Accelerated Publications

The Place of Inactivated Actin and Its Kinetic Predecessor in Actin Folding−Unfolding†

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ABSTRACT: The kinetics of actin unfolding induced by guanidine hydrochloride of different concentrations was studied. The parametric representation of the kinetic dependencies of tryptophan fluorescence intensity changes recorded at two wavelengths allowed us to detect and characterize a new essentially unfolded kinetic intermediate. Its characteristics suggested that this intermediate state is a premolten globule. It was shown that the equilibrium transition between inactivated and completely unfolded states is also a two-step process and proceeds via an essentially unfolded kinetic intermediate. The new kinetic pathway of actin unfolding−refolding was proposed. According to it, the founded essentially unfolded kinetic state is the on-pathway intermediate, while inactivated actin is the off-pathway misfolded state stabilized by aggregation of partially folded macromolecules of protein.

Actin is one of the main proteins of the muscle system and cytoskeleton of nonmuscle eukaryotic cells (*1*−*3*). At low ionic strength, actin exists as a monomer (G-actin). In the presence of neutral salts, it polymerizes, forming double-stranded polymer (the so-called fibrous form of actin, or F-actin). F-actin forms the backbone of thin filaments of muscle fibers. The 3D structure was determined for complexes of actin with proteins which prevent its polymerization, namely, with DNase I (*4*), helsolin (*5*, *6*), and profilin (*7*), as well as for ADP-actin, modified by tetramethylrhodamine-5-maleimide (*8*). The G-actin globule (42 kDa) is formed by a single polypeptide chain containing 375 amino acids. The actin monomer consists of two major domains, each of which is divided into two subdomains. Actin is known to bind one molecule of ATP (or ADP) and one divalent cation. In vitro, Mg^{2+} is usually changed by Ca^{2+}. Nucleotide and metal ion are located in the cleft between the two domains. Actin contains four tryptophan residues. All tryptophan residues are located in subdomain I.

The release of calcium ion by EDTA or EGTA treatment leads to the transformation of G-actin into the inactivated form (I), in which the protein molecule loses its capability to polymerize (*9*, *10*). Inactivated actin also can be obtained as a result of heat denaturation (*9*−*21*), moderate urea or GdnHCl[1] concentration (*10*, *18*), dialysis from 8 M urea or 6 M GdnHCl (*18*, *19*), or spontaneously during storage (*18*). It has been shown that inactivated actin is a thermodynamically stable monodisperse associate, consisting of 15 monomers (*22*), and that properties of inactivated actin are invariant to the way of denaturation (*10*, *22*, *23*). Inactivated actin has a unique structure: the hydrophobic clusters are on the surface of the associate, while the polar regions, including tryptophan residues, are in the interior, inaccessible to solvent regions of the associate (*10*, *22*, *23*).

The study of the structure and pathways of the formation of denaturated partially folded aggregated (associated) forms of proteins not only is important for solving fundamental problems of protein folding, but also is essential practical value for medicine (the reasons for diseases connected with the disruption of protein folding) and for biotechnology (the formation of misfolded aggregated forms of recombinant proteins and their accumulation in inclusion bodies). Inactivated actin is a very promising model for such type of investigation.

The kinetic study of the change of actin structure induced by GdnHCl of different concentrations allowed us to

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[1] Abbreviations: UV, ultraviolet; GdnHCl, guanidine hydrochloride; N, native; U, unfolded; I, inactivated actin.

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conclude that essentially unfolding occurs in the pathway of the transition from the native to the inactivated state, and that the inactivated state is not intermediate between the native and completely infolded states, as has been believed previously (*24*).

The aim of present work was to examine the properties of the found kinetic intermediate, the predecessor of inactivated actin, and to elucidate the place of inactivated actin and its kinetic predecessor in the processes of actin folding−unfolding.

