractions (different preparation from *A*) applied to the affinity column (10 μg); lane 2, fractions eluted with 2 mM $CaCl_2$ in 0.6 M NaCl/20 mM Tris·HCl (pH 7.8) (7 μg); lane 3, brevin eluted with 5 mM ethylene glycol bis(β-amino-ethyl ether)-*N, N, N', N'*-tetraacetic acid (EGTA) in 0.15 M NaCl/1 mM dithiothreitol/20 mM Tris·HCl (pH 7.8) (4 μg).

Effect of Brevin on Filament Length and Critical Concentration. The reduction of viscosity produced by brevin results primarily because the inhibitor decreases the lengths of filaments (Fig. 2) rather than the total amount of actin in polymeric form. Shortening of filaments is dependent on the concentration of inhibitor (Fig. 3). To determine the extent to which brevin changes the total amount of actin in the form of filaments, we measured the effect of the inhibitor on critical concentration (Fig. 4). Using inhibition of DNase I (13), we could detect only a small change in the critical concentration, which appeared to increase with the amount of brevin. The change was significant ($P < 0.05$) only when inhibitor was added at a concentration greater than 0.22 unit/ml. Even the highest concentration of brevin, however, increased the critical concentration by less than 2% of the total amount of actin, and this increment would make an insignificant contribution to the changes in viscosity observed. Analysis of monomeric actin by sedimentation (see legend to Fig. 4) did not reveal any effect on critical concentration, although the data were quite scattered. Nevertheless, even lower values were obtained by sedimentation than by the assay with DNase I. [The discrepancy is probably a consequence of the small contribution of F-actin to the inhibition of DNase I (13).]

Stoichiometry of Brevin Action. A small amount of purified brevin is sufficient to inhibit the polymerization of a much larger molar amount of G-actin: 2 μg of our most highly purified prep-

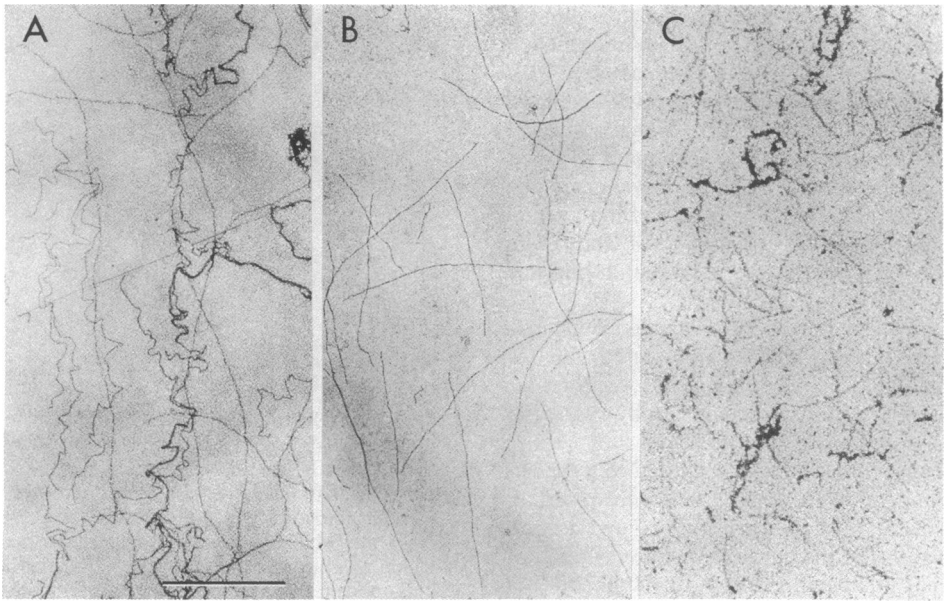

FIG. 2. Electron microscopic examination showing that brevin produces shorter actin filaments. Rabbit G-actin at 0.43 mg/ml was polymerized for 1 hr at 25°C in buffer A (9) containing 0.1 M KCl. Purified brevin was not added (*A*), or was present from the start of polymerization at a concentration (units/ml) of 0.10 (*B*) or 0.34 (*C*). Samples were examined after negative staining. Bar = 1 μm; *A–C* are shown at the same magnification.

aration (Table 1) reduced the final viscosity of 215 μg of actin by 50% in the standard assay. Because this preparation was 81% pure, however, the actual molar ratio of inhibitor to actin monomer would be 1:281. The small amount of brevin required to affect the viscosity suggested that each elongating filament interacts with only a few molecules of inhibitor.

