The Effect of the 540-Kilodalton Actin Cross-linking Protein, Actin-binding Protein, on the Mechanical Properties of F-actin*

Ken Scott Zaner
From the Hematology-Oncology Unit, Massachusetts General Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114

This study describes the effect of actin-binding protein derived from rabbit lung macrophages on the mechanical properties of F-actin. The dynamic storage modulus, \( G'(\omega) \), and loss modulus, \( G''(\omega) \) of F-actin, at concentrations from 1 to 4 mg/ml, in the absence or presence of actin-binding protein at molar ratios to actin of 1:1000 to 1:125, were measured at frequencies ranging from \( 3 \times 10^{-3} \) to 0.5 Hz. Actin-binding protein increased the dynamic moduli of F-actin, but this increase was much greater as either the actin-binding protein/actin ratio or the total protein concentration increased. Moreover, there was a convergence of the values of \( G' \) and \( G'' \) at high frequencies for F-actin which became more prominent upon the addition of actin-binding protein. The value of the modulus obtained by an extrapolation of these data to actin concentrations similar to that found in the cell cortex was close to values which have been obtained by direct measurements. The addition of actin-binding protein to an F-actin solution enabled it to reach an equilibrium strain following the application of a stress, in contrast to pure F-actin. These data allow a more rigorous definition of the “sol” to “gel” transition and suggest that the cross-linking of actin filaments by actin-binding protein leads to the formation of a network structure whose underlying mechanism of mechanical behavior is short range intrafilament bending in contrast to the classical rubber network.

Actin is a principal component of the peripheral cytoplasm of many eukaryotic cells (Poste and Nicolson, 1981). The reversible assembly of actin into linear filaments probably plays an important role in the consistency changes, often termed sol-gel transformations, which have been inferred to take place in this region of the cell (Stossel, 1984). Since there is a complex array of factors in cells that contribute to the mechanical properties of cytoplasm, it seems reasonable to define some first principles based on the behavior of pure actin and to examine the effects of the many actin-modulating proteins (Stossel et al., 1985) on these properties.

A number of groups have studied the mechanical properties of F-actin solutions (Kasai et al., 1960; Maruyama et al., 1974; Jen et al., 1982; Zaner and Stossel, 1982, 1983; Sato et al., 1985). We have found that the behavior of these solutions can be well approximated as a system of interpenetrating and relatively stiff rods. We consider the close agreement to the predictions of a theoretical treatment based only on such geometrical considerations (Zaner and Stossel, 1983) to be persuasive evidence that there is little role for the existence of additional interfilament bonds, although others have expressed different views (Sato et al., 1985).

F-actin solutions have an elasticity. In our opinion, this elasticity is not due to the long range configurational changes of individual chains, as is true for a rubber or gel composed of freely flexible units, but rather results from the storage of energy in the rotational motion of the topologically constrained filaments, which can be recovered when they randomize their orientations. Under this conceptual framework, increasing length and concentration of actin filaments should affect the degree of interpenetration and cause an increase in the observed apparent rigidity. Moreover, the range of frequency or time over which elastic behavior is observed would be expanded, and viscous flow properties of the system should be seen only at very low oscillation frequencies or at long times for dynamic or time-dependent measurements, respectively. Most experimental results are consistent with these predictions (Zaner and Stossel, 1983; Sato et al., 1985).

Despite the evidence for definite elastic properties of pure actin, actin filament cross-linking proteins have a strong influence on the mechanical properties of pure F-actin. Some of these proteins promote the alignment of actin filaments into bundles, whereas others lock actin filaments into more isotropic structures. The mechanical effect of cross-linking has been inferred from the ability to define discrete gel points, i.e. a large increase in static rigidity or viscosity occurring at a critical cross-link to filament ratio (Stossel et al., 1985). As was the case for experiments conducted with synthetic polymers, the phase transitions occurring at the gel point between “sol” and “gel” states were initially defined phenomenologically.

