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Actin Folding, Structure and Function: Is It a Globular or an Intrinsically Disordered Protein?

Olga I. Povarova¹, Anna I. Sulatskaya¹,
Irina M. Kuznetsova^{1,2} and Konstantin K. Turoverov¹

¹Laboratory of Structural Dynamics, Stability and Folding of Proteins,
Institute of Cytology RAS,

²St. Petersburg State Polytechnical University, St. Petersburg,
Russian Federation

1. Introduction

Actin is the most abundant protein in eukaryotic cells. This protein is found in every living cell, but it is most plentiful in muscle cells, from which it was isolated more than 60 years ago (Straub, 1942). Since that time the continuous flow of investigations was started, involving more and more scientists. Even today, scientific interest in actin has not died down but is, rather, continuing to make noteworthy advances, as can be observed from the Scopus database (Figure 1). Initially, these investigations focused on muscle actin, with an emphasis on its ability to polymerize and to interact with the other main muscle protein, myosin, and several other regulatory proteins that control muscle relaxation and contraction. In addition, researchers examined the role of ATP hydrolysis as a source of energy and that of the bivalent cation Mg^{++} , an essential component of the polymerization-depolymerization process, that is replaced by Ca^{++} in vitro. In the early 1970s, it became evident that actin is an abundant protein, not only in muscle cells but also in every other type of eukaryotic cell. The main role of actin in non-muscle cells is the formation of the cytoskeleton, which functions to enable cell motility and inter-cell interaction. Further investigations showed that actin participates in many other vitally important cellular processes. Although the first report of actin in the nucleus appeared at virtually the same time as the discovery of the protein in the cytoplasm, the former observation was presumed to be an artifact. It was only recently that actin was proven to be an integral component that plays a key role in the nucleus, as it does in the cytoplasm. The actin role in nucleus begins with the nuclear scaffold formation and ends with the transcription process. Furthermore, it became evident that globular (G-) actin in nuclei participates in transcription and chromatin remodeling.

A large amount of data regarding actin has accumulated, with specific topics that include the structure of G-actin; the polymerization of G-actin to form fibrillar (F-) actin and the depolymerization of F-actin; the participation of the protein in muscle contraction; in the formation of the cytoskeleton in non-muscle cells; the localization of actin in different cell

compartments and its role there; and its interactions with numerous partners, of which more than 60 classes are currently known. These topics are discussed in multiple research works and a series of modern reviews (see, e.g., (dos Remedios et al., 2003; Winder & Ayscough, 2005; Reislter & Egelman, 2007; Schleicher & Jockusch, 2008; Vartiainen, 2008; Zheng et al., 2009; Skarp & Vartiainen, 2010; Wang et al., 2010; Dominguez & Holmes, 2011; Schoenenberger et al., 2011)). Therefore, in the present work, these questions will be discussed rather briefly, and the primary focus will be on the processes of actin folding and unfolding and on the validation of the hypothesis that G-actin belongs to the so-called “intrinsically disordered” (ID) proteins.

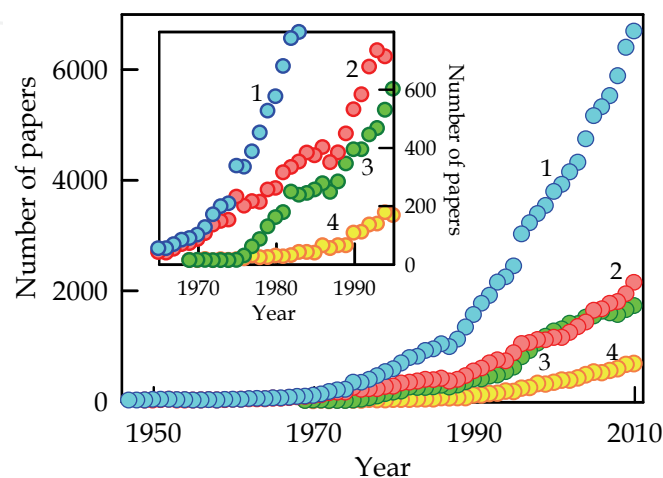


Fig. 1. The increase of researchers' interest in actin. The graph illustrates the increase in the total number of publications on actin (1, blue), with separate tracks showing the number of publications on muscle (2, red), cytoplasmic (3, green) and nuclear (4, yellow) actin from 1950 to the present.