To determine the stoichiometry of binding directly, we sedimented F-actin that had been polymerized in the presence of inhibitor and analyzed the pellets by polyacrylamide gel electrophoresis (Fig. 5). Brevin that sediments must be bound to filaments, because brevin alone does not sediment. By densitometry of the stained gels, we estimated how many millimoles of brevin were bound per mole of sedimented actin protomer (Table 2). Using the lengths in Fig. 3 to determine the average number of protomers per filament, we then calculated the molar ratio of brevin to actin filaments. This ratio was constant, averaging 1.17 ± 0.1 (SEM) over a 6-fold range of brevin concentration and a 7-fold range of filament length. Similar results were obtained by assaying the supernatants for brevin activity before and after centrifugation and calculating by difference the amount of sedimented inhibitor.

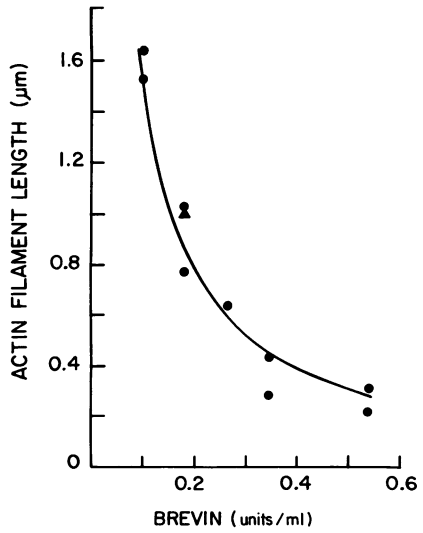

FIG. 3. Filament length depends on the concentration of brevin present during polymerization. Actin was polymerized in the presence of the indicated amounts of purified inhibitor, using the same conditions given in the legend to Fig. 2. Samples were examined by electron microscopy after negative staining; each sample was applied to one or two grids and the number-average filament length was measured on each grid separately (●). The hyperbolic curve was fit by least-squares analysis. In the absence of brevin, the average length of filaments was greater than 5 μm. The triangle (▲) represents the average filament length produced when brevin at 0.18 unit/ml was added to actin that had already been polymerized for 1 hr in the absence of inhibitor.

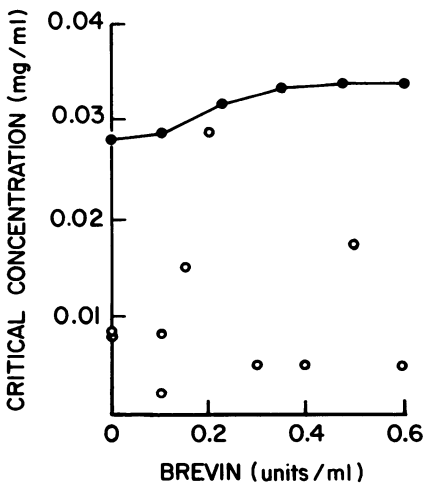

FIG. 4. Effect of brevin on critical concentration. Rabbit G-actin at 0.43 mg/ml was polymerized for 1 hr at 25°C in the presence of various amounts of purified brevin in buffer A (9) containing 0.1 M KCl. The critical concentration of monomeric actin was estimated by inhibition of DNase I (13) (●, average of triplicate determinations) or by measurement of the amount of actin that did not sediment after centrifugation at 230,000 × *g* (○). To sediment actin filaments completely it was necessary to centrifuge samples for 3 hr. When centrifugation was carried out for only 1 hr, much more actin remained in the supernatant, particularly when larger amounts of brevin were added, probably because short oligomers of F-actin do not sediment.

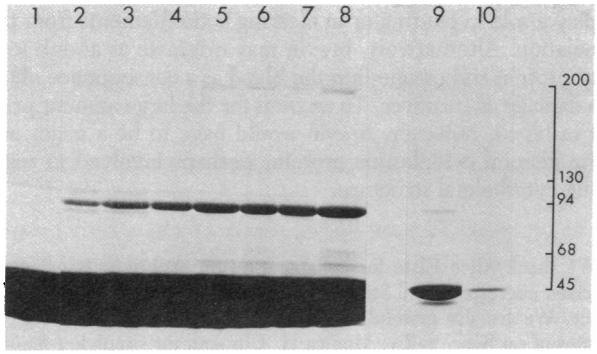

FIG. 5. Electrophoretic analyses of samples after sedimentation of brevin with F-actin. Rabbit G-actin (0.86 mg) was polymerized at 25°C in the presence of various amounts of purified brevin in 2 ml of buffer A (9) containing 0.1 M KCl. After 1 hr, samples were centrifuged at 230,000 × *g* for 3 hr at 25°C, and the supernatants and pellets were analyzed on 5% polyacrylamide gels in $NaDodSO_4$. The molecular weight markers are the same as in Fig. 1. Lanes 1–8, pellets (half the total) from samples containing the following initial concentrations of purified brevin (in units/ml): 0, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, and 0.6. Lane 9, 40 μl of a sample containing 0.6 unit/ml of brevin before centrifugation. Lane 10, 40 μl of supernatant from the same sample after centrifugation. Lanes 1–8 are overloaded with actin in order to visualize the small amounts of brevin that sedimented. Smaller volumes of the same samples were electrophoresed in parallel in order to quantitate densitometrically the amounts of actin that sedimented (not shown).