An actin filament cross-linking molecule of interest to us because of evidence that it has an important role in the apparent gelation of macrophage, granulocyte, platelet, and toad egg extracts (Stossel and Hartwig, 1976; Boer and Stossel, 1976; Rosenberg et al., 1981; Corwin and Hartwig, 1983) is actin-binding protein.

1 Actin-binding protein from these cells is related in structure and function to a smooth muscle protein called "filamin." Although there are compelling similarities between these proteins and some authors use the name filamin to describe the nonmuscle homologue, important functional variations have been described for the two proteins, and the name "actin-binding" protein has been used by investigators studying leukocyte and platelet cytoskeletal proteins. For historical purposes and pending further clarification of the relationship between these high M, actin gelation proteins, we continue to use the name actin-binding protein.

*This work was supported by United States Public Health Service Grants HL-19429 and HL-00912 and a grant from the Edwin S. Webster foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
forming an orthogonal network of relatively straight actin filaments branched at predominantly right angles. In this paper, the rheological parameters of actin filaments cross-linked by actin-binding protein are reported. The data provide physical properties of these actin networks at various actin concentrations and at differing extents of cross-linking and more rigorous definitions of the sol and gel states of F-actin.

**MATERIALS AND METHODS**

Proteins—Actin was purified by the method of Spudich and Watt (1971). Purified actin was stored in the depolymerized form at 4°C and was used within 4 days of purification. Actin-binding protein (ABP) was purified from rabbit lung macrophages by the method of Hartwig and Stossel (1981). Protein concentrations were measured by the method of Lowry et al. (1951).

Rheologic Measurements—The dynamic storage and loss modulus, $G'(\omega)$ and $G''(\omega)$, and the compliance, $J(\omega)$, were measured by methods previously described (Zaner et al., 1981; Zaner and Stossel, 1983). The previously described single channel viscoelastometer and a new version which measures five samples simultaneously were used for the measurements. The principle of operation of both devices is the same. The rheologic properties are determined by observing the motion of a mica strip through a sample. The mica strip is suspended from a rod attached to a tilt table on which the sample is placed. The rheologic parameters are determined by tilting the table in a time-dependent manner, depending on the experiment, to induce a stress, and observing the time-dependent movement of the probe which represents the strain.

If the stress, $\sigma$, is applied with frequency, $\omega$, and amplitude, $A$, it will induce a strain, $\gamma$, which will be of the same frequency but shifted in phase by an amount, $\phi$, and decreased in amplitude to $H$. From these values it can be shown that $G' = C(A/H \cos \phi - 1)$ and $G'' = A/H \sin \phi$, where $C$ is constant for the instrument which depends on the size of the cuvette, and the width and dimensions of the probe. If the table is tilted in a single step and the movement of the probe is observed, the compliance, $J(t)$, which is defined as the ratio of time-dependent strain, $\gamma(t)$, to the constant stress, $\sigma_0$, can be measured. The initial stepped stress reaches its full amplitude within 0.4 s, but because of the probe movement, the stress slowly decreases over time, which is corrected for by an algorithm previously discussed and experimentally verified (Zaner and Stossel, 1983).

$G$-actin, in buffer $A$ of Spudich and Watt (1971), was diluted into buffer containing 10 mM imidazole, 2 mM ATP, 0.2 mM CaCl$_2$, pH 7.5, with or without ABP. The salt concentration was adjusted to 0.1 M KCl and 2 mM MgCl$_2$ to induce polymerization, and the mixture was immediately placed in the viscoelastometer. The dynamic moduli were monitored at a frequency of 0.03 Hz at approximately 30-min intervals. The measurement of the frequency spectra and/or compliance was begun when the moduli were constant to within 5%/h. The stress amplitude for the frequency-dependent measurements was adjusted to maintain the strain below 10%.

