Effect of Self-Association on the Structural Organization of Partially Folded Proteins: Inactivated Actin

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ABSTRACT The propensity to associate or aggregate is one of the characteristic properties of many nonnative proteins. The aggregation of proteins is responsible for a number of human diseases and is a significant problem in biotechnology. Despite this, little is currently known about the effect of self-association on the structural properties and conformational stability of partially folded protein molecules. G-actin is shown to form equilibrium unfolding intermediate in the vicinity of 1.5 M guanidinium chloride (GdmCl). Refolding from the GdmCl unfolded state is terminated at the stage of formation of the same intermediate state. An analogous form, known as inactivated actin, can be obtained by heat treatment, or at moderate urea concentration, or by the release of Ca^{2+}. In all cases actin forms specific associates comprising partially folded protein molecules. The structural properties and conformational stability of inactivated actin were studied over a wide range of protein concentrations, and it was established that the process of self-association is rather specific. We have also shown that inactivated actin, being denatured, is characterized by a relatively rigid microenvironment of aromatic residues and exhibits a considerable limitation in the internal mobility of tryptophans. This means that specific self-association can play an important structure-forming role for the partially folded protein molecules.

INTRODUCTION

It is known that protein association represents an essential problem in biomedicine and biotechnology. In particular, the association and/or aggregation of specific proteins results in the development of such diseases as Down's syndrome, Alzheimer's disease (Massry and Glassock, 1983), cataracts (Thompson et al., 1987; Clark and Steele, 1992) and many others, including the so-called transmissible encephalopathies. The formation of inclusion bodies (specific *in vivo* protein aggregates) is a major problem in the overexpression of recombinant proteins (Marston, 1986; Schein, 1989; Wetzel, 1992a,b), while the production and *in vivo* delivery of protein drugs is often complicated by association (Lougheed et al., 1980). It is also known that protein refolding is often accompanied by transient association of partially folded intermediates (Tanford, 1968; London et al., 1974; Clark et al., 1981; Mitraki and King, 1989; De Young et al., 1993a,b; Oberg et al., 1994; Fink, 1995a,b; Semisotnov et al., 1996). Moreover, the propensity for association or aggregation is believed to be a general characteristic of the nonnative protein molecule (Tanford, 1968; London et al., 1974; Clark et al., 1981; Mitraki and King, 1989; De Young et al., 1993a,b; Eliezer et al., 1993; Oberg et al., 1994; Fink, 1995a,b; Ptitsyn, 1995; Uversky, 1998; Uversky and Fink, 1998a,b; Uversky et al., 1998b, 1999). Despite the crucial importance of protein aggregation, there are only a few studies that consider the details of the process and the effect of association on the structural properties and conformational stability of proteins. It has been established that, under some conditions, self-association can represent an additional structure-forming factor resulting in the appearance of new "structural levels" in a partially folded protein (Uversky, 1998; Uversky and Fink, 1998a,b; Uversky et al., 1998b, 1999). We show here that inactivated actin represents another interesting system for the investigation of the problem of self-association of partially folded protein molecules.

Actin, one of the major proteins of muscle tissue, is a globular protein with a molecular mass of 42 kDa that is known to bind one divalent cation and one molecule of ATP (or ADP). At low ionic strength actin exists as a monomer (G-actin), but in the presence of neutral salts it is polymerized into a double-stranded polymer (the so-called fibrous form of actin, or F-actin). F-actin forms the backbone of thin filaments in muscle fibers. It is known that the release of cation by EDTA or EGTA treatment leads to the *irreversible* transformation of G-actin into the inactivated form, in which the protein molecule loses its capability to polymerize (Lehrer and Kerwar, 1972; Strzelecka-Golaszewska et al., 1985; Levitsky et al., 1995). Inactivated actin can also be obtained in several other ways: 1) as the result of heat denaturation (Nagy and Jencks, 1962; West et al., 1967; Lehrer and Kerwar, 1972; Nagy and Strzelecka-Golaszewska, 1972; Strzelecka-Golaszewska et al., 1974, 1985; Contaxis et al., 1977; Levitsky et al., 1995; Kuznetsova et al., 1988; Bertazzon et al., 1990); 2) at moderate urea (Kuznetsova et al., 1988) or GdmCl concentration (see

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below); 3) by dialysis from 8 M urea or 6 M GdmCl (Kuznetsova et al., 1988; Bertazzon et al., 1990); and 4) spontaneously during storage (Kuznetsova et al., 1988). In all of these cases it was shown that the denaturation of G-actin is an irreversible process accompanied by changes in the secondary structure of the protein. The irreversibility of G-actin denaturation and unfolding was assumed to be due to the association of inactivated protein molecules (see, e.g., Kuznetsova et al., 1988; Bertazzon et al., 1990). To understand the structural cost of self-association we have focused our attention on the characterization of the structural properties of inactivated actin.

MATERIALS AND METHODS

Sample handling

Rabbit skeletal muscle G-actin was extracted as described previously (Spundich and Watt, 1971). The protein was purified by one or two cycles of polymerization-depolymerization, using 30 mM KCl to initiate polymerization to F-actin. G-actin in buffer G (0.2 mM ATP, 0.1 mM $CaCl_2$, 0.4 mM β-mercaptoethanol, 5 mM Tris-HCl, pH 8.2, 1 mM NaN_3) was stored on ice and used within a week of preparation. The A parameter introduced by Turoverov et al. (1976) was used to determine the quality of actin. The A parameter was defined as $A = (I_{320}/I_{365})_{297}$, where I_{320} and I_{365} are fluorescence intensities at $\lambda_{em} = 320$ and 365 nm. Only samples of actin with $A > 2.56$ (corresponds to the content of inactivated actin not higher than 2%; Turoverov et al., 1976) were used. Inactivated actin was prepared by heating G-actin to 60°C and incubation a this temperature for 30 min, by dialysis from 6 M GdmCl, by incubation with 4 M urea or with 1.5 M GdmCl; or by incubation with 5 mM EDTA. The samples of inactivated actin had $A = 1.30 \pm 0.05$. The actin concentration was determined on a Cecil 2000 spectrophotometer, using a molar extinction of $E_{280}^{1cm} = 1.09$ (Rees and Young, 1967). The protein concentration was varied from 0.0005 to 1.5 mg/ml. Variations of the actin concentration within this range did not affect the experimental data.

Acrylamide (Wako, Osaka, Japan), P-terphenyl (Sigma, St. Louis, MO), *N*-acetyl-tryptophanamide (Serva, Heidelberg, Germany), and ANS, 8-anilino-1-naphthalene sulfonate (Serva) were used without additional purification.

Circular dichroism studies

Circular dichroism spectra were recorded with a Jasco-600 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) equipped with a temperature-controlled cell holder. The protein concentration for both near and far UV CD measurements was 1.0 mg/ml. The cell path length was 0.148 and 10 mm for far and near UV CD measurements, respectively.

Fluorescence measurements

Fluorescence experiments were performed with spectrofluorimeters with steady state and pulsed excitation (Turoverov et al., 1998), as well as with a SPF-1000^{CS} Aminco spectrofluorimeter (American Instrument Co., Silver Spring, MD). Fluorescence was excited at the long-wave absorption edge (297 nm), where the contribution of tyrosine residues can be ignored.

The position and form of the fluorescence spectra were characterized by the parameter $A = (I_{320}/I_{365})_{297}$, where I_{320} and I_{365} are fluorescence intensities at $\lambda_{em} = 320$ and 365 nm, respectively, and $\lambda_{exc} = 297$ nm. The values of parameter A and of the fluorescence spectrum intensity were corrected by instrument sensitivity. ANS fluorescence was excited at 390 nm.

