Investigation of the Actin–Deoxyribonuclease I Interaction Using a Pyrene-Conjugated Actin Derivative†

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ABSTRACT: The interaction of deoxyribonuclease I with muscle actin was studied with the aid of a pyrenyl derivative of the actin [Kouyama, T., & Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33–38] that increases its quantum yield by an order of magnitude on polymerization. It is shown that this derivative copolymerizes with unlabeled G-actin in a random manner and will also bind to deoxyribonuclease with inhibition of enzymic activity. The derivative affords a highly sensitive means of following nucleated polymerization. Preincubation of F-actin with deoxyribonuclease at a concentration of 5% or less of that of total subunits causes inhibition of polymerization of additional G-actin onto the filaments. In red cell membranes that contain stabilized short filaments of actin such that the concentration of filament ends is large relative to monomers, complete inhibition of nucleated polymerization of G-actin is achieved by preincubation with deoxyribonuclease. The results indicate that binding of DNase occurs at the "plus" ends of the actin filaments. Competition with cytochalasin E, which is known to have a high affinity for the plus or preferentially growing ends of F-actin, can be observed. Whereas the activity of deoxyribonuclease in the 1:1 complex with G-actin is inhibited, the enzyme attached to the ends of filaments appears to be fully active. This causes a reduction in the inhibition of enzymic activity with increasing F-actin concentration, presumably by reason of a change in the partition of the enzyme between monomers and filament ends. The degree of inhibition increases with time, however, as the actin depolymerizes. Implications for measurements of actin monomer concentrations by the deoxyribonuclease assay procedure are considered.

The formation of a 1:1 complex between G-actin and pancreatic deoxyribonuclease I (DNase) with inhibition of enzymic activity (Lazarides & Lindberg, 1974; Mannherz et al., 1975; Hitchcock et al., 1976) is the basis of a sensitive, specific, and widely used assay for G-actin (Blikstad et al., 1978). The dissociation of F-actin appears in most circumstances to be sufficiently slow to permit the estimation of G-actin in the presence of polymer. After depolymerization with dilute guanidine hydrochloride, all the subunits of F-actin become available for assay (Blikstad et al., 1978). Mannherz et al. (1980) have determined a value of 5×10^8 M^{-1} for the association constant of the 1:1 complex at low ionic strength; under the same conditions a very weak interaction with F-actin, characterized by an apparent binding constant of 1.2×10^4 M^{-1} and of undetermined stoichiometry, was reported. Evidence for binding of DNase to F-actin was also adduced by Hitchcock (1980). Because of the strong cooperativity of the actin self-association process (Oosawa & Kasai, 1971; Wegner & Engel, 1975), which results in a high degree of polymerization at concentrations above critical, strong binding specifically to filament ends would have escaped detection and can indeed most often be disregarded. However, when the DNase assay is used, as it has been (MacLean-Fletcher & Pollard, 1980a), to determine the critical concentration or analyze the state of actin in a system such as the red cell cytoskeleton (Pinder et al., 1981) in which short actin filaments ["protofilaments" in the terminology of Brenner & Korn (1980)] can be inferred to prevail (Brenner & Korn, 1980; Ungewickell et al., 1979; Lin & Lin, 1979), such an interaction could become significant.

We show here, with the aid of a pyrenyl derivative of actin (Kouyama & Mihashi, 1981), the quantum yield of which increases by more than an order of magnitude on polymerization, that DNase binds with high affinity to the ends of filaments and blocks further addition of subunits. It competes with cytochalasin E and may thus be inferred to associate with the "plus", or preferentially growing, end of the F-actin. The activity of the DNase thus attached appears to be undiminished, in contrast with the strong inhibition that characterizes the complex with G-actin. We analyze the implication of these results for DNase assays when applied to systems containing short F-actin filaments and to determination of critical concentration.

