A new calcium-sensitive actin filament cross-linking protein has been purified from *Acanthamoeba*. By gel electrophoresis in sodium dodecyl sulfate, the apparent subunit molecular weight varies depending on the concentration of sulfhydryl reducing agent in the sample. The major band is 60,000 in 10% β-mercaptoethanol, 85,000 in 1% mercaptoethanol, and 90,000 without β-mercaptoethanol. By electron microscopy, the molecule is a rod about 3 nm wide and 55 nm long with a 5.5-nm globular region at one end, which accounts for its large Stokes radius of 8.5 nm. At low concentrations, the gelation protein cross-links actin filaments to form a solid gel. This gelation reaction is inhibited by micromolar concentrations of Ca²⁺, by cytochalasin B, and by capping protein and is promoted by ATP, MgCl₂, and KCl.

An detailed understanding of the molecular basis of cellular motility and cytoplasmic structure requires the purification of the various proteins which interact with actin. This work is being done on a variety of organisms (reviewed by Taylor and Condeelis, 1979), one of which is *Acanthamoeba castellanii*. The initial description of *Acanthamoeba* cytoplasmic extract gelation and the demonstration that actin filaments are the major structural component of these gels (Pollard, 1976a, and 1976b), Maruta and Korn (1977) purified four small proteins with Mᵣ = 23,000, 28,000, 32,000, and 38,000, each of which caused pure actin filaments to gel. It was stated that these proteins account for >95% of the gelation factor activity in the extract. The existence of these proteins was confirmed by MacLean-Fletcher and Pollard (1980a), who also presented preliminary evidence for additional small gelation proteins. Although these small gelation proteins have a high specific activity for cross-linking actin filaments, it was not clear that they alone could account for the gelation reaction in the crude extract or in the cell. One problem was that Ca²⁺ inhibits the gelation reaction in the extract but not in the reconstituted system with either crude or purified gelation proteins (MacLean-Fletcher and Pollard, 1980a). The present report describes the discovery, purification, and initial characterization of a new gelation protein from *Acanthamoeba*. It is considerably larger than the previously described *Acanthamoeba* gelation proteins and its actin cross-linking activity is inhibited by Ca²⁺. A preliminary account of this work has been presented (1980 Annual Meeting of the American Society for Cell Biology (Pollard et al., 1980)).

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**EXPERIMENTAL PROCEDURES**

**Purification**—Two different methods were used to purify the calcium-sensitive gelation protein from sucrose extracts of *Acanthamoeba*. The first step of both methods is ion exchange chromatography of the extract on DEAE-cellulose column like that originally used to purify actin (Gordon et al., 1976) and myosin II (Pollard et al., 1978). As illustrated by MacLean-Fletcher and Pollard (1980a), this large column separates three peaks of gelation factor activity from myosin I, myosin II, and actin. Overlapping the peaks of myosin II and actin, there is a peak of “capping protein” which inhibits the low shear viscosity of actin filaments (MacLean-Fletcher and Pollard, 1980a; Isenberg et al., 1980). The new gelation protein is found in the same fractions as the capping protein but is not detected in the falling ball assay because of the capping protein. When actomyosin II is precipitated from the combined myosin II and actin fractions, part of the gelation protein precipitates with the actomyosin II and part remains in the supernatant.

When the actomyosin II precipitate is fractionated by gel permeation chromatography, a peak of gelation factor activity, coincident with a peak of protein, elutes between and well separated from the myosin II and actin peaks (Fig. 1). These fractions are enriched in polypeptides with apparent Mᵣ = 85,000 and 90,000 in 1% β-mercaptoethanol (Fig. 2B). Chromatography on hydroxylapatite (Fig. 2) removes the minor contaminants (Fig. 2C). The yield is about 30 mg/g of wet cells. There are major polypeptides in the crude extract with the same molecular weights as those of the isolated polypeptides (Fig. 3A).