Brevin Shortens Already-Polymerized Actin Filaments. The experiments described above indicate that shorter actin filaments result when brevin is present during the course of polymerization. By electron microscopy we have also found that brevin shortens filaments when added after polymerization is already complete. This effect is dependent upon the amount of inhibitor used. Within 1 min, brevin at 0.18 unit/ml shortened actin filaments to an average length of 1.02 ± 0.07 μm (SEM), a value not significantly different from the length obtained when the same amount of inhibitor was present during polymerization of G-actin (Fig. 3, ▲). With 1.2 or 2.4 units/ml (corresponding to a molar ratio of brevin to actin monomer of 1:23 or 1:11) only very short filaments were observed. With 6 or 60 units/ml (corresponding to molar ratios of 1:4.5 or 1:0.5) no filaments at all were visible. At the magnifications used, the length of the shortest filament that could be detected was 0.05 μm.

The results obtained by using electron microscopy were confirmed by viscometric measurements. Within 1 min, brevin at 0.1 unit/ml reduced the specific viscosity of F-actin by 50%, and at 0.20 unit/ml, by 73%. The same final values of viscosity were obtained when inhibitor was present in similar quantities during polymerization of a comparable amount of G-actin. Thus, brevin produces the same final viscosity and filament lengths independent of the initial state of the actin.

Brevin Binds to Monomeric Actin. We subjected the inhibitor to affinity chromatography on G-actin-agarose to determine whether brevin binds to monomeric actin. Brevin did not bind, but we could not be certain whether coupling to agarose had denatured the actin. We therefore performed affinity chromatography on actin-DNase I-agarose with elution conditions similar to those used by Bretscher and Weber (11) to purify villin from extracts of intestinal brush border. Brevin is bound quantitatively to this affinity column in the presence of Ca^{2+} and can be released by washing the column with EGTA (Fig. 1*B*). The inhibitor was not retained by a DNase-agarose column to which actin had not previously been bound (data not shown).

Brevin probably binds to monomeric actin, because this is the form usually found complexed with DNase (13, 15). From the amounts bound, we estimate that one or two molecules of actin are required to bind one molecule of the inhibitor. This estimate is tentative, however, because proteolysis occurs during passage through the column (despite treatment with protease inhibitor), and the overall recovery of brevin is only 50%. Small amounts of contaminating proteins also bind and are eluted by EGTA, but densitometry of gel electropherograms reveals that only brevin is enriched by passage through the column.

Binding of brevin to the affinity column appears to depend on Ca^{2+}, and this is consistent with two additional observations suggesting that Ca^{2+} interacts with the inhibitor. First, 1 mM Ca^{2+} stabilizes brevin against heat denaturation, prolonging its half-life at 50°C from 2–10 min to greater than 30 min. Second, inhibition of actin polymerization is dependent upon Ca^{2+}. In the presence of 0.5 mM EGTA, brevin activity in the viscometric assay is reversibly abolished. Inhibitor that had been incubated with EGTA becomes fully active when subsequently assayed in the presence of 1 mM Ca^{2+}.

Table 2. Stoichiometry of binding of brevin to actin filaments

Initial brevin concentration, units/ml	Ratio of brevin to actin protomer, mmol/mol	Protomers per filament	Ratio of brevin to actin filaments, mol/mol
0.10	2.42	586	1.42
0.15	4.22	385	1.63
0.20	4.51	285	1.29
0.30	6.20	184	1.14
0.40	6.48	134	0.87
0.50	8.46	104	0.88
0.60	11.59	84	0.97

Brevin was sedimented with F-actin at 230,000 × *g* for 3 hr and the molar ratio of inhibitor to actin protomer in the pellet was determined by densitometric scanning of stained electropherograms such as that shown in Fig. 5 (lanes 2–8). It was assumed that actin stains twice as darkly as brevin with Coomassie blue, because the amount of actin estimated colorimetrically with this dye (5) is 2 times the true value determined by A_{280}. The number of protomers per filament was calculated from the lengths in Fig. 3, assuming 2.73 nm per protomer (14). The molar ratio of brevin to actin filaments was calculated by multiplying the ratio of brevin to actin protomer by the number of protomers per filament.