Experimental Procedures

Chicken breast muscle actin was prepared by the method of Spudich & Watt (1971) and further purified by column chromatography on Sephadex G-200 (MacLean-Fletcher & Pollard, 1980b). Bovine pancreatic DNase I (Sigma) was purified by chromatography on hydroxylapatite as described by Price et al. (1969), except for the inclusion of 1 mM calcium chloride and 0.15 M sodium chloride in the buffer. Concentrations of G-actin were determined spectrophotometrically, taking $E^{1\%}_{1cm} = 6.2$ at 290 nm (Gordon et al., 1976), and DNase by colorimetric micro-Kjeldahl nitrogen analysis (Jaenicke, 1974). For derivatization, F-actin at 1 mg/mL was treated at a mole ratio of unity with *N*-(1-pyrenyl)iodoacetamide (Molecular Probes, Inc.), exactly as described by Kouyama & Mihashi (1981). After treatment with excess β-mercaptoethanol, the protein was dialyzed against a depolymerizing buffer of 2 mM tris(hydroxymethyl)aminomethane (Tris), 0.2 mM calcium chloride, 0.2 mM ATP, and 0.2 mM dithiothreitol, pH 8.0. In most preparations the actin was passed through another polymerization–depolymerization cycle. The G-actin was then further purified by passage through a Sephadex G-200 column (50 × 2.5 cm) and eluted with the depolymerization buffer. The concentration of pyrene in the modified actin was determined spectrophotometrically (Kouyama & Mihashi, 1981) and that of protein by the method of Bradford (1976), calibrated with native actin.

Assays for DNase activity were performed by the spectrophotometric (Kunitz) procedure, with high-molecular-weight calf thymus (type 1) DNA (Sigma) as the substrate, under

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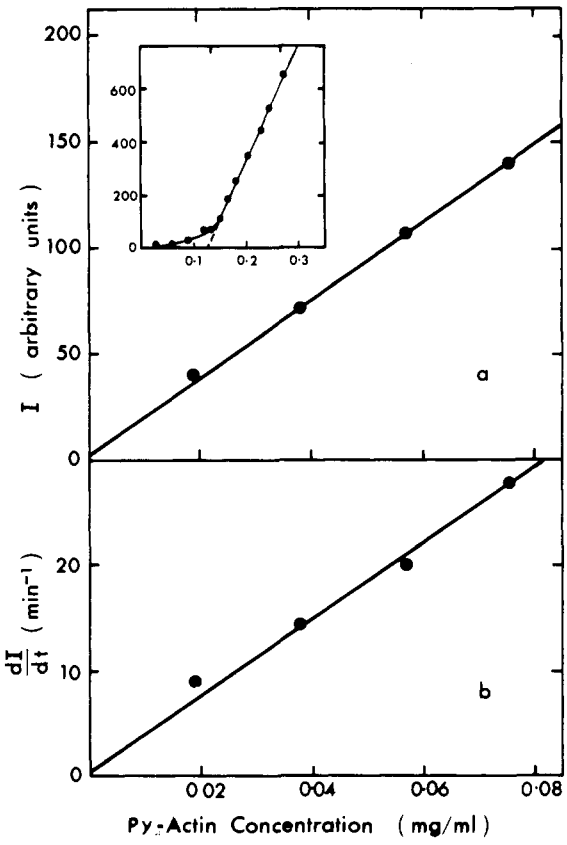

FIGURE 1: Copolymerization of pyrene-labeled and unlabeled actin at constant total actin concentration (0.5 mg/mL) and varying proportion of labeled actin. Monomers were mixed in a depolymerizing medium (see text), and polymerization was initiated by addition of sodium chloride to 0.1 M and magnesium chloride to 1 mM. (a) Final fluorescent intensity (408 nm, excited at 366 nm) after completion of polymerization. (b) Rate of linear change of fluorescence during polymerization. (Inset) Determination of critical monomer concentration of pyrene-labeled actin. Fluorescent intensity after polymerization is shown as a function of total actin concentration. Solvent: 12 mM Tris, 0.1 M sodium chloride, 1 mM magnesium chloride, 0.2 mM calcium chloride, 5 mM ATP, 0.2 mM dithiothreitol, and 3 mM sodium azide, pH 7.5. Temperature 4 °C.

