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Changes of structure and intramolecular mobility in the course of actin denaturation

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Rabbit skeletal muscle G-actin on heating is transformed into the G_t-state in which the intrinsic fluorescence spectrum is shifted to a longer wavelength compared with that of native actin, but of much shorter wavelength than that of actin in 8 M urea. A structure with fluorescence characteristics identical to those of the G_t-form appears upon the removal of Ca^{2+}, upon partial denaturation in 3–5 M urea and renaturation from the completely unfolded form in 8 M urea as well as spontaneously during storage of actin solutions. All this allows us to regard the G_t-form of the actin macromolecule as an 'intermediate' state. However, in contrast to other proteins in the intermediate state, a band of the CD spectrum has been observed for G_t-actin, with an amplitude comparable to that of native proteins in the region where aromatic groups absorb. This points to a relatively low level of intramolecular mobility of the side chains in this structural state of actin. Moreover, according to polarized fluorescence measurements, the G-G_t transition is accompanied not by an increase – as would have been expected – but by a decrease in mobility of the tryptophan residues. The data obtained confirm the previously observed regularity of the intramolecular mobility of tryptophan residues in a hydrophobic environment being often greater than that of tryptophan residues whose microenvironment is formed by polar protein groups.

1. Introduction

As is well known, heating, in contrast to denaturation by high concentrations of urea or guanidine hydrochloride (Gdn-HCl), transforms the macromolecules of a protein into a partially denatured state, in which the secondary structure is largely preserved [1]. The appearance of such structures can also be the result of treatment with moderate concentrations of urea or Gdn-HCl, change of pH, removal of ligands stabilizing the native structure, etc. In view of the fact that the physico-chemical properties of partially denatured states of protein macromolecules obtained under different perturbations proved to be similar, the possibility of the existence of a stable intermediate state of protein macromolecules has been suggested [2–4]. Recently, considerable attention has been paid to this intermediate state, because it is believed to play an important role in the process of protein folding and functioning [3]. The experimental data obtained by Lehrer and Kerwar [5] show that skeletal muscle G-actin is transformed, upon heating or removal of Ca^{2+}, into a state which differs from the completely unfolded form in 8 M urea. Contaxis et al. [6] reported an example of such a state under heating for bovine cardiac G-actin.

The purpose of the present work is to study the intramolecular mobility of actin in various struct-

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ural states by the method of polarized ultraviolet fluorescence and to analyse the physico-chemical properties of inactivated actin in the light of modern ideas about the intermediate state of protein macromolecules.

2. Materials and methods

G-Actin extracted from rabbit skeletal muscle was prepared according to a method described previously [7]. It was purified by one or two polymerization-depolymerization cycles using 30 mM KCl for polymerization [8]. For the removal of Ca^{2+}, F-actin was suspended in a solution containing 5 mM EDTA (pH 7.5) and dialyzed against this solution overnight. Depolymerized actin was centrifuged for 1 h at 150000 × g. All fluorescence measurements were recorded with a spectrofluorimeter described earlier [9]. The position and form of the fluorescence spectra were characterized by the parameter A, which is equal to the ratio of the emission intensities at 320 and 365 nm [10]. The degree of polarization was measured with an accuracy ±0.002. The given temperature was maintained to an accuracy of ±0.5° C. In measurements of the heat-denaturation curves, the rate of variation of the temperature was 0.5–2.5° C/min. In the ultraviolet-fluorescence experiments the concentration of actin was varied from 0.3 to 2.0 mg/ml. CD spectra were recorded using a mark III Jobin-Yvon dichrograph (France). In measurements of the CD spectrum in the near-ultraviolet region, where aromatic groups absorb, solutions of protein with an absorbance equal to unity were used ($A_{280} = 1.0$). Such solutions were prepared by diluting concentrated protein solutions with distilled water five times, just before experiments. The sedimentation constants were measured on an analytical ultracentrifuge (MOM 3170, Hungary) with Filpot-Svenson optics at 40000 rpm.