Gel filtration

Gel filtration measurements were carried out on a Superose-6 column, using a Pharmacia fast protein liquid chromatography apparatus. To determine the molecular mass of inactivated actin, the gel filtration column was calibrated by standard procedures (Ackers, 1967, 1970). Proteins from a standard molecular mass (M) marker set were passed through the column, and the retention coefficient (K_d) for each individual protein was determined:

$$K_d = (V - V_O)/(V_t - V_O), \quad (1)$$

where V_O is the column void volume determined as the elution volume of blue dextran, V_t is the total solvent-accessible column volume determined as the elution volume of acetone, and V is the elution volume of the given protein. Least-squares analysis was used to fit the data to generate the calibration curve:

$$K_d = (1.73 \pm 0.03) - (0.26 \pm 0.007)\log(MW). \quad (2)$$

Hydrodynamic dimensions (Stokes radius, R_S) of actin in different conformational states were also measured by size exclusion chromatography. In this case a specific calibration curve, $1000/V$ versus R_S dependence, was used (Ackers, 1967; Corbett and Roche, 1984; Uversky, 1993, 1994). A set of globular proteins with known R_S was used for column calibration (Uversky, 1993).

Analysis of fluorescence decay

The fitting routine was based on the nonlinear least-squares method. Minimization was performed according to the method of Marquardt (1963). P-Terphenyl in ethanol and *N*-acetyl-tryptophanamide in water were used as reference compounds (Zuker et al., 1985). The decay curves were analyzed by a multiexponential approach:

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i), \quad (3)$$

where α_i and τ_i are the amplitude and the lifetime of component i, $\Sigma\alpha_i = 1$.

S_i, the contribution of component i to the total emission, was calculated as

$$S_i = \frac{\alpha_i \int_0^\infty \exp(-t/\tau_i)dt}{\Sigma\, \alpha_i \int_0^\infty (-t/\tau_i)dt} = \frac{\alpha_i\tau_i}{\Sigma\, \alpha_i\tau_i}. \quad (4)$$

The root mean square value of fluorescent lifetimes, $\langle\tau\rangle$, for biexponential decay was determined as

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} = \sum f_i\tau_i. \quad (5)$$

Fluorescence quenching by acrylamide

The fluorescence quenching constants were evaluated by Stern-Volmer equations for time-resolved measurements (Eftink and Ghiron, 1981):

$$\bar{\tau}_0/\bar{\tau} = 1 + \langle K\rangle[Q]. \quad (6)$$

Here $\bar{\tau}$ and $\bar{\tau}_0$ are weighted mean lifetimes ($\bar{\tau} = \alpha_i\tau_i$) determined in the absence and in the presence of quencher at molar concentration $[Q]$, and $\langle K\rangle$ is the collisional quenching constant. The collisional constant is $k_q\langle\tau_0\rangle$, where k_q is the bimolecular rate constant for the quenching process and $\langle\tau_0\rangle$ is the root mean square value of fluorescence lifetime in the absence of quencher (Eftink and Ghiron, 1981). The acrylamide concentration was varied from 0.0 to 0.8 M.

RESULTS

GdmCl-induced unfolding of G-actin: evidence for intermediate state accumulation

Fig. 1 *A* illustrates GdmCl-induced changes in the near-UV CD spectrum of actin. It can be seen that the $[\theta]_{272.5}$ versus [GdmCl] dependence represents two well-separated cooperative transitions, reflecting the fact that the GdmCl unfolding of G-actin is a two-step process. This conclusion is confirmed by the GdmCl-determined changes in the far-UV CD spectrum (Fig. 1 *B*) and the intrinsic fluorescence parameters of the protein, such as the fluorescence intensity at 320 nm, the *A* parameter ($(I_{320}/I_{365})_{297}$, which reflects the fluorescence spectrum position), and the degree of fluorescence anisotropy, *r* (Fig. 1 *C*). It can be seen that changes in all of the parameters studied can be described by two noncoinciding transition curves. It is obvious that if there are two cooperative transitions, the existence of at least three different conformational states separated by these transitions can be expected. This is consistent with the assumption of the accumulation of an intermediate state during GdmCl-induced unfolding of actin. Such an intermediate state, being stable between 1 and 2 M GdmCl (cf. Bertazzon et al., 1990), is characterized by the relatively well-developed secondary and tertiary structures and by the unusual combination of a *red shift* of the fluorescence spectrum with a considerable *increase* in the fluorescence anisotropy.

Finally, it is necessary to emphasize that the second GdmCl-induced transition was completely reversible, whereas the first one was irreversible (see *black symbols* in Fig. 1). In other words, the process of equilibrium actin refolding from the GdmCl-unfolded conformation is terminated at the stage of formation of an intermediate state analogous to that described above. The same behavior had been observed for urea-induced unfolding or heat denaturation of G-actin (Kuznetsova et al., 1988; Turoverov et al., 1999). The shape of GdmCl unfolding-refolding curves was not affected by the addition of 50 mM dithiothreitol (DTT) to the protein solution (data will be published elsewhere), suggesting that inappropriate disulfide bridges are not responsible for the irreversibility of actin unfolding.

Inactivated actin as a specific associate

It has been assumed that the formation of soluble aggregates (or associates) was the reason for the irreversibility of G-actin denaturation (Strzelecka-Golaszewska et al., 1985; Kuznetsova et al., 1988). This assumption was confirmed by sedimentation measurements, which showed that the heat-inactivated actin produced homogeneous stable associates with a sedimentation constant of ~20 S (Kuznetsova et al., 1988). Additional indirect evidence of self-association was derived from the analysis of far-UV CD spectra for inactivated actin (see below, Nagy and Strzelecka-Golaszewska, 1972; Strzelecka-Golaszewska et al., 1985; Kuznetsova et al., 1988; Bertazzon et al., 1990).