In order to determine if the measurement had disrupted the actin filaments, an additional measurement at 0.03 Hz was obtained subsequently to measuring the frequency spectra or compliance and was found to differ by less than 5% from the measurement obtained prior to determining the frequency spectra or compliance. In addition, the compliance and some of the points on the spectra were measured at different stress amplitudes and were found to agree closely.

**RESULTS**

Dynamic Moduli Measurements—The dynamic moduli, $G'(\omega)$ and $G''(\omega)$, in dynes/cm$^2$, as a function of frequency in radians/s, for 1, 2, and 4 mg/ml F-actin in the presence of ABP at molar ratios ranging from 1:125 to 1:1000 (ABP/actin) are shown in Figs. 1–3. The in-phase component $G'$ rises as the frequency increases, over the entire frequency range for all of the samples. However, the $G'$ of 4 mg/ml F-actin in the presence of high concentrations of ABP increases much more steeply as a function of frequency than that of the lower concentrations of actin containing lower amounts of ABP or of lower actin concentrations. In addition, the increment in $G'$ as the ABP/actin ratio increases becomes larger with increasing actin concentration.

The out-of-phase component $G''$ increases more steeply than $G'$ with increasing frequency for the 1 and 2 mg/ml samples and much more steeply for the 4 mg/ml samples. The
The effect of ABP on the mechanical properties of actin is illustrated in Fig. 3, which shows the dynamic moduli of 4 mg/ml F-actin in the presence of ABP. The labels have the same meaning as in Fig. 1.

The alteration in the frequency spectrum of F-actin produced by the addition of ABP is brought out in Fig. 4 where $G'$ and $G''$ for F-actin with and without ABP at a 1:250 molar ratio are plotted for 1, 2, and 4 mg/ml F-actin. ABP causes an upward displacement of the $G'$ and $G''$ curves at 1 mg/ml and progressively larger increments at F-actin concentrations of 2 and 4 mg/ml. $G'$ increases to a greater extent than $G''$ as the F-actin concentration increases, which has the effect of moving $G'$ and $G''$ closer together. Although there is some difference in the absolute value of the moduli obtained from different protein preparations, a consistent finding is that the dynamic moduli converge at progressively lower frequencies as the actin concentration increases.

The effect of ABP concentration on the normalized moduli of F-actin at concentrations of 1, 2, and 4 mg/ml at a frequency of 0.03 Hz is shown in Fig. 5. The normalized moduli $G'_N$ and $G''_N$ are the moduli for ABP/actin mixtures divided by the value for their respective actin control. The actual value of $G'$ at an F-actin concentration of 4 mg/ml and an ABP/actin ratio of 0.008 is about 25 dynes/cm². $G'_N$ increases slowly with increasing ABP concentration, for the 2 mg/ml and the 1 mg/ml F-actin samples at low ABP/actin ratios, and then much more sharply at higher ratios. This behavior is much more obvious for 4 mg/ml F-actin where $G'_N$ increases linearly at low concentrations of ABP but then very sharply above an ABP/actin ratio of 1:500. This shows that there is a critical concentration of ABP above which there is a very large increase in rigidity for a small increase in ABP. The effect of ABP on $G''_N$ is the same or slightly greater than $G'_N$ for the 1 and 2 mg/ml F-actin but is substantially greater at 4 mg/ml F-actin. In addition $G''_N$ increases with increasing frequency for the 4 mg/ml F-actin sample, but remains the same or decreases slightly for the 1 and 2 mg/ml sample (not shown).