The calcium-sensitive gelation protein can also be purified from the actomyosin II supernatant using the initial steps employed in capping protein isolation (Isenberg et al., 1980). Both proteins precipitate between 1.5 and 2.5 M ammonium sulfate and are then separated by gel permeation chromatography.
Acanthamoeba Gelation Protein

Fig. 1. Gel permeation chromatography of actomyosin II on a column (2.6 × 91 cm) of Bio-Rad A15m (200–400 mesh). Equilibrating buffer: 0.6 M KCl, 10 mM imidazole, pH 7.0, 1 mM dithiothreitol. The sample in 20 ml of 0.6 M KCl, 20 mM imidazole, pH 7.0, ATP, 0.5 mM dithiothreitol, 2 mM MgCl₂, was applied to the column behind 50 ml of the same buffer. Az, Ca²⁺-ATPase, nanomoles·min⁻¹·ml⁻¹; low shear viscosity assay of 0.05 ml of column fraction in 1 ml containing 0.5 mg of actin, 10 mM imidazole, pH 7, 2 mM MgCl₂, 1 mM EGTA incubated for 10 min at 25 °C, °—○, cp, centipoise.

Fig. 2. Hydroxylapatite chromatography of the pooled gelation factor peak from an A15 column. Equilibrating buffer: 20 mM imidazole, pH 7.5, 1 mM dithiothreitol, column size, 1.5 × 8 cm; gradient, 0 to 250 mM potassium phosphate, pH 7.5, in equilibrating buffer, total volume of 300 ml, measured by conductivity (Δ—Δ). The small peak of conductivity in the void volume is due to salt in the sample, A₁₀₀. Low shear viscosity assay of 0.05 ml of column fraction in 1 ml containing 0.5 mg of actin and buffer as in Fig. 1, ○—○, cp, centipoise.

Fig. 3. Polacrylamide gel electrophoresis in sodium dodecyl sulfate. A, extract; B, gelation factor peak from the A15 column; C, purified gelation protein from hydroxylapatite chromatography of B; D, purified gelation protein boiled in 10% β-mercaptoethanol; E, purified gelation protein boiled in 10% β-mercaptoethanol, dialyzed against 1% β-mercaptoethanol, and then boiled again, have the Mᵣ = 85,000 and 90,000 bands and little fraction is about 30 µg/g of wet cells. No consistent differences have been found in the protein purified by the two methods.

Physicochemical Properties—The electrophoretic mobility of the purified gelation protein polypeptides in sodium dodecyl sulfate varies considerably depending on the way that the sample is prepared. When the native protein is boiled in 0 or 1% β-mercaptoethanol, there are two bands with apparent Mᵣ = 85,000 and 90,000 (Fig. 3, B to D). The Mᵣ = 85,000 band predominates in 1% β-mercaptoethanol; the Mᵣ = 90,000 band predominates in its absence. When the native protein is boiled in 10% β-mercaptoethanol, most of the protein runs as a Mᵣ = 60,000 band (Fig. 3E). Samples boiled in 10% β-mercaptoethanol, dialyzed against 1% β-mercaptoethanol, and then boiled again have the Mᵣ = 85,000 and 90,000 bands and little

1 The abbreviation and trivial name used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

Fig. 4. Electron micrographs of purified calcium-sensitive gelation protein. A sample of protein at 33 µg/ml was adsorbed to a grid, washed, and negatively stained. Top, 7 selected particles at ×340,000; bottom, a field of particles at ×250,000.
or no $M_r = 60,000$ band. The converse experiment confirmed that the electrophoretic mobility is determined by the final concentration of $\beta$-mercaptoethanol, showing that the $M_r = 60,000$, 85,000, and 90,000 bands are interconvertible and therefore presumably consist of the same material. After dansylation by the method of Stephens (1975), all of the stained protein runs as a single band of $M_r = 90,000$. Neither 1 mM CaCl$_2$ nor 1 mM EGTA affected the electrophoretic mobility of samples boiled in 1% $\beta$-mercaptoethanol.

The Stokes radius of native gelation protein is 8.5 nm in both 0.6 M KI/KCl buffer (Fig. 1) and in 20 mM KCl. Since a spherical molecule with a Stokes radius of 8.5 nm would have a molecular weight of more than $2 \times 10^6$, it seemed likely that the gelation protein is highly asymmetric even if it contained multiple subunits. Direct visualization of the gelation protein by electron microscopy revealed that it is rod-shaped (Fig. 4). The mean length is 55 nm (S.D., 6.7 nm) and the mean maximum width is 3.0 nm (S.D., 0.4 nm). The width of the molecule is reasonably uniform along most of its length, but it can be bent into a variety of different shapes, suggesting that it is flexible. In the best preparation, 44 of 51 molecules had a distinct globular region with a mean diameter of 5.5 nm (S.D., 0.7 nm) at one end.