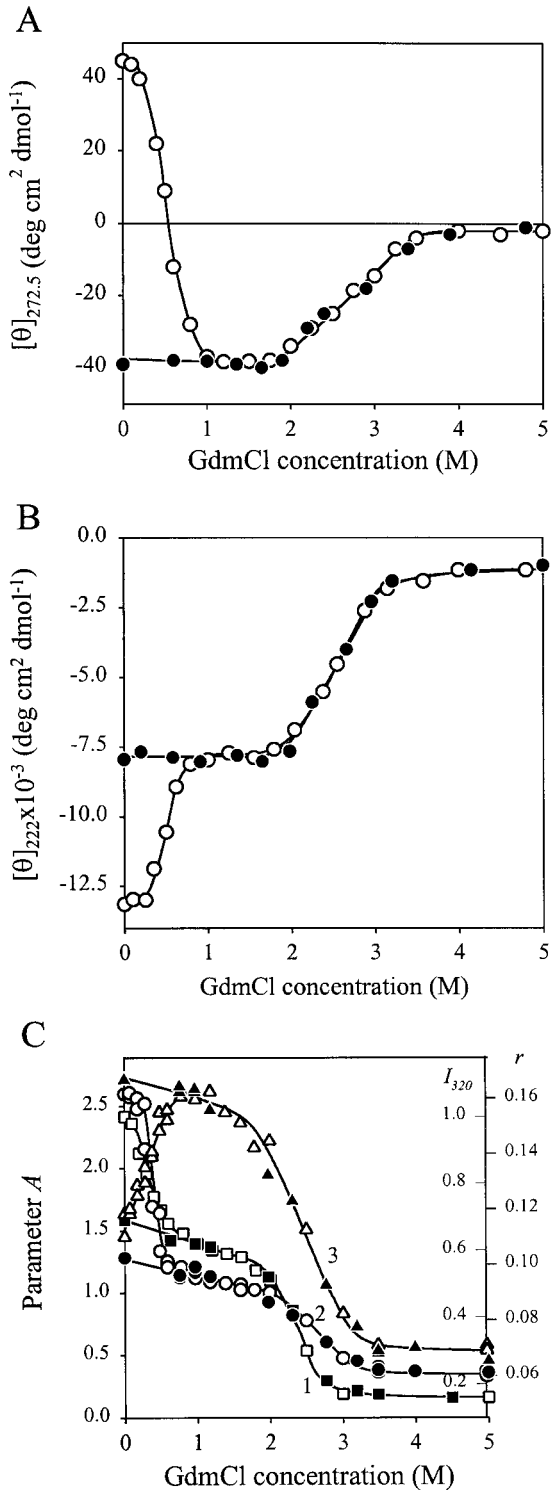

FIGURE 1 GdmCl-induced unfolding of G-actin. (*A*) Effect of increased GdmCl concentration on the near-UV CD spectra of the protein. (*B*) GdmCl-induced changes in the far-UV CD spectra of actin. (*C*) GdmCl-induced changes in the intrinsic fluorescence of actin. $A = (I_{320}/I_{365})_{297}$ (●, ○); degree of fluorescence anisotrophy, *r* (▲, △); and fluorescence intensity at λ = 320 nm, I_{320} (■, □). Open and black symbols correspond to the unfolding and refolding experiments, respectively. The protein concentration was 0.1 and 0.6 mg/ml for fluorescence and CD experiments, respectively. The cell path length was 0.148 and 10 mm for the far- and near-UV CD experiments, respectively. All measurements were carried out at 23°C.

However, the question of the specificity of association has to be clarified. To answer this question, the hydrodynamic dimensions of actin in different inactivated forms were compared, and the problem of stability of associates was studied. Gel filtration is one of the most efficient techniques used for the estimation of both the molecular mass of globular proteins (Andrews, 1965; Ackers, 1970) and their hydrodynamic dimensions in different conformational states (Ackers, 1967; Corbett and Roche, 1984; Uversky, 1993, 1994). The results of gel filtration analysis of G-actin conformers are summarized in Table 1. It must be stressed that in all cases the chromatographic profile contained a sole symmetrical sharp peak, reflecting the homogeneity of the samples. Interestingly, the hydrodynamic dimensions of GdmCl-unfolded actin were independent of the presence or absence of the reductant (50 mM DTT), suggesting the absence of disulfide cross-links in the actin molecule (see Table 1). This conclusion correlates well with the results of previous studies (Martonosi, 1968; Strzelecka-Golaszewska et al., 1985) and the crystal structure of the protein (Kabsch et al., 1990). Table 1 shows that inactivated actin is characterized by a Stokes radius of ~80 Å. This value essentially exceeds that of the unfolded protein (~61 Å), reflecting the association state of the inactivated form. The values of apparent molecular mass, measured for different forms of inactivated actin, were relatively close (710 ± 10 kDa; see last four lines in Table 1). Interestingly, the same molecular mass, 720 kDa, could be expected for the heat-inactivated actin from the published value of the sedimentation constant (s = 20 S; Kuznetsova et al., 1988), assuming that we are dealing with spherical particles, for which $s \approx M^{2/3}$ (Svedberg and Pedersen, 1940). These results clearly show that the inactivation of actin is accompanied by the association of protein molecules.

Chromatographic analysis of inactivated actin was performed at protein concentrations between 0.0005 and 1.0 mg/ml. It was established that there is a single elution peak with a constant position for all of the protein concentrations. This means that the degree of association remains constant after 2000-fold dilution of the inactivated actin solution. Additional confirmation of the high stability of associates was obtained by analysis of their urea-induced unfolding, measured for heat-inactivated actin at different protein concentrations. We have established that the unfolding curves measured for 1.0, 0.1, and 0.05 mg/ml solutions of the heat-inactivated actin coincide. These data are consistent with the suggestion that inactivation of actin is accompanied by specific association, resulting in the formation of soluble oligomers with conserved stoichiometry and a high stability. It would be interesting to understand how such specific association affects the structural properties of inactivated actin.

Tertiary structure of inactivated actin

Inactivated actin was unable to polymerize under conditions favorable for polymerization of G-actin, independent of the method of inactivation (Kuznetsova et al., 1988). Scanning microcalorimetry is a powerful tool in the investigation of thermal denaturation of globular proteins (Privalov, 1979; Privalov and Potekhin, 1986). The heat-induced melting of G-actin at pH 7.2 was investigated earlier, and it was shown that this protein is characterized by the calorimetric curve with a single peak of heat absorption (Tatunashvili and Privalov, 1984; Bertazzon et al., 1990; le Bihan and Gicquaud, 1993). It was also established that thermal denaturation of G-actin is an irreversible process (Tatunashvili and Privalov, 1984; Bertazzon et al., 1990; le Bihan and Gicquaud, 1993; Turoverov et al., 1999, and references therein). Interestingly, specific association of the denatured actin molecules was not accompanied by the appearance of a cooperatively melted structure, at least in the temperature range from 0° to 100°C (Tatunashvili and Privalov, 1984; Bertazzon et al., 1990). The possibility of such a structural formation was noted for the Cro repressor of phage λ (Gitelzon et al., 1991; Griko et al., 1992; Filimonov and Rogov, 1996).

Probing of inactivated actin structure by limited proteolysis

Denaturation of most proteins is known to enhance proteolytic degradation (Mihalyi, 1972). In intact G-actin, only a small number of potential proteolytic sites are exposed on the surface of the protein. Most of them are located within subdomain 2 of the monomer (Mornet and Ue, 1984). To reveal the nature of new cleavage sites in the inactivated actin, the heat-inactivated protein was treated with trypsin,

TABLE 1 Hydrodynamic dimensions and apparent molecular masses estimated for different conformations of G-actin by gel filtration

Conformational state, conditions	R_S (Å)	Apparent molecular mass (kDa)
Native state: pH 7.2	28.0	40.8
Unfolded state: 6 M GdmCl, 50 mM DTT, pH 7.2	61.0	42.6*
Unfolded state: 6 M GdmCl, pH 7.2	60.0	41.3*
Intermediate state: pH 7.2, after 30-min incubation at 70°C (G_t-actin)	80.4	710.6
Intermediate state: pH 7.2, 1.5 M GdmCl	79.9	698.7
Intermediate state: pH 7.2, 4 M urea	80.8	720.2
Intermediate state: pH 7.2, 0 M GdmCl, refolded from 6.0 M GdmCl	80.5	713.0

*The value of the apparent molecular mass of the urea-unfolded actin was estimated from the known dependence of R_S, for urea-unfolded proteins, on the molecular mass, M, $\log R_S = 0.533 \log M - 0.682$ (Uversky, 1993).

subtilisin, and *E. coli* protease ECP32. Trypsin attacks peptide bonds involving Arg or Lys, whereas subtilisin and protease ECP32 have a preference for the hydrophobic amino acid residues. It is known that trypsinolysis of intact G-actin yields a stable fragment of 33 kDa because of the cleavage of the peptide chain between Lys^{68} and Tyr^{69} (Jacobson and Rosenbush, 1976). Fig. 2 *A* shows that the formation of such a fragment is a rather slow process when the trypsin/actin ratio is low (1/100). In contrast, incubation of inactivated actin with trypsin at the same trypsin/actin ratio results in a rapid fragmentation of the protein (see Fig. 2 *A*). Similar results were observed when the inactivated actin was treated with subtilisin: in intact actin subtilysin cleaves the bond between Met^{47} and Gly^{48}, producing a stable fragment of 35 kDa (Schwyter et al., 1989), whereas in denatured actin numerous unstable fragments were generated (see Fig. 2 *B*). Finally, digestion of actin by specific bacterial metalloprotease ECP32 (Khaitlina et al., 1991; Matveyev et al., 1996) also showed considerable changes in the proteolitic pattern connected with the inactivation (Fig. 2 *C*). All of these data clearly show that in inactivated actin the accessibility of both the polar and the nonpolar amino acid residues to proteolytic digestion increases, suggesting that the conformation of the protein is nonnative.