**Compliance Measurements**—The compliance, $J(t)$, is the ratio of the time-dependent strain to the stress, applied as a single step at time 0. Since, for a fluid, the rate of strain becomes constant following the application of a fixed stress, the compliance of such a material will become linear at very long times. On the other hand, a solid will reach a constant strain following the application of a stress, which will correspond to an equilibrium modulus. If the measurements are in the linear viscoelastic range, i.e., they do not significantly alter the structure of the solution, the compliance measures the same molecular events as the inverse of the dynamic moduli with the inverse of the time replacing the frequency.
Effect of ABP on the Mechanical Properties of Actin

**DISCUSSION**

It is generally accepted that as the number of cross-links between polymer chains exceeds a critical value, there is the abrupt formation of an infinite molecule, which is defined as a polymer gel. Based on this fact, it has been previously claimed that ABP is an actin gelation protein by the demonstration of its ability to cause an abrupt increase in the apparent modulus of rigidity (Zaner and Stossel, 1978) or apparent viscosity (Zaner and Stossel, 1982) of an F-actin solution at a critical concentration of ABP. This paper reports the rheologic values for F-actin/ABP mixtures at three different total actin concentrations and at different actin to ABP ratios in an effort to define further the alterations in mechanical behavior caused by the introduction of ABP. The data show, as expected, that the effect of ABP depends on the time scale over which the measurement is performed, as well as on the actin to ABP ratio and the total actin concentration.

ABP has its most pronounced effect on the mechanical properties of F-actin at high frequencies, especially at high total protein concentrations. There is a 10-fold increase in $G'$ at the highest frequency measured, whereas only a 5-fold increase at lower frequencies. There is an even larger increase...
Effect of ABP on the Mechanical Properties of Actin

in $G''$ than $G'$ at very high frequencies, which causes a convergence of $G'$ and $G''$ at high frequencies. This characteristic is also observed in the mechanical spectra of synthetic, flexible polymer systems and reflects a change in the mechanism responsible for the rheologic properties from long range translational and rotational motions, which are dominant at low frequencies, to shorter range bending motions, which predominate at high frequencies. The frequency at which this convergence occurs for flexible polymers, however, is generally much higher than that observed for F-actin (Ferry, 1980). The spectrum observed for the F-actin samples is, therefore, consistent with an increase in the contribution of bending motions at high frequencies. Moreover, the decrease in the frequency at which this convergence occurs upon addition of ABP probably represents a further increase in the contribution of filament bending to the rheologic properties, which is apparent at progressively lower frequencies. This behavior is different from flexible polymer systems wherein cross-links usually have little effect on the contribution of short range motions. ABP most likely accomplishes this increase by the formation of rigid, perpendicular branches in the actin solution (Hartwig et al., 1980; Niederman et al., 1983) which drastically reduces the rotational freedom of the filaments and eliminates their ability to respond to the stress by rotational motions. Additionally, these changes may be due to the incorporation of the shorter filaments, which contribute little to the behavior of pure F-actin solutions, into the network.

The effect of ABP on both the compliance and the dynamic moduli varies directly with the total actin concentration and is much greater at 4 than at 1 or 2 mg/ml actin. This dependence is probably due to the fact that at the lower mass concentrations of F-actin, the filaments are widely separated and have relatively few overlap points per filament. Since ABP can only cross-link two actin filaments together at their overlap points, the quantity of ABP which is effective will decrease with decreasing actin concentration. A way to quantitate this effect is to calculate the minimum total length of F-actin required to connect together all of the ABP molecules into a network. If the ABP concentration in milligrams/milliliter is $C$, then there are $1.11 \times 10^{16} \times C$ molecules of ABP/ml. If the molecules are uniformly distributed throughout the solution, then each molecule is surrounded by a spherical volume of $8.9 \times 10^{-16}/C$ ml. This sphere has a radius of $5.96 \times 10^{-7}/C$ µm. Since ABP is at the center of a four-armed actin structure, each molecule requires four times this radius, and the total length, in microns, is $2.65 \times 10^4 \times C$. Converting this quantity to milligrams, about $6.9 \times 10^6$ mg/ml of F-actin is required to form a complete network with an ABP concentration of C. For example, if C is 0.2 mg/ml then 2.4 mg/ml of F-actin is required, failing which some of the ABP would be ineffective as a cross-linking protein and bind to only one filament or create a highly branched but incompletely aggregated structure. It is therefore possible that at the lower actin concentrations, a disconnected, branched structure is formed which has a slightly larger rigidity than actin alone due to an increase in topological constraints, but is not a complete network and therefore lacks the high rigidity characteristic of a structure the elasticity of which is derived primarily from intramolecular filament bending.