3. Results and discussion

3.1. Spectral characteristics of inactivated actin

On heating, actin is transformed into state G_t, in which the fluorescence maximum is at a longer wavelength compared with the spectrum of native actin, but at a shorter wavelength than in 8 M urea (fig. 1). This indicates that the environment of the tryptophan residues of G_t-actin is more polar than that of native actin but less polar than that of actin in 8 M urea. From the far-ultraviolet CD spectra (185–250 nm), it is clear that the secondary structure of G_t-actin, in contrast to that of actin in 8 M urea, is largely preserved. The minor differences in the CD spectra of G- and G_t-actin in this spectral region [11] are caused by the untwisting of some α-helices into randomly coiled chains. The CD spectrum of G_t-actin differs sharply from that of actin in 8 M urea (fig. 2), in which the secondary structure is completely destroyed. A structure with spectral characteristics identical to those of the G_t-form was obtained upon removal of Ca^{2+} from the solution (fig. 3) or in moderate concentrations of urea (fig. 4). Spontaneous transition of actin into this state occurs on storage [10] or on renaturation from the completely unfolded state in 8 M urea (fig 4). This

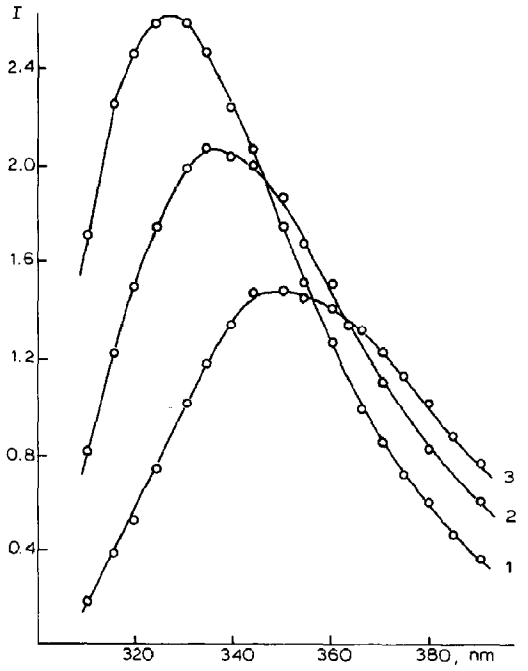

Fig. 1. Fluorescence spectra of actin. (1) G-Actin, (2) actin inactivated by heating up to 70° C (G_t-actin), (3) actin in 8 M urea. $\lambda_{ex} = 296.8$ nm.

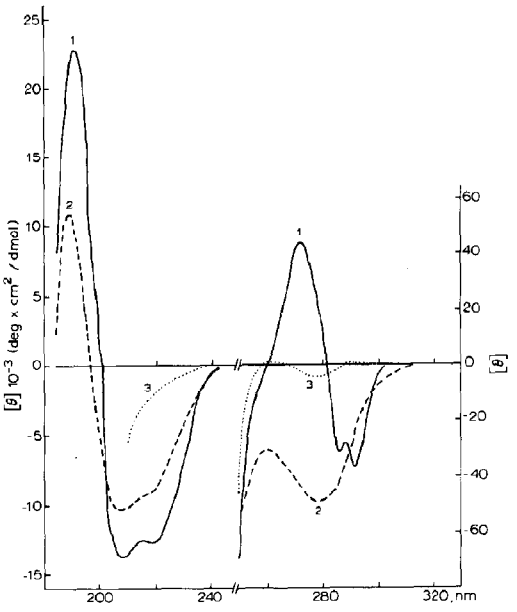

Fig. 2. CD spectra of actin. (1) G-Actin, (2) G_t-actin, (3) actin in 8 M urea.