**Interaction with Actin**—The gelation protein can cross-link actin filaments to form a solid gel. A sensitive way to evaluate this reaction is to measure the dependence of the low shear viscosity of actin filament solutions on the gelation protein concentration (Fig. 5). Here, as in other actin gelling systems (Brotchi et al., 1978; MacLean-Fletcher and Pollard, 1980a and 1980b), the curves are hyperbolic. In our standard buffer with 2 mM MgCl$_2$, 1 mM ATP, 1 mM EGTA, the “critical gelling concentration” of the gelation protein is about 11 $\mu$g/ml (Table I). Some preparations of the protein isolated from the actomyosin precipitate had a somewhat higher critical gelling concentration, that is, a lower specific activity (Table I).

The critical gelling concentration depends on the composition of the buffer. The gelation of actin by freshly prepared gelation protein is inhibited by free Ca$^{2+}$ (Fig. 5) in a concentration-dependent fashion in the 1 to 100 nM range. It is also inhibited by micromolar concentrations of cytochalasin B (Fig. 5) and by 0.5 to 1 pg/ml concentrations of capping protein (Isenberg et al., 1980). Gelation of actin by the gelation protein does not require MgCl$_2$ or ATP, but the critical gelling concentration was usually higher without ATP or without MgCl$_2$ (Fig. 5). In the range of 20 to 200 mM KCl, KCl lowers the critical gelling concentration, probably because KCl promotes self-association of the actin filaments. The critical gelling concentration does not depend on the actin concentration in the range of 0.4 to 1.6 mg/ml, but below the critical gelling concentration, the low shear viscosity is proportional to the actin concentration.

Table I

| Preparation     | Gelation protein from actomyosin precipitate | Gelation protein from actomyosin supernatant |
|-----------------|---------------------------------------------|----------------------------------------------|
| GP              | G                | GP                             |
| AM-52           | 17.6             | 10.0                          |
| AM-53           | 21.6             | 10.0                          |
| AM-54           | 9.3              | 10.0                          |
| AM-55           | 12.5             | 10.0                          |

The gelation protein accelerates the polymerization of actin

![Fig. 5](image-url)

**Fig. 5. Low shear falling ball viscometry of actin-gelation protein mixtures.** Conditions included buffer (10 mM imidazole pH 7.0, 2 mM MgCl$_2$, 1 mM ATP, 1 mM EGTA), 0.5 mg/ml of actin, 10-min incubation at 25 °C. The gelation protein (GP) concentration is varied as indicated. Complete buffer, O—O; buffer with 50 mM KCl substituted for MgCl$_2$, O—O; buffer minus ATP, O—O; buffer plus 2 $\mu$M cytochalasin B (CB) ——; buffer with 100 $\mu$M CaCl$_2$ substituted for EGTA, Δ—Δ. cp, centipoise.

![Fig. 6](image-url)

**Fig. 6. Ostwald capillary viscometry analysis of the time course of actin polymerization.** Buffer and actin as in Fig. 5. Gelation protein (GP) concentration, 25 $\mu$g/ml.
Acanthamoeba Gelation Protein

judged from the time course of the high shear viscosity change (Fig. 6). In addition, the maximum high shear viscosity is greater with the gelation protein. The fact that the final high shear viscosity of actin filaments is essentially the same with or without the gelation protein (Fig. 6) is thought to be due to the mechanical disruption of the gel by flow through the viscometer.

When actin filaments and the gelation protein are mixed and spread on an electron microscope grid, most of the specimen appears as a random array of separate actin filaments accompanied by some 3-nm fibers (Fig. 7a). Some of the 3-nm fibers are attached at one end to the actin filaments. In addition there are a few small, doughnut-shaped objects about 8.5 nm in diameter which do not appear to be associated with the filaments. These same doughnuts were occasionally observed on grids of gelation protein alone. In some areas of the grid, the actin filaments are gathered in small bundles (Fig. 7b). Control samples of actin filaments alone do not have the bundles, 3-nm fibers, or the 8.5-nm doughnuts.