DISCUSSION

The idea that control of actin polymerization might be an important aspect of intracellular motility was suggested by Tilney's discovery of proteins in echinoderm sperm that serve to maintain actin unpolymerized until the moment of fertilization (16). Many other proteins have now been described from diverse cells and tissues that can influence the state of actin polymerization. In general, these cells and tissues were chosen because intracellular motility is a prominent feature of their physiological functioning. Several of these intracellular proteins have properties that are similar to those that we have found for brevin (11, 17–22). Because brevin is an extracellular protein, however, it cannot play a direct role in intracellular motility. But, because it constitutes 0.3% of plasma protein, brevin is probably the actin-binding protein that occurs in largest amounts in the body. It would therefore be interesting to ask whether brevin is identical to any of the intracellular proteins thought to regulate polymerization within cells, or whether it belongs to a family of proteins that, while not identical, all operate by a similar mechanism.

Most likely to be related are proteins that have been isolated from blood cells, for example platelets (19), and alveolar macrophages (18), which are thought to derive from monocytes. The molecular weight of these proteins is about 90,000, and they bind actin and produce effects in substoichiometric amounts. It is unlikely that brevin is derived from blood cells, however, because only 1% of the total brevin activity in blood is in cells. On the other hand, it is possible that these intracellular proteins are actually brevin, acquired by adsorption or pinocytosis. For example, macrophages may engulf plasma proteins from blood or lymph and thereby accumulate brevin. These proteins are probably not derived from brevin, however, because they seem to be major constituents of the cytoplasm of the cells in which they are found. Thus the specific brevin activity we found in platelets was 4 times greater than that in serum, and gelsolin appears to constitute about 2% of the protein in macrophages (18).

Brevin is probably the same protein that was described in sera as an F-actin-depolymerizing factor (ADF) (1, 2, 23, 24), and which has been recently purified (25). Brevin and ADF have similar molecular weights (2, 23, 25), both are labile to mild heat treatment (2, 24) and are stabilized by the presence of Ca^{2+} (24), and both bind to actin-DNase agarose (25). Yet the principal action of brevin is not to change the amount of actin in monomeric form (critical concentration; see Fig. 4). Thus, if ADF and brevin are identical, "ADF" is not an appropriate name because the protein shortens actin filaments but does not depolymerize them. Intracellular proteins with a similar action (18, 26) are thought to regulate gel–sol transformations of the cytoplasm (27).

Mechanism of Action. Using viscometry, we previously observed that brevin accelerates the initial phase of salt-induced polymerization (3), and this suggests that the inhibitor promotes the nucleation step of filament growth. We also found that brevin inhibits polymerization that is nucleated by sonicated fragments of F-actin or by protamine (unpublished experiments), suggesting that the inhibitor also interferes with elongation of the polymer. We propose that brevin blocks elongation by binding to one end of growing filaments, as suggested for the platelet factor (19) and a capping protein from amoeba (20) and for the cytochalasins (28–31). This idea is supported by the observation that a single molecule of the inhibitor is bound to each actin filament (Table 2). Brevin might also stabilize actin nuclei by binding to one end, thereby promoting formation of more numerous and hence shorter filaments.

Work on several of the factors that are thought to regulate the state of actin suggest that the same protein might fragment, depolymerize, nucleate or inhibit gelation depending on the assay conditions used (2, 17, 18, 20, 26). At relatively high concentrations, brevin might also depolymerize actin filaments. The amount of brevin used in our experiments was always less than 1/50 of the molar amount of G-actin. It is possible that in larger amounts brevin could substantially increase the critical concentration by binding to actin monomers as they leave the ends of the filament. During sedimentation of brevin and F-actin (Fig. 5), some of the inhibitor remains in the supernatant, perhaps complexed with G-actin. The ability of brevin to bind monomeric actin (Fig. 1*B*) and the small but significant increase in critical concentration produced by the inhibitor (Fig. 4) are also consistent with the possibility that brevin might sequester some actin in an unpolymerized form.

Physiological Role of Brevin. Its abundance in serum and plasma suggests that brevin plays an important physiological role. Preliminary experiments using perfusates from isolated rat liver (7, 8) indicate that brevin is synthesized by the liver, and it is possible that the inhibitor is specifically secreted into blood to play a role in clotting or in clearing actin filaments from the circulation. Alternatively, brevin may originate as an intracellular protein and escape into the blood as a consequence of tissue damage or turnover. To account for the large amount present in blood, however, brevin would have to be a major and quite general cytoplasmic protein, perhaps involved in regulating cytoskeletal structure.

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