Near-UV CD spectra

Fig. 3 *A* represents near-UV CD spectra of actin in different conformational states. The spectrum of native protein is characterized by one positive (at ~273 nm) and two negative (at ~288 and ~293 nm) bands, which are due to the tyrosine and tryptophan residues (Adler et al., 1973). On the other hand, the presence of a longer wavelength peak (~293 nm) in the actin near-UV CD spectrum may also reflect the existence of specific clusters of aromatic amino acid residues. This assumption is consistent with the results of the analysis of the 3-D actin structure (e.g., the nearest environment of Trp^{356} is characterized by the presence of aromatic rings of Tyr^{133} and Phe^{352}) and with the unusually large blue shift of the native protein intrinsic fluorescence. The pronounced signals in the near-UV CD spectrum of G-actin indicate that this protein has a unique tertiary structure. Fig. 3 *A* shows that such a structure can be completely destroyed by adding 6 M GdmCl. One could expect the same behavior for the inactivated actin. Indeed, data on biological activity, scanning microcalorimetry, and limited proteolysis are consistent with the suggestion that the inactivated actin molecule did not have a rigid tertiary structure. However, different forms of inactivated actin (heat- or EDTA-denatured protein, actin in the presence of 4.0 M urea or 1.5 M GdmCl) are characterized by relatively similar near-UV CD spectra with an intensive broad negative band at 280 nm (see Fig. 3 *A*). The near-UV CD spectra of both G-actin and inactivated actin are intense but differ substantially in shape, suggesting that the aromatic residues in these two forms have an asymmetrical but quite different microenvironment.

To illustrate in more detail the effect of inactivation on the near-UV CD spectrum of G-actin, Fig. 3 *B* represents spectra measured at different GdmCl concentrations. One can see that there is considerable change in the spectrum shape during the increase in denaturant concentration. Analysis of the spectral changes allows us make some important conclusions. First of all, the presence of the isodichroic point in the vicinity of 289 nm (Fig. 3 *B*) shows that inactivation of actin represents a two-state transition. Second, the disappearance of the longest wavelength band (294 nm) probably reflects the GdmCl-induced disruption of the specific clusters of aromatic amino acid residues (see above). Third, the reduction in intensity of the positive band at 273 nm is consistent with GdmCl-induced perturbations of the environment around the tyrosines. Fourth, the presence of a negative band at 280 nm in the spectrum shows that the tryptophan environment in inactivated actin is relatively rigid. Fifth, the shape of the inactivated actin near-UV CD spectrum is considerably simplified and does not have fine structure, suggesting that the overall environment of aromatic residues in this conformation is much less specific than that in the native molecule.

Secondary structure of inactivated actin

Fig. 4 shows that inactivation of actin is accompanied by substantial changes in the far-UV CD spectrum of the protein. It can also be seen that different forms of inactivated actin are characterized by a relatively close secondary structure. Far-UV CD changes produced by the inactivation of actin can be explained in terms of partial transition of the α-helical structure to unordered structure and by the formation of some β-structure (Nagy and Strzelecka-Golaszewska, 1972; Strzelecka-Golaszewska et al., 1985; Kuznetsova et al., 1988; Bertazzon et al., 1990). The appearance of a β-structure upon denaturation has been described for a number of proteins and has been correlated with protein aggregation (Joly, 1965; Strzelecka-Golaszewska et al., 1985).

Interaction of inactivated actin with ANS

ANS fluorescence spectra measured for different actin conformations are shown in Fig. 5. It can be seen that fluorescence emission intensity is minimal in the solution of native actin and in completely unfolded actin, whereas any kind of protein inactivation leads to a considerable increase in the emission intensity (see Fig. 5). Interestingly, the ANS fluorescence intensity reaches a maximum value at a protein/ANS molar ratio of 1/14, regardless of the inactivated actin concentration.

Fig. 6 represents ANS fluorescence decay curves measured for native (Fig. 6 *A*) and inactivated actin in the presence of 0.8 M GdmCl (Fig. 6 *B*). It can be seen that the longest component of fluorescence decay of the 8-ANS-native protein complexes is characterized by $\tau = 11.1 \pm 0.5$

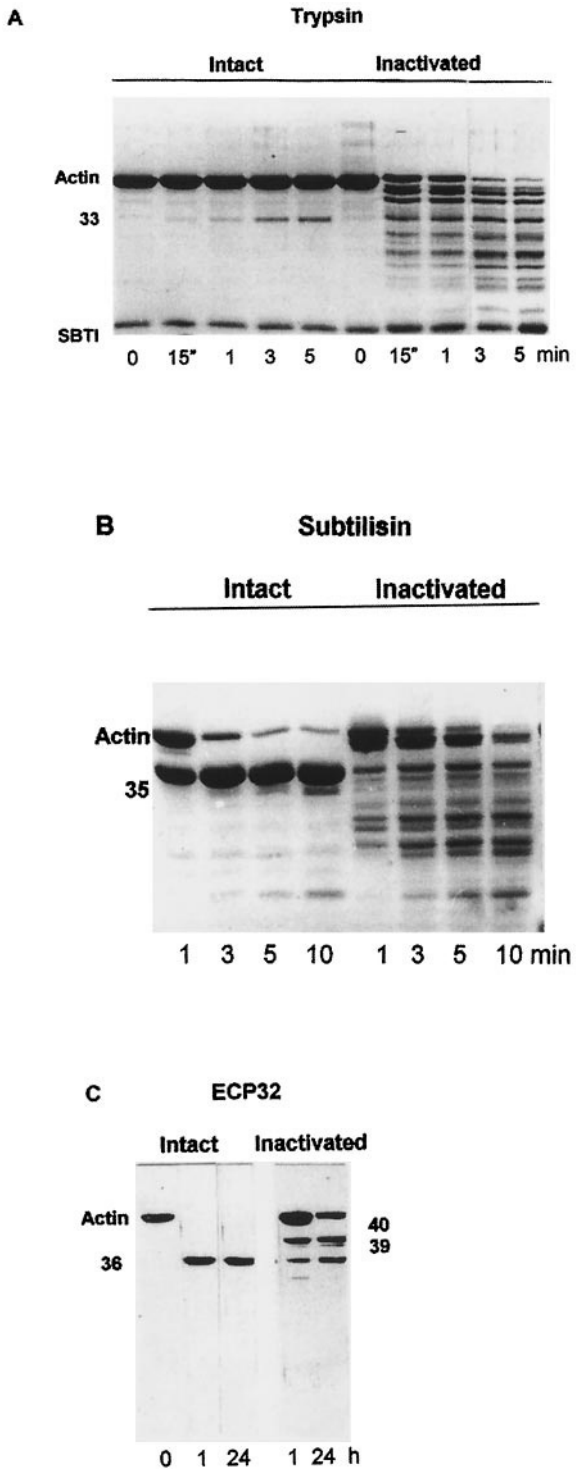

FIGURE 2 Fragmentation of intact and inactivated actin with proteases of different substrate specificity. Intact or heat-inactivated actin (24 μM) was digested with trypsin (*A*), subtilisin (*B*), or protease ECP 32 (*C*). The enzyme/actin mass ration was 1/100. All measurements were carried out at room temperature. At the points indicated, digestion was stopped by the addition of soybean trypsin inhibitor, 1 mM phenylmethyl sulfonyl fluoride, or sodium dodecyl sulfate-containing buffer for *A*, *B*, and *C*, respectively. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (1970), using a 12% acrylamide slab gel. Positions of the 40, 39, 36, and 33 kDa actin fragments are denoted by the respective numerals. SBTI, Soybean trypsin inhibitor.