DISCUSSION

The isolation and this initial characterization of the new calcium-sensitive gelation protein is part of the continuing effort to establish the molecular basis of contractile protein function in Acanthamoeba. This gelation protein is particularly interesting because it is present in a higher concentration than any of the low molecular weight gelation factors isolated previously (Maruta and Korn, 1977; MacLean-Fletcher and Pollard, 1980a) and because it is the first from Acanthamoeba to be Ca²⁺-sensitive like the crude extracts (Pollard, 1976a; MacLean-Fletcher and Pollard, 1980a). Although present in high concentrations in the extract, the new gelation protein was missed in the earlier studies because it elutes from the initial DEAE-cellulose column in a complex mixture with actin and capping protein, which is a strong inhibitor of gelation.

By electron microscopy, the new protein appears to be a rod (3 × 55 nm) with a globular region at one end. These dimensions suggest that the rod region may be an α-helical coiled coil like tropomyosin (Cohen and Holmes, 1963). If the 55-nm rod is a coiled coil, it would have a $M, = ~90,000$ and could be constructed either of two separate polypeptides like tropomyosin or a single polypeptide folded back on itself like staphylococcal A-protein (Diesenhofer et al., 1978). If the subunit $M, = 60,000$ obtained by electrophoresis in 10% mercaptoethanol is correct, the two-polypeptide model is favored and two $M, = 15,000$ polypeptide regions would be available to form the globular region. However interesting these speculations may be, it must be realized that there may be substantial errors in measuring the dimensions of such small negatively stained particles in addition to the uncertainty about the subunit molecular weight. Further physical characterization, especially the native molecular weight and α-helix content, should clarify the structure of the molecule.

In some ways, the Acanthamoeba gelation protein resembles macrophage actin-binding protein and smooth muscle filamin, two vertebrate gelation proteins. All are elongated flexible rods (Tyler et al., 1980) capable of cross-linking actin filaments. The vertebrate proteins are considerably larger, however. They consist of two $M, = 250,000$ polypeptides and are longer (80 nm) and probably wider (Tyler et al., 1980) than the Acanthamoeba gelation protein.

The new Acanthamoeba gelation protein cross-links actin filaments in similar but distinct ways when compared with the smaller gelation proteins isolated previously from Acanthamoeba and the gelation proteins from other cells. In every case, there is a sharp transition from a liquid to a solid gel at a critical geling concentration as predicted by Flory (1953). As in the case of the $M, = 116,000$ "actinogelin" from Ehrlich ascites cells (Mimura and Asano, 1980), Ca²⁺ appears to have a direct effect on the cross-linking of actin filaments by the new Acanthamoeba gelation protein. In contrast, purified gelation proteins from other cells such as macrophage actin-binding protein (Brotschi et al., 1978), smooth muscle filamin (Brotschi et al., 1978), and sea urchin egg fascin (Bryan and Kane, 1978) are not directly calcium-sensitive. A final similarity is that gels formed from mixtures of actin with calcium-sensitive gelation protein, or the $M, = 33,000$ Acanthamoeba gelation factor or aldolase have the same structure. All consist of random networks of filaments which are indistinguishable from the networks in low viscosity solutions of actin filaments alone. This was established by electron microscopy of specimens prepared by quick-freezing, etching, and rotary shadowing. This observation and the fact that the various gelation factors are active at very low concentrations show that the actin filament networks in the gels are stabilized by relatively few widely spaced interfilament bonds.

The demonstration that an isolated protein like the calcium-sensitive gelation protein can cross-link actin filaments makes it a candidate for a structural protein in the cell. This is insufficient evidence, however, because a number of basic macromolecules, including polylysine (MacLean-Fletcher and Pollard, 1980b), histone, ribonuclease, lysozyme, and aldolase,³ can cross-link actin filaments. With the exception of aldolase, none of these molecules is likely to be present with actin in the cytoplasmic matrix. Some of the low molecular weight

³T. D. Pollard and J. E. Heuser, unpublished observations.
gelation proteins from *Acanthamoeba* (Maruta and Korn, 1977; MacLean-Fletcher and Pollard, 1980a) may also fall into this class of basic, possibly nonspecific, actin cross-linkers, because they do not bind to DEAE at pH 7.5. The fact that the new *Acanthamoeba* gelation protein described here is acidic, calcium-sensitive, and present in high concentration certainly suggests that it is a physiologically important component of the cytoplasmic actin network, but more direct evidence is still needed.

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