MATERIALS AND METHODS

Preparations. Rabbit skeletal muscle actin was purified by the standard procedure of (*25*). 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. Actin was purified by one or two cycles of polymerization−depolymerization, using 30 mM KCl for polymerization. Actin samples with the *A* parameter not lower than 2.53, which corresponds to the content of inactivated actin not higher than 4% (*26*), were used. Actin concentration was determined by a spectrophotometer (Hitachi, Japan). The molar extinction constant for actin was taken as $E_{280} = 1.09$ (mg/mL)$^{-1}$·cm^{-1} (*27*). The final actin concentration in kinetic experiments varied from 0.1 to 0.44 mg/mL. GdnHCl (Nacalai Tesque, Japan) was used without additional purification. The concentration of GdnHCl was determined by refraction index with an Abbe refractometer (LOMO, Russia). In the refolding experiments, the appropriate concentrations of GdnHCl were obtained by dilution of actin solution in 4 M GdnHCl.

Fluorescence Measurements. Fluorescence experiments were carried out using the spectrofluorimeter with steady-state excitation (*28*). Fluorescence was excited at the long-wave absorption edge where the contribution of tyrosine residues in the bulk protein fluorescence is negligible. 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 $\lambda_{ex} = 297$ nm (*28*). The values of parameter *A* and of the fluorescence spectrum were corrected by the instrument sensitivity.

Kinetics Measurements of Actin Folding−Unfolding. All kinetic experiments were performed in micro cells 101.016-QS 5 × 5 mm (Hellma, Germany). Unfolding of the protein was initiated by manual mixing of protein solution with buffer containing the corresponding amount of GdnHCl or EDTA; 350 μL of the GdnHCl or EDTA solution of appropriate concentration was injected in the cell with 50 μL of the solution of native protein. The experiments on inactivated actin folding−unfolding were performed similarly; 350 μL of the GdnHCl solution of appropriate concentration, or buffer was injected in the cell with 50 μL of the solution of inactivated (1.8 M GdnHCl) or completely unfolded (4.0 M GdnHCl) actin, respectively. The dead time was determined from the control experiments. To this end, 350 μL of buffer G was injected in the cell with 50 μL of the solution of native protein, and 350 μL of 4.0 M GdnHCl was injected in 50 μL of the solution of protein in 4.0 M GdnHCl. These experiments showed that the dead time of kinetic measurements was less than 4 s. The spectrofluorimeter was equipped with a thermostat that held a constant temperature of 23 °C in the cell and in the special box where the solutions are held before mixing. The parametric relationship between I_{320} and I_{365} was constructed using the experimental kinetic curves of fluorescence intensities successively registered at 320 and 365 nm.

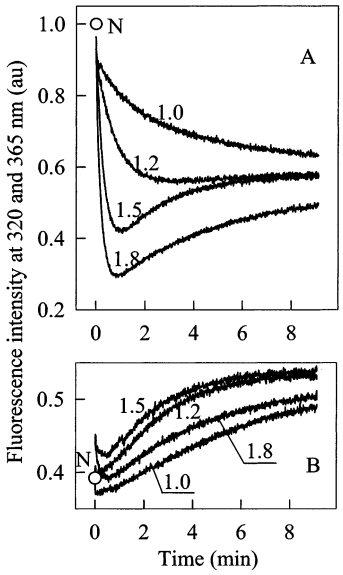


FIGURE 1: Kinetics of actin unfolding induced by GdnHCl. The change of intrinsic fluorescence intensity was monitored at 320 (A) and 365 nm (B). The values on the curves are the concentrations of GdnHCl. Protein concentration was 0.44 mg/mL, $\lambda_{ex} = 297$ nm.

RESULTS

The kinetics of actin unfolding induced by GdnHCl or EDTA of different concentrations and the kinetics of reversible transition inactivated−unfolded actin induced by the change of GdnHCl of different concentrations were studied. The kinetics of the fluorescence intensity changes at 320 and 365 nm were recorded.

Figure 1 shows the kinetic curves of fluorescence intensity changes induced by GdnHCl of different concentrations recoded at 320 and 365 nm. It was found that the changes at 320 nm are much greater than those at 365 nm. The kinetic curves of fluorescence intensity at 320 nm have a minimum at a GdnHCl concentration in the range 1.2−1.8 M.