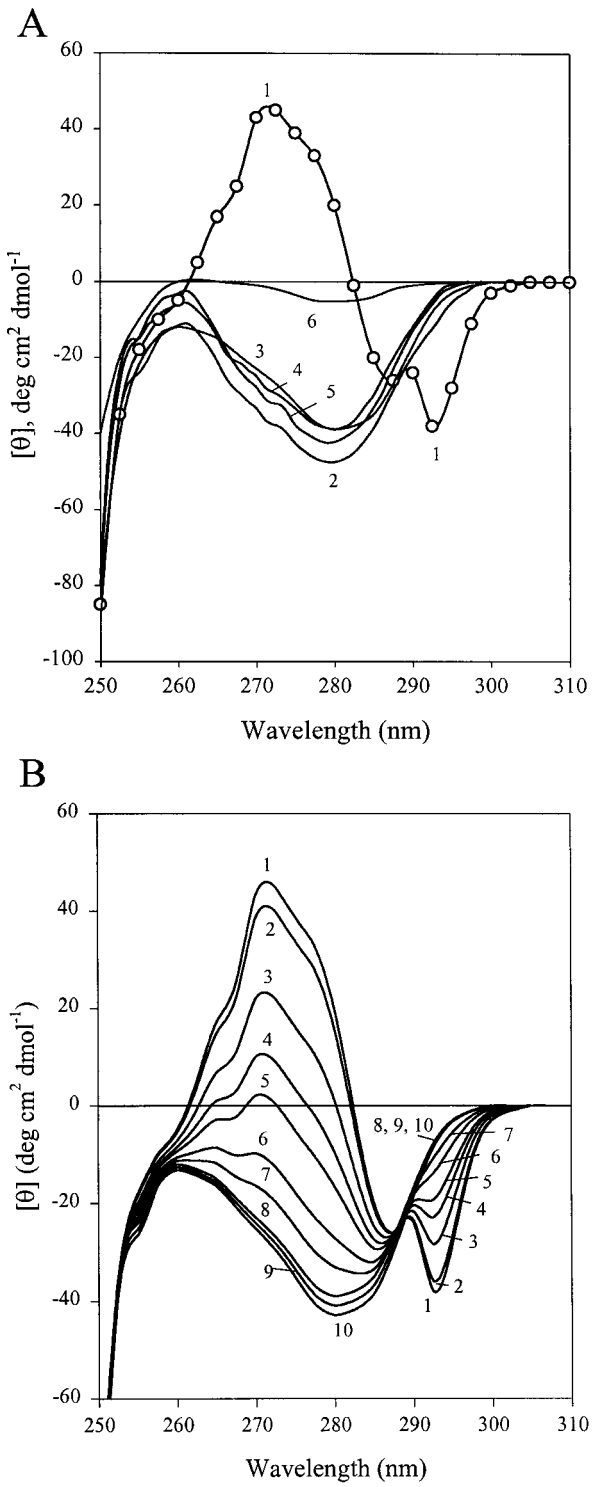

FIGURE 3 (*A*) Near-UV CD spectra of G-actin in different conformational states: 1, native protein; 2, thermal inactivated actin; 3, actin at 1.5 M GdmCl; 4, EDTA-inactivated protein; 5, G-actin at 4.0 M urea; 6, completely unfolded protein (in the presence of 6 M GdmCl). (*B*) Near-UV CD spectra of G-actin measured within the first GdmCl-induced transition. Denaturant concentrations were 1, 0.0 M; 2, 0.2 M; 3, 0.4 M; 4, 0.5 M; 5, 0.55 M; 6, 0.6 M; 7, 0.8 M; 8, 1.0 M; 9, 1.2 M; 10, 1.4 M. All measurements were carried out at a protein concentration of 1.0 mg/ml; a cell path length of 10.0 mm, and 25°C.

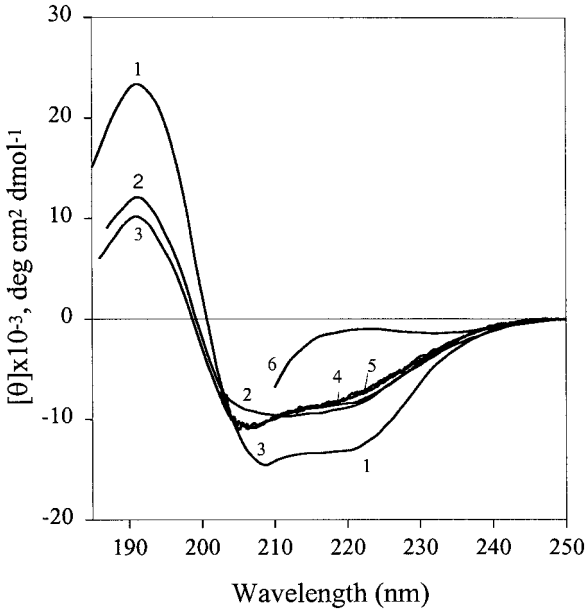

FIGURE 4 Far-UV CD spectrum of G-actin in different conformational states. 1, Native protein; 2, heat-inactivated actin; 3, EDTA-inactivated protein; 4, actin at 1.5 M GdmCl; 5, actin at 4.0 M urea; 6, completely unfolded protein (in the presence of 6 M GdmCl). All measurements were carried out at a protein concentration of 1.0 mg/ml, a cell path length 0.148 mm, and 25°C.

ns, whereas in the presence of moderate GdmCl concentrations a ~1.5-fold increase of this value is observed (τ = 16.7 ± 0.5 ns).

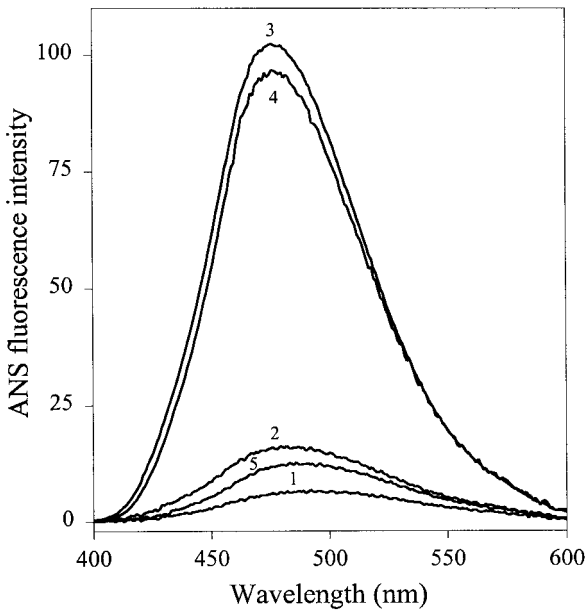

FIGURE 5 ANS fluorescence spectra in the presence of different conformational states of G-actin. 1, free ANS; 2, native protein; 3, actin at 1.0 M GdmCl; 4, actin at 4.0 M urea; 5, completely unfolded protein (in the presence of 6 M GdmCl). Protein and ANS concentrations were 0.1 and 0.02 mg/ml, respectively. Fluorescence was excited at 350 nm. All measurements were carried out at 25°C.