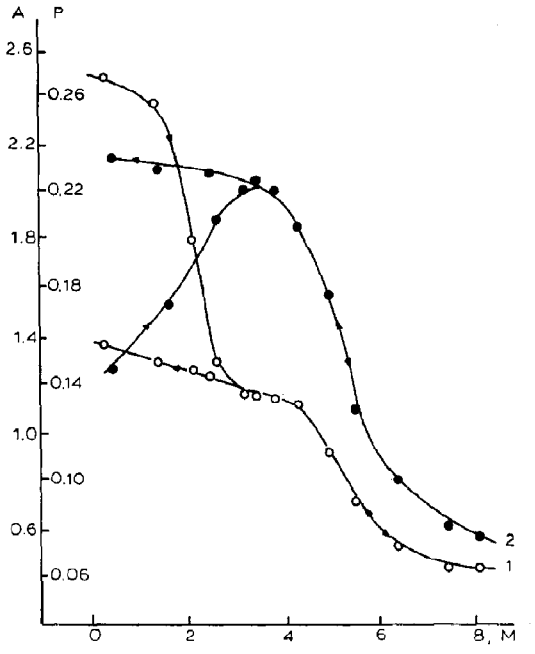

Fig. 4. Change of actin structure under the influence of urea. (1) Parameter *A*, (2) degree of fluorescence polarization, *P*. Arrows indicate the variation of urea concentration.

form always exists in actin preparations before purification by cycles of polymerization-depolymerization [10].

The above spectral characteristics of G_t-actin, as well as the fact that the G_t-form is a stable structural state, to which actin is transformed under various perturbations, have made it possible to consider the G_t-form as an intermediate state that was previously observed for macromolecules of a number of other proteins [2–4]. According to the literature [2–4], the intermediate state is characterized by a significant mobility of the side chains of protein. Due to averaging with time and all kinds of conformations, this should have led to disappearance of the asymmetry of the microenvironment of aromatic residues and, consequently, to the disappearance of the band in the CD spectrum at 250–320 nm. However, this is not the case with G_t-actin. The long-wavelength band of the CD spectrum of native actin, that is mostly formed by tryptophan residues [12], has a maximum at 272 nm and two minima at 286 and 292 nm (fig. 2). After the G-G_t transition, a broad structureless band with an amplitude comparable to that of the native protein appears at 280 nm in place of this band (fig. 2). This means that the intramolecular mobility in inactivated actin is not sufficient to destroy the asymmetrical microenvironment of the tryptophan residues. The existence of a band with a significant amplitude in the region of 250–320 nm in the CD spectrum of G_t-actin disagrees with

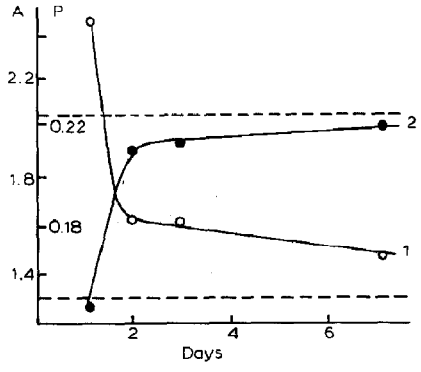

Fig. 3. Actin inactivation by the removal of Ca^{2+}. Abscissa: time after addition of 5 mM EDTA in solution. Ordinate: parameter *A* (1) and the degree of fluorescence polarization, *P* (2).

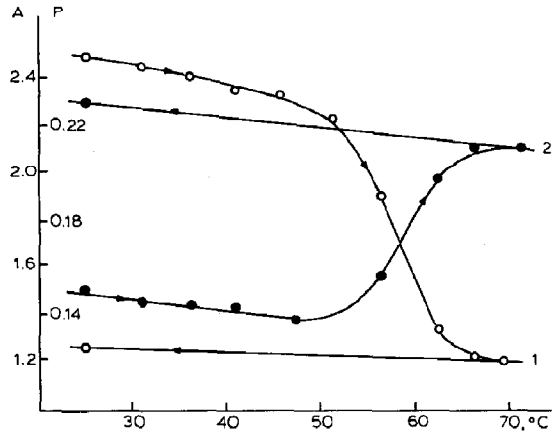

Fig. 5. Heat denaturation of actin. (1) Parameter A, (2) degree of fluorescence polarization, P. Heating rate 0.5° C/min. Arrows indicate the variation of temperature changes.