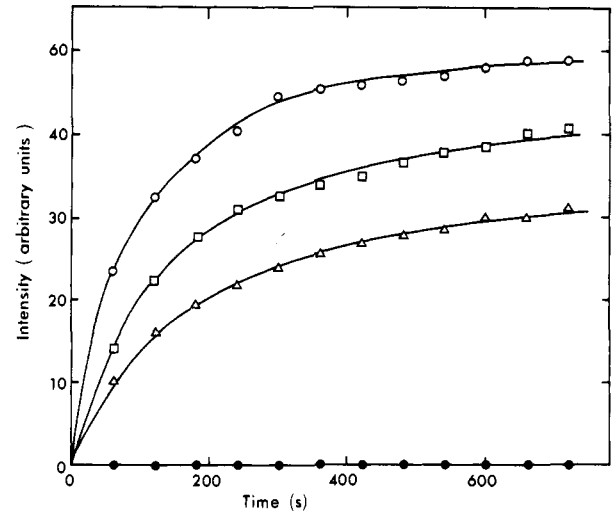

FIGURE 2: Polymerization of pyrene-labeled G-actin (52 μg/mL) nucleated by unlabeled F-actin (0.11 mg/mL), measured by pyrene fluorescence enhancement, in the absence of DNase (○) and after preincubation of the F-actin for 5 min at 30 °C with 190 ng/mL DNase (□) and 2.3 μg/mL DNase (△). The protein concentrations are final values resulting from the addition of 0.3 mL of labeled G-actin to 2.0 mL of solution containing the F-actin and DNase. The DNase alone at 52 μg/mL shows no fluorescence increase (●). The same concentration of DNase mixed with the labeled G-actin before addition to the F-actin led to a fluorescence profile almost indistinguishable from the upper curve.

the conditions described by Blikstad et al. (1978). The polymerization of the labeled actin was followed by fluorometry, using a Perkin-Elmer Hitachi MPF 3L instrument. Excitation and emission wavelengths were 366 and 408 nm, respectively, and both slits were set at 10 nm. The cell housing was thermostated at 30 ± 0.1 °C.

Ghosts were prepared from the washed red cells of human blood, less than 1 week old, by lysis with saponin in isotonic phosphate-buffered saline at 25 mg/mL. This process was designed to ensure that no resealing occurred when the ionic strength was raised. Membranes were incubated with DNase for 5 min to give equilibrium binding. Spectrin extracts containing oligomers with actin and protein 4.1, which are able to induce nucleation of G-actin polymerization, were prepared as described elsewhere (Pinder et al., 1979).

Results

As Kouyama & Mihashi (1981) have reported, the quantum yield of the pyrenyl G-actin undergoes a large increase on polymerization. This is accompanied by a change in the vibronic structure of both the emission and absorption spectra. The change in the shape of the emission and excitation envelopes therefore allows one to discriminate between polymerization and other effects such as elevated Rayleigh scattering, even at extremely low levels of polymerization. Because of the high quantum yield, all observations could be performed at very low absorbance levels at the excitation wavelength, so as to avoid inner-filter effects. Polymerization experiments with different proportions of labeled actin were performed in order to establish that the derivatized actin monomers are neither preferentially excluded from nor incorporated into copolymers with unlabeled G-actin. The results (Figure 1) show that both the rate and extent of polymerization of the labeled monomers were unchanged within the limits of error with increasing proportions of untreated, native G-actin. The critical monomer concentration, determined from measurements of fluorescent intensity, does not differ significantly from that of unlabeled actin at the same temperature (4 °C in the experiment depicted in Figure 1, inset).

By addition of the fluorescent monomer to unlabeled F-actin at equilibrium, rapid nucleated polymerization could be observed (Figure 2). The monomer at the total concentration of actin used in these experiments does not polymerize measurably on this time scale in the absence of preformed nuclei. One may also add the fluorescent monomer to unlabeled F-actin at a final total monomer concentration equal to or just below the critical value. An increase in fluorescence ensues, which must reflect exchange of subunits between F-actin and the monomer pool. As shown in Figure 2, the rate of nucleated polymerization of fluorescent G-actin at 50 μg/mL could be reduced by some 40% by prior incubation of the initiating F-actin (130 μg/mL) with DNase (2.5 μg/mL). This suggests that the DNase acts by blocking filament ends, for if it were merely binding to monomer, less than 5% of the latter would be rendered unavailable and the rest should and indeed does polymerize normally.