Figure 2 represents the change of fluorescence intensity recoded at 320 nm in the processes of inactivated actin unfolding, when the concentration of GdnHCl was changed from 1.8 to 4.0 M (panel B), and partial folding, when the GdnHCl concentration was diminished from 4.0 to 1.8 M by dilution (panel C). For comparison, the change of fluorescence intensity on the unfolding of native actin (concentration of GdnHCl changed from 0.0 to 4.0 M) is presented (panel A). Figure 2 shows that the process of inactivated actin unfolding is much slower than that of native actin, and even after 24 h it is not completely unfolded. The fluorescence characteristics of partially folded actin obtained by protein transition from 4.0 to 1.8 M GdnHCl are the same as for the inactivated one.

The obtained kinetic data of fluorescence intensity were used for construction of the parametric relationship between I_{320} and I_{365} nm (Figures 3−5). This allowed us to evaluate

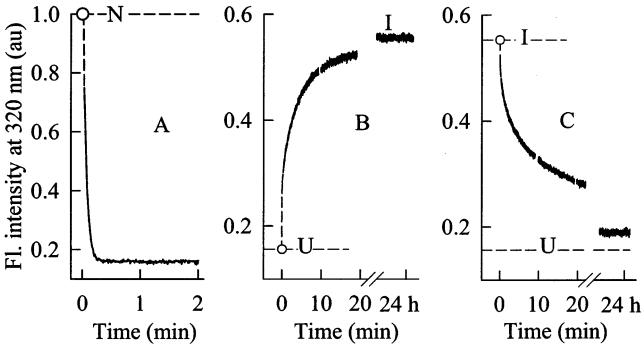


FIGURE 2: Inactivated actin unfolding−refolding induced by the change of GdnHCl concentration. Kinetics of the change of fluorescence intensity at 320 nm are shown. (A) Native actin unfolding induced by 4.0 M GdnHCl; (B) unfolded actin refolding induced by the change of GdnHCl concentration from 4.0 to 1.8 M; (C) inactivated actin unfolding induced by the change of GdnHCl concentration from 1.8 to 4.0 M GdnHCl. Protein concentration was 0.44 mg/mL, $\lambda_{ex} = 297$ nm.

whether the transition is one- or multi-step and to characterize the kinetic intermediate (see below).

DISCUSSION

In our previous work (*24*), it has been shown that when native actin (N) is treated with GdnHCl an essential unfolding of the protein's macromolecule and the appearance of a kinetic intermediate (U*) precede the formation of inactivated actin (I):

$$\mathrm{N} \xrightarrow{k_1} \mathrm{U^*} \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \mathrm{I} \qquad (1)$$

This conclusion was done on the basis of the character of kinetic curves of fluorescence intensity recorded at 320 nm, parameter *A*, and fluorescence anisotropy. All these dependencies at the final concentration of GdnHCl in the range of 1.2−1.8 M had a minimum, the formation of which was induced by the accumulation of the kinetic intermediate U* in the early stage of protein unfolding. In comparison with native and inactivated actin, this intermediate has considerably lower fluorescence intensity I_{320}, a considerably lower value of fluorescence anisotropy, and a considerably more red fluorescence spectrum (lower value of parameter *A*). The calculation of the rate constants k_1, k_2, and k_3 was done with the assumption that the fluorescence intensity I_{320} of the kinetic intermediate U* and that of the completely unfolded state of actin U coincide and give a good congruence between simulated and experimental kinetic dependencies of fluorescence intensity. One of the aims of this work was to characterize the fluorescence properties of actin in the state U* as far as possible, and to elucidate whether the fluorescence spectra of actin in the states U and U* coincide or are distinguishable. To this end, the kinetic curves of fluorescence intensity were recorded (Figure 1), and on the basis of these curves, the parametric relationship between fluorescence intensity at 320 and 365 nm was constructed (Figure 3). Before analyzing the obtained dependencies, it is necessary to elucidate the main point of approach used in this work.