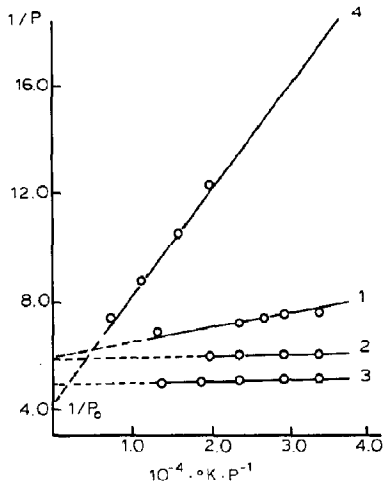

Fig. 6. Perrin plots for actin in various structural states (1) G-Actin, (2) F-actin, (3) G_t-actin, (4) actin in 8 M urea. $1/P_0$, reciprocal of the polarization of the fluorescence of immobilized tryptophan; $1/P_0 = 3.91$ [18,20]. $\lambda_{ex} = 296.8$ nm, $\lambda_{em} = 365$ nm.

the notion of the intermediate state as being a 'molten globule' state [3] which, while retaining a secondary structure and globular construction, has its tertiary structure destroyed due to intramolecular mobility.

3.2. Characteristics of heat denaturation of actin

An interesting feature of the heat inactivation of actin consists of the long-wavelength shift of the fluorescence spectrum (the decrease in parameter A) being accompanied not by a decrease – as might be expected – but by an increase in the degree of polarization (fig. 5). Although the interaction with urea is a different denaturation process, the increase in urea concentration from 0 to 3 M also leads to a long-wavelength shift of the fluorescence spectrum and to an increase in polarization (fig. 4). Further increase in urea concentration is accompanied by a long-wavelength shift of the fluorescence spectrum and a decrease in polarization. With urea concentration equal to 3–4 M, a maximum of the polarization and a bend

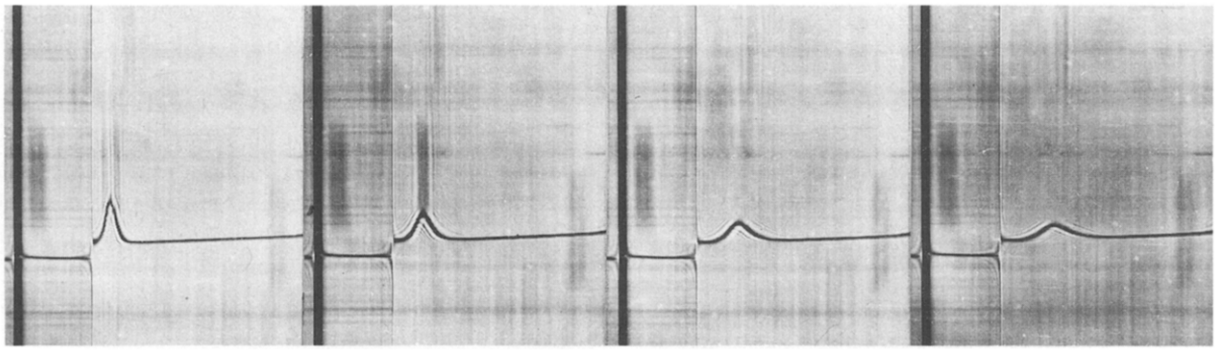

Fig. 7. Ultracentrifugation patterns of G_t-actin. Protein concentration was 3.0 mg/ml. Photographs were taken every 4 min after reaching 40000 rpm at 20 ° C.