Stronger evidence for this conclusion was obtained by recourse to a system in which the concentration of filament ends is high, rather than, as in F-actin, some orders of magnitude below that of the monomer in equilibrium with the filaments. Such a system is the red cell cytoskeleton, which contains numerous protofilaments of actin that bind cytochalasins (Lin & Lin, 1979; Brenner & Korn, 1980), nucleate G-actin po-

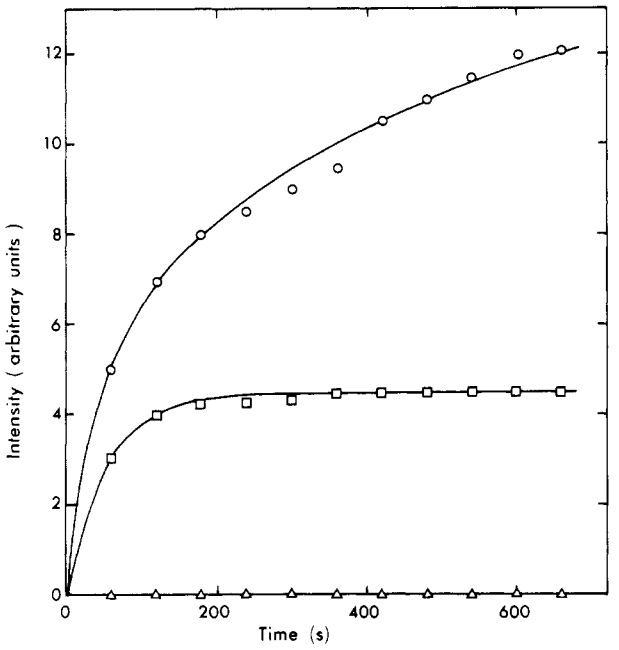

FIGURE 3: Polymerization of pyrene-labeled G-actin, nucleated by red cell ghosts that had been preincubated with or without DNase for 5 min at 30 °C. Polymerization was followed by increase in fluorescent intensity. The assay mixture contained 10 μL/mL pelleted ghosts and 52 μg/mL added fluorescent actin. The rate curves refer to no DNase (○), 190 ng/mL DNase (□), and 2.3 μg/mL DNase (△).

lymerization with great efficiency (Cohen & Branton, 1979), and are stabilized by their interactions with other proteins to the extent that a sizable proportion of the subunits is resistant to dissociation with 0.75 M guanidine hydrochloride (Pinder et al., 1981). Results are shown in Figure 3. The molar concentration of DNase with which the ghosts were incubated was small compared to that of the added G-actin. Polymerization was, however, strongly inhibited, indicating that the membrane-associated nuclei were blocked. This confirms that the DNase binds to filament ends and in particular to the plus ends, which, as the properties already mentioned demonstrate, are free in the membranes. Furthermore, the enzymic activity of the DNase was undiminished after incubation with red cell membranes that had been washed free (Pinder et al., 1981) of all dissociable actin monomers. Similar inhibition of nucleated polymerization of fluorescent G-actin was exerted by DNase (Figure 4) when the nucleating species was a spectrin oligomer fraction liberated from the red cell membrane and containing actin and protein 4.1; these oligomers are presumed to represent junction points of the cytoskeleton (Pinder et al., 1979; Lin & Lin, 1979; Brenner & Korn, 1980). The oligomers again did not measurably inhibit the DNase activity. It thus seems inescapable to conclude that whereas in the 1:1 complex with G-actin the activity of DNase is strongly inhibited, binding to the ends of filaments causes no measurable inhibition.

The dependence of inhibition of DNase activity by G-actin on the concentration of the latter under conditions in which no polymerization occurs is shown in Figure 5. If we take the fractional inhibition as a measure of the proportion of DNase complexed, the curve can be satisfactorily fitted in terms of 1:1 association with a binding constant of 1×10^9 M^{-1}, in good agreement with Mannherz et al. (1980).