Construction of the Parametric Relationship between Two Independent Extensive Characteristics of the System as an Approach for Protein Folding−Unfolding Examination. Any extensive characteristic of the system consisting of two components is determined by the simple equation:

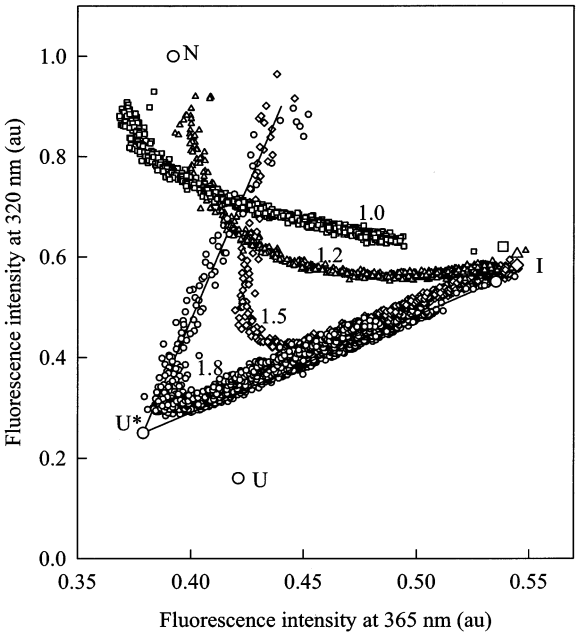


FIGURE 3: Parametric relationships between I_{320} and I_{365} nm, characterizing kinetics of actin unfolding induced by GdnHCl. The values on the curves are the concentrations of GdnHCl. The parameter is the time interval from the beginning of unfolding. The majority of experimental points correspond to the first 10 min after mixing (the corresponding kinetic curves are shown in Figure 1). The final points (symbols of larger size) correspond to 24 h. Fluorescence characteristics of native (N), essential unfolded intermediate (U*), inactivated (I), and completely unfolded (U) actin are indicated. The two solid lines approximate the linear portions of the parametric dependence, which characterize the processes N* → U* and U* → I at 1.8 M GdnHCl. Protein concentration was 0.44 mg/mL, $\lambda_{ex} = 297$ nm. The fluorescence intensity of native actin at 320 nm was taken as unity.

$$I(\theta) = \alpha_1(\theta)I_1 + \alpha_2(\theta)I_2 \qquad (2)$$

where I_1 and I_2 are the values of $I(\theta)$ at 100% content of the first and the second component, respectively, and $\alpha_1(\theta)$ and $\alpha_2(\theta)$ are the relative fractions of the components in the system, $\alpha_1(\theta) + \alpha_2(\theta) = 1$, where θ is any parameter depending on which content of the components is changed. Denaturant concentration, temperature, pH of the solution, etc. can be taken as a parameter. Only for extensive characteristics which give quantitative characterization of the system is eq 2 valid, and the fraction of components in the system, as well as the equilibrium constant *K*, can be determined by simple equations:

$$\alpha_1(\theta) = \frac{I(\theta) - I_2}{I_1 - I_2}$$

$$\alpha_2(\theta) = \frac{I_1 - I(\theta)}{I_1 - I_2}$$

$$K(\theta) = \frac{I_1 - I(\theta)}{I(\theta) - I_2} \qquad (3)$$

If intensive characteristics [such as fluorescence spectrum

position, parameter *A* (*28*), fluorescence anisotropy, etc., which characterize the system qualitatively] are used, these equations for the determination of $\alpha_1(\theta)$, $\alpha_2(\theta)$, and $K(\theta)$ are not valid (*29*), although often no account is taken of this in the investigations of protein conformation transition.