in the curve of parameter A are observed. Moreover, the values of P and A for actin at this urea concentration are practically the same as those of actin after heat denaturation. The same values of A and P were recorded during the final stage of actin renaturation from the completely unfolded state in 8 M urea. All these data indicate that in the course of urea denaturation the protein structure passes through the intermediate state G_t. In order to study this phenomenon, a comparative analysis of Perrin plots of actin in different structural states was performed. It turned out that the slope of the Perrin plot for actin in the G_t-form is equal to zero, as in the case of F-actin (fig. 6). This means that the relaxation time of the G_t-actin macromolecule is much longer than the lifetime of the excited state of the tryptophan residues responsible for the protein fluorescence. The reason for this might only lie in the aggregation of the macromolecules of actin during the G-G_t transition. The appearance of large aggregates was confirmed by sedimentation analysis. The sedimentation constants obtained for G- and G_t-actin equalled 3 and 20 S, respectively. The aggregates (20 S) are stable with respect to time and their characteristics do not depend upon the concentration of ATP and Ca^{2+}, nor do they depend on whether heat denaturation was performed in the presence or absence of dithiothreitol. The fluorescence characteristics of G_t-actin are not affected either by the concentration of G-actin from which it was prepared or by the dilution of G_t-actin from 2.0 to 0.3 mg/ml. The ultracentrifugation patterns (fig. 7), as well as the independence of the sedimentation constants and some fluorescence characteristics from the conditions of heat denaturation, lead to the conclusion that G_t-actin represents not a statistical collection of aggregates of different molecular weights, but rather a fairly homogeneous supermolecular structure. Aggregation apparently accounts for the complete irreversibility of the process of thermal inactivation of rabbit skeletal muscle actin . The transition of the G_t-form into native G-actin did not occur even under the conditions in which it had been reportedly [6] observed for heat-inactivated bovine cardiac actin. According to recent data [13], actin consists of two domains. However, the G-G_t transition cannot be viewed as the complete unfolding of one of the domains with the other remaining compact, because in this case the G-G_t transition will inevitably lead to an increase in mobility of tryptophan. As shown in ref. 14, both actin domains participate in the G-G_t transition. Thus, the two-step character of the dependence of the fluorescence spectral position upon urea concentration (see also ref. 15) is caused not by the successive unfolding of the two domains of the actin macromolecule, but by the transition into a completely unfolded state in 8 M urea via the intermediate state G_t.

3.3. Intramolecular mobility of the tryptophan residues in G_t-actin

The increased degree of fluorescence polarization in the case of denatured actin is due not only to the aggregation of separate molecules – that excludes the motion of macromolecules – but also to a decrease in the level of tryptophan residues' intramolecular mobility in G_t-actin as compared to that in native actin. The independence of the fluorescence polarization of G_t-actin from the viscosity of the solution indicates that there is no intramolecular mobility of tryptophan residues, the correlation time of which is of the same order as the lifetimes of their excited states. The absence of such intramolecular mobility in the G_t-form is the most striking difference between the G_t-state and the unfolded form in 8 M urea (fig. 6).

The zero slope of the Perrin plot facilitates the precise determination of the segment intersecting the ordinate ($1/P_0'$). The value of $1/P_0'$, which is a direct feature of the amplitude of the high-frequency intramolecular mobility of tryptophan residues [16], proved to be lower for actin in the G_t-form ($1/P_0' = 4.9$) than for G- and F-actin ($1/P_0' = 5.9$) (fig. 6). This means that the G-G_t transition is accompanied by a decrease in the intramolecular mobility of tryptophan residues.

The most probable cause of the differences between $1/P_0'$ and $1/P_0$ (P_0, fluorescence polarization of immobilized tryptophan; $P_0 = 0.256$ [18]) is the high-frequency rotational oscillations of the indole ring of tryptophan residues about the C_β–C_γ

bond [16,18]. The amplitude of such oscillations $\sqrt{\overline{\phi^2}}$ can be evaluated from the equation [16–18]:

$$\frac{1/P_0' - 1/3}{1/P_0 - 1/3} = \frac{1}{1 - (3\sin^2\alpha)\overline{\phi^2}}, \quad (1)$$

where α is the angle between the axis of rotation and the direction of oscillator ^1L_a (see also fig. 2 in ref. 18). $\sqrt{\overline{\phi^2}}$ was shown to be approx 22° for G- and F-actin and 17° for G_t-actin. The lack of dependence of the intramolecular mobility upon solvent viscosity and the smaller amplitude of the high-frequency mobility, as compared with that of the native state, sharply differentiate the properties of G_t-actin from those of other proteins in the intermediate state. According to data in the literature [2–4], the intermediate state is characterized by a strongly fluctuating tertiary structure which is also called the molten globule state [3,4]. The reason for this difference probably lies in the additional structural formation associated with molecular aggregation in the G_t-state.

The results of a comparison of the intramolecular mobility of various forms of actin confirm the previous conclusion [20] that inner tryptophan residues are often more mobile than those with a microenvironment composed of polar protein groups, the interaction with which ensures the structure's high rigidity.

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