From the level of inhibition of nucleation by DNase seen in Figure 3 (>95%), it would appear that the binding constant for DNase to filament ends must be greater than 10^8 M^{-1}. It is evidently much larger in fact (Figure 2) than that for binding

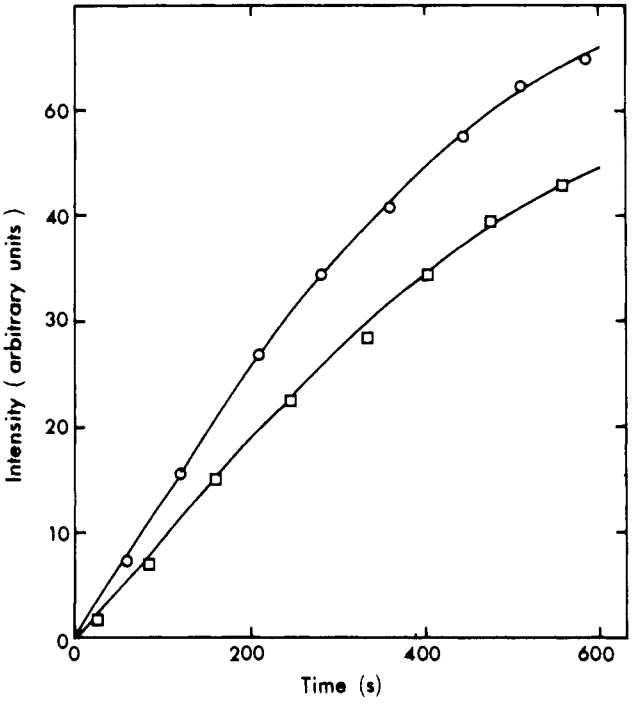

FIGURE 4: Polymerization of pyrene-labeled G-actin, nucleated by column-purified oligomer fraction (containing spectrin, actin, and protein 4.1) extracted from red cell membranes. Oligomers at 15 μg/mL total protein concentration were preincubated without (○) or with DNase at 9.27 μg/mL (□), and labeled G-actin was added at 0.13 mg/mL. The G-actin alone under the same conditions showed no fluorescence increase over the duration of these experiments.

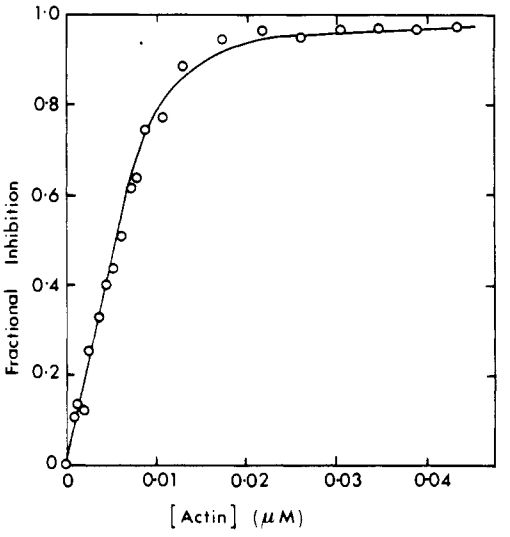

FIGURE 5: Inhibition of enzymic activity of DNase by monomeric actin under the conditions of Blikstad et al. (1978). The DNase concentration was 8.6 nM. The curve is calculated for a 1:1 association with a binding constant of 1.1×10^9 M^{-1}.

to G-actin, which should be present in equilibrium with F-actin in large molar excess over filament ends. One might therefore expect that with increasing actin concentration the number of filament ends will eventually become sufficient to cause a significant change in the partition of bound DNase between filament ends and monomers, with consequent relief of inhibition: Figure 6 shows what seems to be an effect of this nature. If the actin is first equilibrated with cytochalasin E, the maximum in the inhibition profile vanishes. Thus at low actin concentrations the cytochalasin causes relief of the inhibition of DNase activity. One conjectural explanation suggests itself in terms of the expected and observed propensity