Although the measurements reported here use actin concentrations of up to 4 mg/ml, it has been estimated recently that the cortical cytoplasm of the macrophage has an F-actin concentration of at least 8 mg/ml. Although technical difficulties preclude the direct measurement of actin samples at 8 mg/ml at this time, a lower limit for the value of $G'$ for 8 mg/ml actin with a 1:125 molar ratio of ABP to F-actin can be estimated. By an extrapolation of the data of Fig. 5, it is found to be about 150 dynes/cm². The extrapolated value is almost 800 dynes/cm² if the values of $G'$ at 0.3 Hz are used. This value is similar to the values obtained from in vivo measurements of human leukocytes (Schmid-Schonbein et al., 1981).

It is important to consider the data presented in relation to the sol to gel transition which is considered to be an important mechanism by which contractile proteins are involved in cell movement. The designation of a protein as an F-actin gelation factor implicitly assumes that F-actin is a sol. Although the compliance data suggest that F-actin is a sol by a rigorous definition, it has significant elastic properties at biologically relevant stresses and rates of shear (Zaner and Stossel, 1983; Sato et al., 1985). It would therefore be important to consider alternative ways of defining the sol to gel phenomenon.

An important characteristic of the sol to gel transition is that a small increase in cross-linker concentration causes a large increase in the mechanical rigidity of the system. This kind of behavior is shown in Fig. 5 and, in this sense, represents a sol to gel transition and shows that ABP is a gelation factor. An additional characteristic of this phenomenon is that the addition of cross-linker changes a material which can flow into one which cannot. As shown previously and in this report, flow is observed for 1, 2, and 4 mg/ml F-actin at long times following the application of the stress. However, the compliance eventually approaches a constant value in the presence of ABP at an actin concentration of 4 mg/ml. Therefore, ABP generates an F-actin structure which, in contrast to F-actin alone, withstands a finite stress and reaches an equilibrium strain without flowing, at least within the time frame of the experiment. An alteration in behavior of this kind represents, phenomenologically, a sol to gel transition and is consistent with electron micrographs, indicating that ABP cross-links actin into an orthogonally branched structure (Hartwig and Stossel, 1981; Niederman et al., 1983).

It should be pointed out that the ability of an actin-ABP structure to achieve a constant strain in response to a stress does not require that the actin filaments be connected together into a true gel, defined as an “infinite” network. For example, if the filaments are extensively branched, the time required for flow to be observed would be well beyond any reasonable experimental time frame and would therefore have the identical behavior of an “infinite network.” The differentiation of an extensively branched structure from an infinite network would therefore be quite difficult for the actin system, when rheologic measurements are made over a limited time or frequency range. A branched but not infinite network could be important under some circumstances. For example, the cytoskeleton is required to engage in large deformations in order for the cell to move, yet have a degree of mechanical integrity. A true gel would deform to a finite extent then either reach an equilibrium strain or break. A highly branched but not infinite network could have a high apparent rigidity in response to a discontinuous stress, but could deform to a large extent in response to a continuous stress. Moreover, such a structure would respond more easily to a molecular force, such as generated by myosin, and would more easily swell or contract in response to osmotic stresses.