For any two independent extensive characteristics, we have

$$I_1(\theta) = \alpha_1(\theta)I_{1,1} + \alpha_2(\theta)I_{2,1} \quad (4)$$

and

$$I_2(\theta) = \alpha_1(\theta)I_{1,2} + \alpha_2(\theta)I_{2,2} \quad (5)$$

Eliminating $\alpha_1(\theta)$ and $\alpha_2(\theta)$ from eqs 4 and 5, we can obtain the relationship between $I_1(\theta)$ and $I_2(\theta)$:

$$I_1(\theta) = a + bI_2(\theta) \quad (6)$$

where

$$a = I_{1,1} - \frac{I_{2,1} - I_{1,1}}{I_{2,2} - I_{1,2}}I_{1,2}$$

and

$$b = \frac{I_{2,1} - I_{1,1}}{I_{2,2} - I_{1,2}}$$

Equation 6 means that if with the change of parameter θ the transition between states 1 and 2 follows the model "all-or-none" without formation of the intermediate states, then the parametric relationship between any two extensive characteristics must be linear. If the experimentally recorded parametric relationship between two extensive characteristics of the system is not linear, it unequivocally means that the process of the transition from the initial to the final state is not a one-stage process but proceeds with the formation of one or several intermediate states.

The analysis of the protein conformational transition based on the construction of a parametric relationship between the fluorescence intensity recorded at two different wavelengths was proposed as early as 1976 by Burshtein (*30*), but in succeeding years was used only a few times (see, e.g., *31*). We used this approach to prove the multitude of the intermediate states which appear on the denaturation of carbonic anhydrase II (*32*) and creatine kinase (*33*) by GdnHCl. To analyze kinetic data, this approach is used for the first time in this work.

Parametric Relationship between I_{320} and I_{365}, Constructed on the Basis of the Registered Kinetic Process of GdnHCl-Induced Actin Unfolding. In these experiments, the fluorescence intensities recorded at wavelengths of 320 and 365 nm were used as independent extensive characteristics, and the time from the moment of protein solution mixture with GdnHCl solution of appropriate concentration was taken as a parameter. The results of previous investigation of the actin denaturation induced by GdnHCl (*24*) suggested that the rate constant of the process N → U* (k_1) increases with the increase of the GdnHCl final concentration, while the rate constant of inactivated actin formation, U* → I (k_2), decreases. The relation between k_1 and k_2 is that the processes N → U* and U* → I are separated in time when the concentration of GdnHCl in solution is 1.8 M. Therefore, the corresponding parametric relationship between I_{320} and I_{365}, given in Figure 3, can be approximated by two straight lines.

It is evident that the intersection point of these two lines characterizes the fluorescence properties of actin in the U* state. The mean values of the fluorescence intensities of actin in the U* state are $(I_{U^*}/I_N)_{320} = 0.25$ and $(I_{U^*}/I_N)_{365} = 0.97$, while for actin in the completely unfolded state these values are 0.16 and 1.08, respectively. The experimental measurements of kinetic dependencies allowed us to conclude that $A_{U^*} > A_U$, $I_{320,U^*} > I_{320,U}$, and $I_{365,U^*} < I_{365,U}$. The last may be because the intrinsic tryptophan residues Trp 340 and Trp 356, which contribute significantly to the fluorescence spectrum of native actin (*34*), retain in this state slightly more blue fluorescence spectrum than that in the unfolded state U, while tryptophan residues Trp 79 and Trp 86, whose fluorescence spectrum is red-shifted, but the contribution to the fluorescence spectrum of native actin is small, in the U* state remain quenched by sulfur atoms of cysteine and methionine residues.

It was found that the parametric relationships between I_{320} and I_{365} originate not from one point that corresponds to the native state of actin in the absence of GdnHCl, but from different points (Figure 3). It means that the change of the solution leads to a rapid change of the fluorescence properties of actin, some of which can be fixed even with manual mixing of the protein solution with GdnHCl by registering the fluorescence intensity at 365 nm (Figure 1B). Now it is not clear whether the registered effects are determined by the structural change of the protein or simply by the change of the solution content, that affects the fluorescence properties of Trp 79, that is partially exposed to the solvent though its contribution to the bulk fluorescence of actin is not large (*34*). The final points corresponding to 24 h after mixing (Figure 3, symbols of larger size) also do not coincide. Most likely, it is caused by the dependence of inactivated actin fluorescence properties from the GdnHCl content in solution. The existence of such a dependence for inactivated actin was shown in the steady-state experiment for GdnHCl concentration from 0 to 1.8 M (*24*).