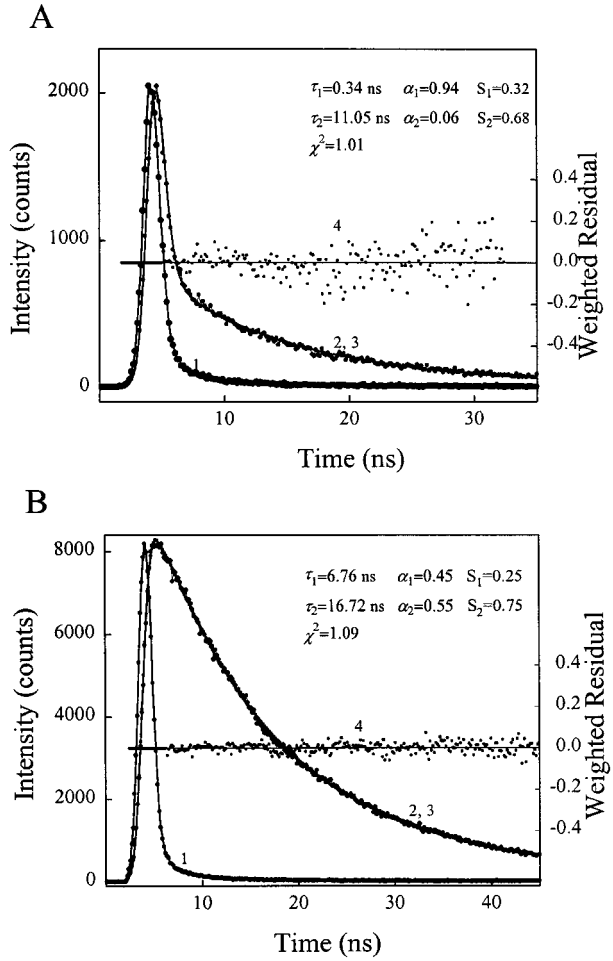

FIGURE 6 ANS fluorescence decay curves measured for native (*A*) and inactivated (*B*) actin. The figure represents the excitation lamp profile (*curve 1*), the experimental decay curve (*curve 2*), the best-fitted calculated fluorescence decay curve (*curve 3*), and the deviation between the experimental and the calculated decay curves (weighted residuals; *curve 4*). The protein/ANS molar ratio was 1/20. The protein concentration was 1.0 mg/ml. The excitation wavelength was 390 nm; the recording wavelength was 480 nm. All measurements were carried out at 25°C.

These observations confirm the suggestion that inactivated actin represents the compact denatured conformation. Indeed, the high affinity for the hydrophobic probe ANS is a well-known characteristic of different intermediates of globular proteins (Rodionova et al., 1989; Semisotnov et al., 1991; Fink, 1995a,b; Ptitsyn, 1995; Uversky et al., 1996, 1998c). Steady-state measurements showed that the formation of complexes between this fluorescent probe and the protein molecule results in a considerable increase in the dye fluorescence intensity and a blue shift of its fluorescence spectrum (Stryer, 1965; Rodionova et al., 1989; Semisotnov et al., 1991; Uversky et al., 1996). The fluorescence decay of free 8-ANS has a uniexponential character, whereas the formation of ANS-protein complexes yields at least a double-exponential fluorescence decay (Uversky et al., 1996, 1998c). In this case the shorter lifetime component of fluorescence decay ($\tau < 6$ ns) is characteristic of the

dye molecules that interact with the surface of a protein molecule, while the longer lifetime component ($\tau > 10$ ns) refers to the protein-embedded 8-ANS molecules (Uversky et al., 1996). A good correlation between changes in the longest lifetime component and overall conformational changes of the protein molecule was shown. The value of this parameter is sensitive whether 8-ANS interacts with the native (*N*) or with the compact denatured (*D*) protein molecule ($\tau_N \leq 12$ ns as compared with $\tau_D \geq 15$ ns) (Uversky et al., 1996, 1998c).

Internal mobility of Trp residues in inactivated actin

Intrinsic fluorescence spectrum of actin and microenvironments of tryptophan residues

The fluorescence spectrum of native G-actin is characterized by $\lambda_{max} = 325$ nm and $A = 2.6$. It is known that the value of parameter *A* is determined by the position and shape of the tryptophan fluorescence spectrum (Turoverov and Shchelchkov, 1970). Fig. 7 *A* shows that the fluorescence spectrum of inactivated actin, being considerably different from that of native protein ($\lambda_{max} = 340$ nm, $A = 1.3$), still remains substantially blue shifted as compared with the spectrum of the completely unfolded protein ($\lambda_{max} = 350$ nm, $A = 0.4$). This indicates that the microenvironment of tryptophan residues of inactivated actin is intermediate in its mobility and polarity between that of the native and unfolded protein. A pronounced red shift of tryptophan fluorescence does not necessarily reflect a considerable increase in its mobility. Some native proteins with a *fixed* but relatively *polar* environment of tryptophan residues also show a characteristic red-shifted spectrum (Kuznetsova and Turoverov, 1998). Additional experiments were required to distinguish the tryptophan residue microenvironment mobility from that of the indole ring of the tryptophan residues.

Accessibility of Trp residues to solvent: acrylamide quenching of actin fluorescence

Fig. 7 *B* shows that the Stern-Volmer plots (the $\bar{\tau}_0/\bar{\tau}$ versus [*Q*] dependence) for the native, completely unfolded, and inactivated (by 0.8 M GdmCl) forms of actin are linear in a large interval of acrylamide concentrations (up to 0.8 M). The slope of this dependence is proportional to the Stern-Volmer constant and reflects the accessibility of the chromophore group to the quencher molecules (Eftink and Ghiron, 1981). The corresponding values of Stern-Volmer constants (*K*), as well as the bimolecular rate constants for the collisional process (k_q) are presented in Table 2. It is seen that the efficiency of dynamic quenching is low not only for the native but also for the inactivated actin. This parameter increases considerably in the completely unfolded protein. Interestingly, the Trp residues of inactivated actin appear to be even less accessible to acrylamaide molecules than those of native protein.

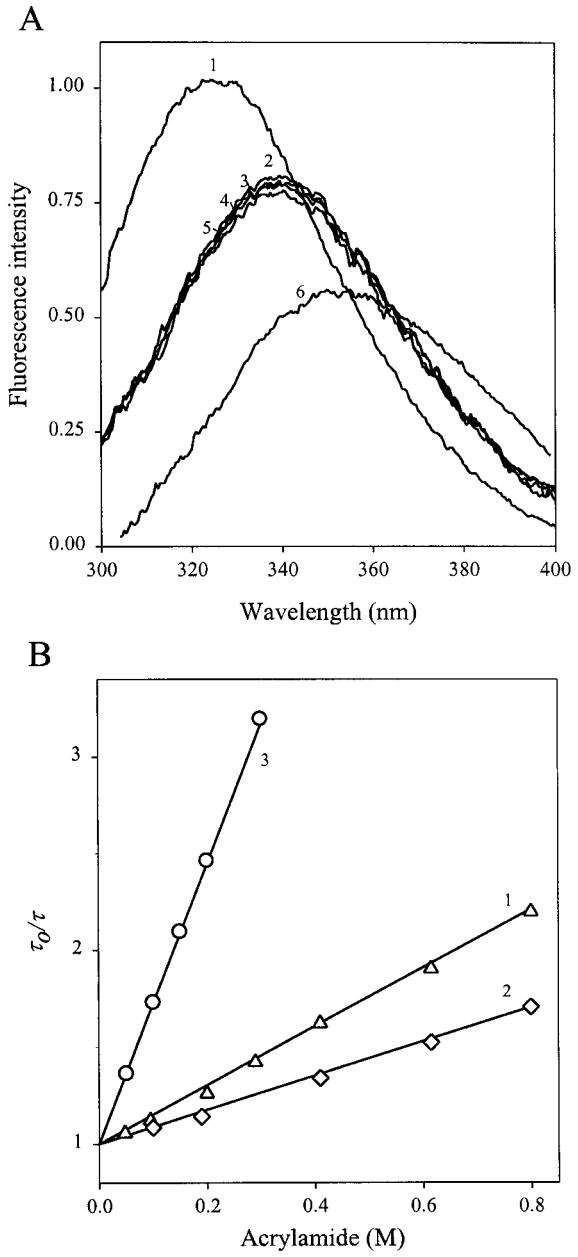

FIGURE 7 (*A*) Tryptophan fluorescence spectra of G-actin in different conformational states. 1, Native protein; 2, heat-inactivated actin; 3, EDTA-inactivated protein; 4, actin at 1.5 M GdmCl; 5, actin at 4.0 M urea; 6, completely unfolded protein (in the presence of 6 M GdmCl). The excitation wavelength was 297 nm. (*B*) Stern-Volmer plots for different actin conformations: Native G-actin (*curve 1*), inactivated actin in 0.8 M GdmCl (*curve 2*), and completely unfolded protein in 5.0 M GdmCl (*curve 3*). The figure represents the results of intrinsic fluorescence quenching by acrylamide, monitored by Trp lifetime changes. The excitation wavelength was 297 nm. All measurements were carried out at a protein concentration of 0.5 mg/ml at 25°C.