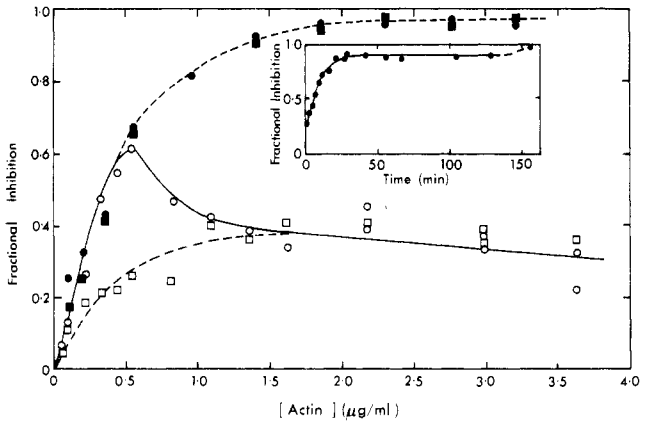

FIGURE 6: Inhibition of enzymic activity of DNase by actin. The curves represent the fractional inhibition of the activity of DNase at a final concentration of 0.27 μg/mL added immediately before assay to actin equilibrated at 4 °C in a polymerizing medium (open symbols) in the absence (○) and presence (□) of 0.1 μM cytochalasin E and of the same concentration of DNase incubated (4 °C) with actin until equilibrium was reached (filled symbols) with (●) and without (■) cytochalasin E. Buffers as in Figure 1 (inset). (Inset) Time course of increase of DNase inhibition after addition of the enzyme (0.27 μg/mL) to preequilibrated actin at 1 μg/mL.

of cytochalasins to promotion of formation of oligomeric seeds from G-actin (Howard & Lin, 1979). DNase added to this system at subcritical actin concentrations, at which no or few oligomers would exist in the absence of cytochalasin, would then suffer a reduced inhibition of activity, either by reason of a dearth of actin monomers or because it can compete successfully with the cytochalasin for the oligomeric nuclei.

When F-actin is exposed to DNase for a longer period, depolymerization occurs (Hitchcock, 1980; Mannherz et al., 1980); under our conditions equilibrium was largely attained in less than 1 h (Figure 6, inset), as judged by the inhibition of enzymic activity. The DNase was presumably then substantially associated with monomers, and as expected its activity in this system was unperturbed by the cytochalasin. The increase in inhibition is accompanied by recovery of the nucleating capacity of the actin, so that the original rate of nucleated polymerization, as characterized by the upper curve of Figure 2, is completely regained.

Discussion

The use of the pyrene derivative of actin for the study of polymerization-linked processes offers several advantages. The large change in fluorescent intensity on polymerization would appear to make it uniquely advantageous for the observation of subunit exchange between F-actin and its monomer pool. For the determination of critical concentrations, spectroscopic methods are likely to be free from the drawbacks associated with sedimentation, viscosity, or light scattering, all of which may be vitiated by the presence of oligomeric material.

The fluorescent derivative is also especially useful for the study of actin polymerization in an inhomogeneous system, such as a membrane suspension. Inhibition of nucleated polymerization by treatment of the membrane or cytoskeletons with cytochalasin E or with DNase could be readily observed. The results allow us to conclude that DNase binds with high affinity to the ends of filaments, as well as to G-actin, and the DNase assay cannot therefore be easily used for the measurement of critical concentration. This factor must also be taken into account in the interpretation of analyses for actin monomer in systems in which short filaments of actin are abundant, and the molar ratio of filaments to monomers cannot be taken as negligible [e.g., as in red cells or intestinal microvilli, which may contain actin nuclei stabilized by villin (Bretscher & Weber, 1980)]. With regard to the interaction of DNase with oligomers derived from the red cell cytoskeleton (Figure 4), we observe binding but no dissociation of the oligomers of the kind reported by Lin (1981), unless DNase preparations that have not been subjected to column purification and therefore contain proteolytic activity are used; neither, by contrast with Lin (1981), do we observe total disruption of isolated cytoskeletons, prepared by extraction with nonionic detergent (unpublished data). It may also be remarked that assay of oligomers by cytochalasin binding would be expected to give erroneous results in the presence of DNase.

We are at this stage unable to provide a satisfactory quantitative description of the actin–DNase interaction. Intuitively a diminution of inhibition of enzymic activity with an increase in actin concentration, and therefore in the number of filament ends, might be expected and appears at first sight to occur (Figure 6). However, the maximum in the inhibition appears only when the assay is performed immediately on addition of the DNase to preequilibrated actin and is thus presumably governed by the state of the unperturbed actin, DNase-induced depolymerization being evidently slow (Blikstad et al., 1978). The number of filament ends can be readily calculated, as follows: If c_1 represents the actin monomer concentration, K the association constant of a monomer with a filament end, and σK the association constant of two monomers to form a dimeric polymerization nucleus, the concentration of dimers is given by $c_2 = \sigma K c_1^2$, that of trimers by $c_3 = \sigma K^2 c_1^3$, and so on. (The formalism is unchanged if the nucleus for polymerization is a trimer or tetramer, rather than a dimer.) The constant σ measures the degree of cooperativity (Oosawa & Kasai, 1971) and $\sigma \ll 1$. Then the molar concentration of filaments of all lengths is

$$c_n = \sum_{i=2}^{\infty} c_i = \sigma K c_1^2 + \sigma K^2 c_1^3 + \ldots + \sigma K^{i-1} c_1^i + \ldots = \sigma \sum_{i=2}^{\infty} K^{i-1} c_1^i$$