The obtained data allowed us to conclude that the fluorescence properties of the kinetic intermediate U* differ from those of actin in 4−6 M GdnHCl. The kinetic intermediate has more blue fluorescence spectrum in comparison with the completely unfolded state of actin. Earlier it has been shown that in the U* state elements of secondary structure are retained (*24*). At the same time, ANS does not bind with actin in this state. In total, these data make it reasonable to suppose that the U* state is a premolten globule (*35*, *36*).

With the decrease of GdnHCl concentration, the parametric relationships between I_{320} and I_{365} differ more and more from that obtained for 1.8 M GdnHCl (Figure 3). At lower concentrations of GdnHCl, linear portions of the parametric relationships between I_{320} and I_{365} could not be distinguished. It means that at any time interval there are more than two components. Nevertheless, their character witness that the process of inactivate actin formation goes via the state of essential protein unfolding, though the lifetime of this state decreases with the decrease of the final concentration of GdnHCl.

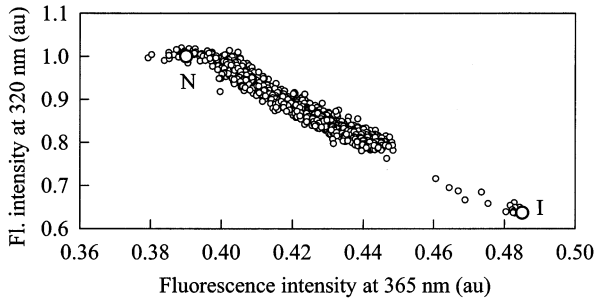


FIGURE 4: Parametric relationship between I_{320} and I_{365}, characterizing the kinetics of native actin unfolding induced by EDTA. The majority of experimental points correspond to the first 10 min after mixing. The final point (symbol of larger size) corresponds to 24 h. The final concentration of EDTA was 15 mM; protein concentration was 0.44 mg/mL, $\lambda_{ex} = 297$ nm. The fluorescence intensity of native actin at 320 nm was taken as unity.

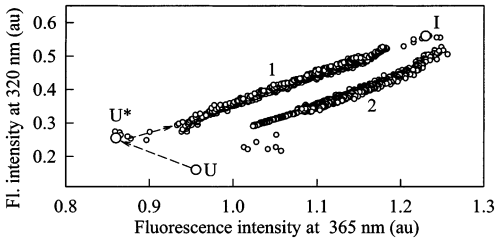


FIGURE 5: Parametric relationships between I_{320} and I_{365}, characterizing refolding of the unfolded actin (curve 1) and unfolding of the inactivated actin (curve 2) induced by the change of GdnHCl concentration from 4.0 to 1.8 M and from 1.8 to 4.0 M, respectively. The time interval of fluorescence intensity recorded at I_{320} and I_{365} is the same as in Figure 2. Protein concentration was 0.44 mg/mL, $\lambda_{ex} = 297$ nm. The fluorescence intensity of native actin at 320 nm was taken as unity.

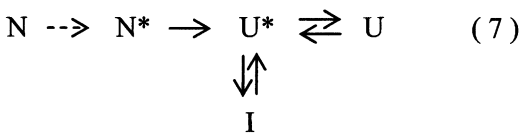
Does the Linearity of the Parametric Relationship between Two Extensive Characteristics Always Suggest One-Step Character of the Conformational Transition? Criteria of One- and Not-One-Step Character of the Transition. It is necessary to mention that the existence of linearity of the parametric relationship does not necessary indicate that the transition is of one-step character. The parametric relationship between two extensive characteristics of the system can be practically linear though the transition between conformational states proceeds via an intermediate state in the following cases: (1) if the values of registered characteristics of the intermediate state are close to those of the initial or final states; (2) if the values of registered characteristics of the intermediate state are somewhat between those of the initial or final states (*32*); (3) if the rate of the transition from the intermediate to the final state is much larger that that of the formation of the intermediate state ($k_2 \gg k_1$).