Intramolecular mobility of tryptophan residues

The mobility of the tryptophan residues can be estimated from fluorescence anisotropy measurements. We have established that the transition of actin from the native to the intermediate state is accompanied by a considerable *in-*

TABLE 2 Parameters of the decay and acrylamide-induced quenching of tryptophan fluorescence of actin in different conformational states

Conformational state	$\langle\tau\rangle$ (ns)	k_q ($\times 10^9$ M^{-1} s^{-1})	K_{SV} (M^{-1})
Native	3.83	0.43	1.65
Inactivated	4.77	0.16	0.76
Unfolded	3.00	2.22	7.29

crease in the anisotropy value. The increased anisotropy value (0.17 ± 0.02) is characteristic of the different forms of inactivated actin (Table 3). Such an anisotropy increase can be due to the considerable decrease in the internal mobility of tryptophan residues, which can be the result of protein association.

Fig. 8 represents Perrin plots ($1/r$ versus T/η dependence) for the native, inactivated, and completely unfolded actin molecules. It can be seen that actin inactivation is characterized by the independence of $1/r$ from T/η. This can reflect the association of partially folded actin molecules in particles whose rotational relaxation time is much greater than the fluorescence lifetime. Confirmation of this idea follows from a comparison of curves 2 and 4 in Fig. 8. Indeed, curve 4 is the Perrin plot measured for F-actin, which, as known, is a long rigid filament comprising numerous actin molecules. In this case again there is no visible dependence of $1/r$ on T/η.

Analysis of Fig. 8 shows that the value of the intercept cut by the Perrin plot on the y axis ($1/r_0'$) for both the native and the inactivated actin exceeds that for low-molecular-weight model compounds, such as *N*-acetyltryptophan (see Table 3). This means that in these two conformations the Trp residues participate in the high-frequency intramolecular motions whose rotational relaxation time is much shorter than the excited-state lifetime (Kuznetsova et al., 1988). Alternatively, these Trp residues can be involved in the intramolecular mobility on the nanosecond time scale; their rotational relaxation time does not depend on the solvent viscosity (Kuznetsova and Turoverov, 1983). The existence of the latter type of intramolecular mobility seems to be possible, because the tryptophan residues of native and inactivated actin are inaccessible to acrylamide molecules (see above). The fact that $(1/r_0')_{\text{inactivated}} < (1/r_0')_{\text{native}}$ indicates that the amplitude of the high-frequency motions or rotational relaxation time of inactivated actin, or both, are much lower than those of native actin.

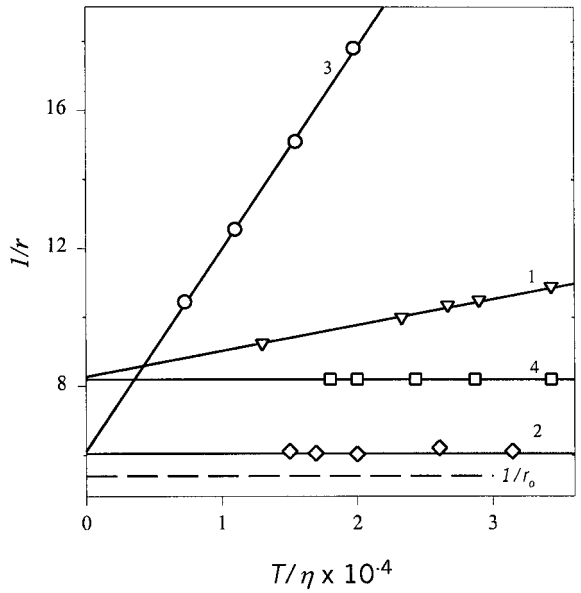

FIGURE 8 Perrin plots ($1/r$ versus T/η dependence) for different conformational states of G-actin. 1, Native protein; 2, actin at 0.8 M GdmCl; 3, completely unfolded protein (in the presence of 8 M urea). A Perrin plot for F-actin (4) is shown for comparison. The excitation wavelength was 297 nm. The recording wavelength of fluorescence anisotropy was 365 nm. The solvent viscosity was varied by the changing water/glycerol ratio. All measurements were carried out at a protein concentration of 0.5 mg/ml at 25°C.

DISCUSSION

Inactivated actin is a specific associated state, comprising partially folded protein molecules with restricted internal mobility

We have established that equilibrium GdmCl-induced unfolding of G-actin is accompanied by the accumulation of an intermediate state whose population reaches 100% in the vicinity of 1.5 M GdmCl (see Fig. 9). It has also been established that the transition from the native to the intermediate state is irreversible, whereas transformation of the intermediate state into the unfolded state is a completely reversible process. In other words, unfolding of actin can be described by the scheme $N \rightarrow I \leftrightarrow U$ (cf. Bertazzon et al.,

TABLE 3 Degree of fluorescence anisotropy (r) and $1/r_o'$ values estimated for different conformations of G-actin

Conformational state, conditions	r	$1/r_o'$
Native state: pH 7.2	0.09 ± 0.01	8.3 ± 0.2
Unfolded state: 6 M GdmCl, pH 7.2	0.07 ± 0.01	6.1 ± 0.2
Intermediate state: pH 7.2, after 30-min incubation at 70°C (G_t-actin)	0.17 ± 0.02	6.7 ± 0.2
Intermediate state: pH 7.2, 1.5 M GdmCl	0.17 ± 0.02	6.1 ± 0.2
Intermediate state: pH 7.2, 4 M urea	0.16 ± 0.02	—
Intermediate state: pH 7.2, 0 M GdmCl, refolded from 6.0 M GdmCl	0.17 ± 0.02	—
N-Acetyl-tryptophanamide	0.00 ± 0.01	5.4 ± 0.2

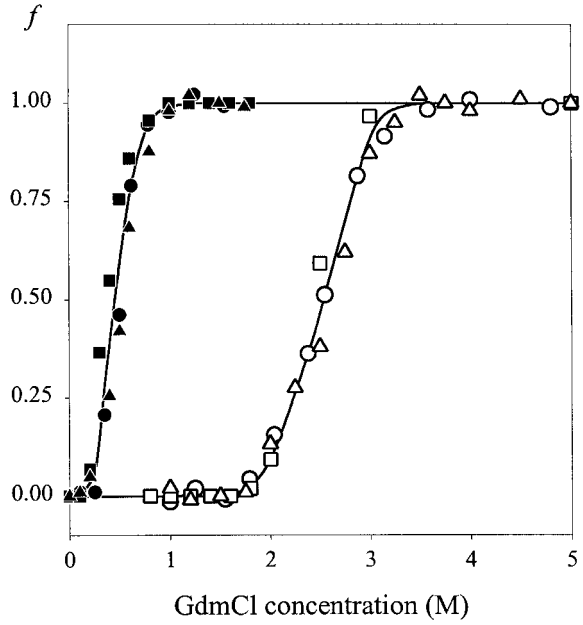

FIGURE 9 Sequential mechanism of GdmCl-induced unfolding of G-actin. Comparison of the effects of the denaturant on the protein far-UV (●, ○) and near-UV (▲, △) CD spectra, and on fluorescence intensity at λ = 320 nm (■, □). Black and open symbols correspond to the first and second transitions, respectively (see Fig. 1).