When summed to infinity, this gives

$$c_n = \frac{\sigma K c_1^2}{1 - K c_1} \text{ for } K c_1 < 1 \qquad (1)$$

The total concentration of actin, $\bar{c}$, expressed as the molarity of subunits, is $\bar{c} = c_1 + 2c_2 + \ldots + ic_i = c_1 + \sigma \sum_{i=2}^{\infty} i K^{i-1} c_i$ which when summed gives

$$\bar{c} = c_1 + \sigma K c_1^2 \left[\frac{1}{1 - K c_1} + \frac{1}{(1 - K c_1)^2} \right] \qquad (2)$$

K is the reciprocal of the critical monomer concentration, say 0.5 μM, and σ has been estimated (under conditions of rather low ionic strength) as 2×10^{-7} (Wegner & Engel, 1975). From eq 1 and 2 we may calculate that the molar ratio of filament ends to monomers in the range of our experiments (Figure 5) does not exceed about 0.005, so that the proportion of DNase bound to filament ends should be very small in this system. At equilibrium, on the other hand, for which data are also shown in Figure 5, the following additional constraints hold:

$$K_d = \frac{c_{1d}}{c_1(\bar{D} - c_{1d} - c_{nd})} \qquad (3)$$

$$K_n = \frac{c_{nd}}{c_1(\bar{D} - c_{1d} - c_{nd})} \quad (4)$$

where K_d and K_n are the association constants for binding of DNase to monomers and filament ends, respectively, c_{1d} and c_{nd} are the concentrations of the corresponding complexes, and $\bar{D}$ is the total concentration of DNase, bound and free. Eliminating c_{1d} and c_{nd} from eq 1–4 and simplifying, we obtain

$$\bar{c} = c_1 + \frac{K_d\bar{D}c_1(1 - Kc_1)}{1 + c_1(K_d - K) - c_1^2K(K_d - \sigma K_n)} + \left[1 + \frac{K_n\bar{D}(1 - Kc_1)}{1 + c_1(K_d - K) - c_1^2K(K_d - \sigma K_n)}\right]\left[\frac{\sigma Kc_1^2}{1 - Kc_1} + \frac{\sigma Kc_1^2}{(1 - Kc_1)^2}\right]$$

in which the second member on the right is c_{1d}; this is proportional to the inhibition of activity. Knowing K, σ, and K_d (Figure 5), we can calculate profiles relating inhibition with actin concentration. It transpires that K_n must be greater than about 10^{11} M^{-1} for an appreciable diminution of inhibition to develop with increasing actin concentration over the range covered. It is therefore not surprising that no such effect can be observed at equilibrium, and in fact this would seem to place an upper limit on K_n. We are left, however, without a quantitative explanation for the maximum in the other curve (measured immediately after addition of DNase to F-actin). Possible explanations might be sought in terms of (a) inadequacy in the equilibrium calculations, due perhaps to scission of filaments, either spontaneously in actin (Wegner, 1982) or under the influence of DNase (cf. Hitchcock, 1980), (b) a grossly incorrect choice of σ for the given solvent conditions, or (c) sequestration of a significant proportion of the DNase by binding along the F-actin filaments (Mannherz et al., 1980; Hitchcock, 1980).

As an actin-binding protein, DNase does not obviously fall into any of the hitherto recognized classes [for a recent review, see Schliwa (1981)]. These include species that bind to the ends of filaments, others that cross-link filaments, in some cases by a calcium-dependent interaction, and such proteins as profilin (Carlsson et al., 1977) that sequester actin monomer. DNase appears to be the only protein now known that binds both to the monomer and to filament ends with high affinity. In the latter type of complex its enzymic activity appears to be, rather surprisingly, totally preserved.

Acknowledgments

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