It should be emphasized that the measurements reported here were performed using actin prepared by the method of Spudich and Watt (1971), without further purification by gel-filtration chromatography. The average length of the filaments in the actin preparation used in this report is in the range of 1.5–2.5 µm which is far shorter than the length of F-
actin that is derived from G-actin that has been further purified by gel-filtration chromatography (Zaner and Stossel, 1982, 1983). From the theory of rods in semi-dilute solution (Doi and Edwards, 1978), it would be predicted that an increase in filament length upon gel filtration would cause a large change in the rheologic properties of F-actin. Recently reported mechanical measurements of this type of actin preparation have demonstrated an extended elastic region with a plateau in $G'$ and $G''$ (Sato et al., 1985), which is consistent with the extreme lengths of the filaments. The mechanical properties of actin with these much longer filament lengths, alone and in the presence of ABP, are presently in progress.\footnote{J. H. Hartwig and K. Zaner, manuscript in preparation.}

Acknowledgments—I wish to thank Dr. Thomas P. Stossel for many valuable discussions and suggestions about this manuscript. In addition I would like to acknowledge the technical assistance of Susan Dalke.

REFERENCES

Boxer, L., and Stossel, T. (1976) J. Clin. Invest. 57, 964–976
Corwin, H., and Hartwig, J. (1983) Dev. Biol. 99, 61–74
Doi, M., and Edwards, S. (1978) J. Chem. Soc. Faraday Trans. II 74, 918–932
Ferry, J. (1980) Viscoelastic Properties of Polymers, 3rd Ed., John Wiley & Sons, New York
Hartwig, J., and Stossel, T. (1978) J. Biol. Chem. 250, 5696–5705
Hartwig, J., and Stossel, T. (1979) J. Mol. Biol. 134, 539–553
Hartwig, J., and Stossel, T. (1981) J. Mol. Biol. 145, 563–581
Hartwig, J., Tyler, J., and Stossel, T. (1980) J. Cell Biol. 87, 841–848
Jain, S., and Cohen, C. (1981) Macromolecules 14, 759–765
Jen, C., McIntire, L., and Bryan, J. (1982) Arch. Biochem. Biophys. 216, 126–132
Kasai, M., Kawashima, H., and Oosawa, F. (1969) J. Polymer Sci. Part D Macromol. Rev. 44, 51–69
Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951) J. Biol. Chem. 193, 265–275
Maruyama, K., Kaibara, M., and Fukada, E. (1974) Biochim. Biophys. Acta 371, 20–29
Niederman, R., Amrein, P., and Hartwig, J. (1983) J. Cell Biol. 96, 1400–1413
Poste, G., and Nicolason, G. (1981) Cytoskeletal Elements and Plasma Membrane Organization, North-Holland Publishing Co., Amsterdam
Rosenberg, S., Stracher, A., and Lucas, R. (1981) J. Cell Biol. 91, 210–211
Sato, M., Leimbach, G., Schwarz, W., and Pollard, T. (1985) J. Biol. Chem. 260, 8586–8592
Schmid-Schoenbein, G. W., Sung, K. P., Tozzen, H., Skalak, R., and Chien, S. (1981) Biophys. J. 36, 243–256
Spudich, J., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
Stossel, T. (1984) J. Cell Biol. 99, (suppl.) 15–21
Stossel, T., and Hartwig, J. (1976) J. Cell Biol. 68, 602–619
Stossel, T., Chapponier, C., Ezzell, R., Hartwig, J., Janney, P., Kwiatkowski, D., Lind, S., Smith, D., Southwick, F., Yin, H., and Zaner, K. (1985) Annu. Rev. Cell Biol. 1, 353–402
Treloar, L. (1975) The Physics of Rubber Elasticity, 3rd Ed., Clarendon Press, Oxford
Yin, H., Zaner, K., and Stossel, T. (1980) J. Biol. Chem. 255, 9494–9500
Zaner, K., and Stossel, T. (1982) J. Cell Biol. 93, 987–991
Zaner, K., and Stossel, T. (1983) J. Biol. Chem. 258, 11004–11009
Zaner, K., Fotland, R., and Stossel, T. (1981) Rev. Sci. Instrum. 52, 85–87