1990). The presence of reductant did not affect this scheme, showing that the irreversibility of actin denaturation cannot be attributed to the formation of intermolecular disulfide cross-links. An analogous conformation, known as inactivated actin, can be populated at an intermediate urea concentration (~4 M) (Kuznetsova et al., 1988), as the result of thermal denaturation (Nagy and Jencks, 1962; West et al., 1967; Lehrer and Kerwar, 1972; Nagy and Strzelecka-Golaszewska, 1972; Strzelecka-Golaszewska et al., 1974, 1985; Contaxis et al., 1977; Levitsky et al., 1995; Kuznetsova et al., 1988; Bertazzon et al., 1990), by dialysis from 8 M urea or 6 M GdmCl (Kuznetsova et al., 1988; Bertazzon et al., 1990), or spontaneously during storage (Kuznetsova et al., 1988). It has also been established that the irreversible removal of tightly bound Ca^{2+} (and, as a consequence, the removal of bound ATP) represents the major reason for the irreversibility of actin inactivation (Strzelecka-Golaszewska et al., 1985; Bertazzon et al., 1990). On the other hand, it follows from our study that inactivation of actin is accompanied by specific association. This allows us to assume that just the association of partially folded protein molecules prevents the binding of Ca^{2+} ions back to actin.

We have shown here that inactivated actin represents a specific stable monodisperse associate, characterized by a Stokes radius of ~80 Å. On the other hand, it has been established that the heat-inactivated form of actin has sedimentation constant of ~20 S (Kuznetsova et al., 1988). This allows us to estimate the molecular mass of inactivated actin, assuming that it represents a rigid sphere of radius R_S. Indeed, it is well known that the sedimentation constant of the given molecule, S, depends on its molecular mass, M, and frictional coefficient, f, as (Svedberg and Pedersen, 1940)

$$S = \frac{M(1 - \bar{\nu}\rho)}{N_A f}, \tag{7}$$

where ν is the partial specific volume of the particle, ρ is the density of the solution, and N_A is Avogadro's number. The frictional coefficient, f, of a rigid sphere of radius R_S moving through a medium of viscosity η is given by (Gosting, 1956)

$$f = 6\pi\eta R_S. \tag{8}$$

This leads to the desired expression for the molecular mass,

$$M = \frac{6\pi\eta R_S S N_A}{1 - \nu\rho}. \tag{9}$$

Assuming that $\nu = 0.72$, the molecular mass can be estimated to be 646 kDa. This means that inactivated actin contains up to 15 monomers. This value can be used as an *upper estimate* of the association degree.

The *lower estimate* of the number of monomers within one multimer can be extracted by taking into account the fact that inactivated actin is denatured. Disruption of the protein tertiary structure (denaturation) is accompanied by an increase in the hydrodynamic dimensions of the protein (Tanford, 1968), even if it is transformed into the molten globule state (Uversky, 1994; Ptitsyn, 1995). Such an increase in hydrodynamic dimensions will obviously lead to an increase in the apparent molecular mass of the protein, as measured by either gel filtration or sedimentation. It has preliminarily been established for a number of proteins that $R_S^{MG}/R_S^N \leq 1.2$ and $R_S^{PMG}/R_S^N \leq 1.5$, where R_S is the Stokes radius, and PMG, MG, and N correspond to the premolten globule, molten globule, and native states, respectively (Uversky, 1994, 1997; Ptitsyn, 1995). The molten globule state is the most compact denatured form of the protein molecule, whereas dimensions of its precursor (PMG) represent the upper limit for compact denatured intermediates. Taking this into account, we can calculate the estimated R_S values of the monomeric form of actin in the molten globule state and in its precursor. It was shown that R_S of globular proteins depends on the molecular mass, M, as $\log R_S = 0.369 \log M - 0.254$ (Uversky, 1993, 1994). This allows us to estimate roughly the apparent molecular masses of the monomeric molten globule and premolten globule-like actin as ~67 kDa and ~122 kDa, respectively. Thus even this rather crude estimation shows that the multimer of inactivated actin must contain at least 6–10 monomers.

One more approach can be used for this estimation. It was shown that the degree of compactness of the partially folded protein molecule is directly proportional to the amount of remaining secondary structure (Uversky and Fink, 1998b; Uversky et al., 1998a). As follows from Figs. 1 and 4, inactivated actin preserves ~65% of the native secondary

structure. This allows us to suggest that between native and inactivated actin there is a 35% difference in the hydrodynamic dimensions as well. This means that the inactivated actin monomer can have $R_S \approx 38$ Å, which corresponds to an apparent molecular mass of ~90 kDa. In other words, this estimation is consistent with the conclusion that inactivated actin contains eight monomers.

All of this means that inactivated actin represents a specific stable oligomer with a conserved stoichiometry. Using the different methods and assumptions (see above), the degree of association of inactivated actin is at least 6–15 monomers. This difference in the number of components is definitely due to the distinction in the estimation approaches. Finally, it is necessary to emphasize that the degree of association is independent of the way in which the actin is inactivated.

As the result of association the inactivated actin is characterized by an unusual combination of structural properties. It loses the capability to polymerize and does not have a rigid cooperatively melted tertiary structure; its secondary structure is considerably distorted; and it has an increased accessibility to proteolytic digestion, a high affinity to ANS, and a strong propensity to associate. On the other hand, we have demonstrated that this conformation has a pronounced near-UV CD spectrum, which, being almost as intensive as that of the native G-actin, is quite different from it in shape. This casual disagreement between the near-UV CD data and the results of fluorescence, calorimetric, activity, and limited proteolysis assays can easily be explained by taking into account the fact of self-association of inactivated protein molecules. Moreover, we can assume that the specific association of the partially folded actin molecule leads to the formation of an asymmetrical microenvironment near the tryptophan residues. This can lead not only to the induction of a pronounced near-UV CD spectrum, but also to the appearance of a substantial limitation in the internal mobility of these residues or their environment.

Analysis of intrinsic fluorescence confirms this supposition and shows that there are considerable limitations in the internal mobility of Trp residues. The decrease in tryptophan residue mobility, observed upon the transition of actin to the intermediate state, fits well with the regularity found in the spectral and polarization characteristics of numerous proteins (Kuznetsova and Turoverov, 1983). The high level of intramolecular mobility was found not only for the external (exposed to solvent) tryptophan residues, but also for the internal ones located within the protein interior in a rather rigid hydrophobic environment. Interestingly, the minimal level of intramolecular mobility was found for tryptophan residues located in the area surrounding polar groups (λ_{max} = 335–345 nm).

It should be emphasized that actin is not the only protein whose transition to the intermediate state is accompanied by a decrease in tryptophan mobility. The same behavior was earlier observed for α-lactalbumin and carbonic anhydrase B (Rodionova et al., 1989; Dolgikh et al., 1981). It is difficult to exclude the possibility that such behavior can be due to the association of protein molecules.

The most intriguing finding of this paper is the observation of a pronounced near-UV CD spectrum for the specific associates consisting of partially unfolded protein molecules. This is a quite impressive confirmation of the idea that self-association of partially folded protein molecules can have a considerable role in structure formation. This hypothesis was proposed for staphylococcal nuclease, for which it was established that dimerization of protein molecules in the premolten globule state is responsible for the appearance of a specific globular structure (Uversky, 1998; Uversky and Fink, 1998a,b; Uversky et al., 1998b, 1999).

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