In such cases, the conclusion about the existence of intermediate states can give only systematic analysis of all experimental data on the structural change of this protein. For example, the conclusion about the processes that go on when actin inactivation is induced by EDTA can be done on the basis of all experimental data on actin unfolding induced by GdnHCl. With the decrease of GdnHCl concentration, the rate of the N $\rightarrow$ U* process also decreases, while the rate of the U* $\rightarrow$ I process increases (*24*). Actin unfolding by EDTA can be regarded as a limiting case of the $N \xrightarrow{k_1} U^* \xrightarrow{k_2} I$ transition, when $k_1 \ll k_2$. This is the reason the parametric relationships between I_{320} and I_{365}, constructed on the basis of kinetic dependencies of fluorescence intensities at these two wavelengths (Figure 4), are linearly independent of EDTA concentration. Similarly, there are no grounds to consider the N $\rightarrow$ U transition (Figure 2A), when native actin is transferred in the solution with high concentration of GdnHCl (4−6 M) by a one-step transition, if kinetic intermediate U* formation is proved for the transition induced by lower GdnHCl concentration.

The registered characteristics are mostly adequate for the determination of intermediate states if the values of these characteristics for the intermediate state are larger (or smaller) than their values for both initial and final states. Particularly, the fluorescence intensity of the hydrophobic dye ANS is extremely sensitive to the formation of intermediate states because its value for the intermediate partially folded states is significantly larger than those for both native and completely unfolded states. The possibility of intermediate state determination essentially depends on its lifetime and the relation between the rate constants of its formation and degradation. The intermediate state can be determined if the rate constant of its formation is larger than that of its degradation.

Kinetics of the Inactivated Actin−Completely Unfolded Actin Transition. The examination of the unfolding−refolding process of actin and the place of inactivated actin and its kinetic predecessor in these processes would be incomplete without the consideration of the transition between inactivated and completely unfolded states. The results of steady-state experiments (*10*, *23*) indicate that this process is reversible and occurs in the interval of GdnHCl concentration from 1.8 to 3.5 M with the middle point at 2.5 M. The kinetic measurements of the changes of fluorescence intensity when the GdnHCl concentration was changed from 1.8 to 4.0 M and vice versa performed in the present work proved the conclusion made earlier (Figure 2B,C). But it was found that U $\rightarrow$ I transition is faster than the inverse process I $\rightarrow$ U. The unfolding of inactivated actin is incomplete even within 24 h after its transformation to 4 M GdnHCl (Figure 2C). Interestingly, the complete unfolding of native actin after its transformation to 4 M GdnHCl is completed within first 10 s. Both I $\rightarrow$ U and U $\rightarrow$ I processes are likely to be not-one-step processes as evidenced by the character of parametric relationships between I_{320} and I_{365} (Figure 5) and by the impossibility of representing them by monoexponential law to which the kinetics of one-step processes are followed. It seems likely that the transition from completely unfolded state to inactivated passes through the kinetic intermediate U* (Figure 5, curve 1). When the mixture of the solution is done manually, this U $\rightarrow$ U* process occurs within the dead time of experiment. Probably, the dependence of inactivated actin fluorescence characteristics on GdnHCl concentration (*24*) dictates the existence of the fast phase of the process I $\rightarrow$ U and the displacement of the plot characterizing unfolding (curve 2) versus that corresponding to the refolding (curve 1) process in Figure 5.

New Kinetic Scheme of Actin Folding−Unfolding. The total of all the obtained data allowed us to propose the new kinetic pathway of actin unfolding−refolding induced by GdnHCl:



(7)

In this scheme, the state N* precedes the transformation of native actin into the essentially unfolded state (U*). There is evidence that the fluorescence properties of N* depend on GdnHCl concentration. However, this state is far from complete understanding and characterization. At the same time, the formation of the essentially unfolded state (U*) preceding the completely unfolded (U) or inactivated actin (I) is proven. In the processes of folding−unfolding, the essentially unfolded state (U*) is an on-pathway intermediate, while inactivated actin (I) is an off-pathway misfolded state, stabilized by aggregation of partially folded macromolecules of protein. These results may also be essential for the search of the pathways of actin folding in vitro.

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