2. Actin structure

Actin is a highly conserved protein of approximately 42 kDa, and its polypeptide chain consists of 375 amino acids (Oosawa, 1971). There are three isoforms of actin that are produced by different genes (alpha, beta and gamma), all of which are polymorphic proteins that are capable of polymerization. The actin isoforms differ by only a few amino acids, with most of the variation occurring toward the N-terminus (Herman, 1993). A distinctive feature of actin is its ability to polymerize. At low ionic strength *in vitro*, actin exists as a monomer (G-actin). In the presence of neutral salts, the protein polymerizes to form a single-stranded polymer (the so-called fibrous form of actin, or F-actin). F-actin forms the backbone of the thin filaments of muscle fibers and of the cytoskeleton. The tendency of actin to polymerize prevents the formation of its crystals, which is a requirement for 3D structure resolution via X-rays. However, actin loses its ability to polymerize after forming a complex with DNase I and/or other actin-binding proteins (ABPs). Actin was crystallized for the first time in complex with DNase I (Kabsch et al., 1990). This accomplishment was an important breakthrough in the study of actin structure. The actin monomer was revealed to be a relatively flat molecule of dimensions 55x55x35 Å. Actin folds into two major α/β -domains (Figure 2), as do other proteins of the structural superfamily to which the sugar kinases, hexokinases and Hsp70 proteins belong (Dominguez and Holmes, 2011). Each large

domain consists of two subdomains, and traditionally, a four-subdomain nomenclature has been adopted (Kabsch et al., 1990). Subsequently, actin structures with certain other ABPs were determined. Nonetheless, it was unclear whether the structure of actin in complex with ABPs differs from that of free, native actin. This problem was solved when the structure of actin with a small molecule (ADP-actin with tetramethyl-rhodamine-5-maleimide, which prevents actin polymerization) was determined (Graceffa & Dominguez, 2003). To date, over 80 structures of actin in complexes with various ABPs have been reported (Dominguez & Holmes, 2011).

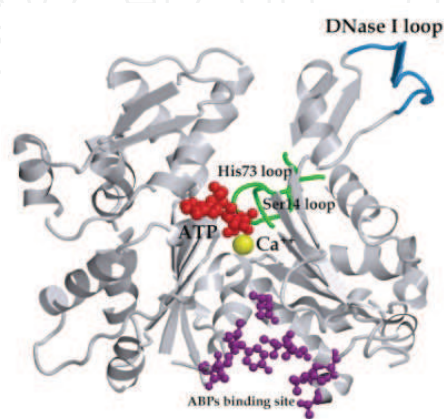


Fig. 2. The structure of the actin molecule. The figure was created on the basis of the PDB data (Dutta et al., 2009), the file 1ATN.ent (Kabsch et al., 1990), using the graphical software VMD (Hsin et al., 2002) and Raster 3D (Merritt & Bacon, 1997). ATP (red), Ca^{2+} (yellow), ATP/ADP sensing loops (green), the DNase I loop (blue) and ABPs binding sites are specially emphasized.

It appeared that irrespectively of modification, bound molecule or nucleotide (ATP or ADP) the conformation of the actin monomer was practically the same except though small but important differences. One of the important variable regions is the so called DNase I loop. This loop includes residues 39-51, which are located at the top of domain 2, and is referred to as the DNase I-binding loop because it is responsible for the formation of the actin complex with DNase I (Kabsch et al., 1990). At the same time this loop plays a critical role in the inter-subunit contacts in the F-actin filament. Any change in this loop, though it may not influence the structure of domain 2, leads to the loss of actin's ability to polymerize. Interestingly, this loop, which forms a β -strand in one crystal structure (Kabsch et al., 1990), was found to be disordered in several other crystal structures (McLaughlin et al., 1993) and to form an α -helix when ADP, rather than ATP, is bound to actin (Otterbein et al., 2001).

The differences between the ATP- and ADP-bound states are relatively minor and primarily involve two loops: the Ser14 β -hairpin loop, which is located in actin subdomain 1, and the sensor loop carrying the methylated His73 (Graceffa & Dominguez, 2003). The nucleotide-dependent conformational changes in these loops, though minor, are very important and can explain how interaction of actin-binding proteins (such as profilin and cofilin) with actin is regulated by the nucleotide bound to actin (Dominguez & Holmes, 2011).

Opposite the large cleft of the actin molecule, there is a smaller cleft. This region participates in the formation of inter-monomer contacts during actin polymerization, when the loop

containing residues 41-45 binds to residues 166-169 and 375 (Kabsch et al., 1990). There are two additional contacts between subdomains 3 and 4: residues 322-325 bind to residues 243-245, and the loop containing residues 286-289 binds to residues 202-204 (Holmes & Kabsch, 1991). This smaller cleft between domains 1 and 3 also appears to be the binding site of several major ABPs. This observation implies that actin bound to an ABP loses its ability to polymerize and can therefore be crystallized. The actin residues that participate in the formation of contacts with ABPs include Tyr143, Ala144, Gly146, Thr148, Gly168, Ile341, Ile345, Leu346, Leu349, Thr351 and Met355 (Figure 2) (Dominguez & Holmes, 2011). This cleft is sometimes referred to as hydrophobic, although not all of the residues mentioned above fit that description. It has been suggested that communication between the two clefts provides the structural basis by which nucleotide-dependent conformation changes modulate the binding affinities of ABPs (Dominguez & Holmes, 2011).

3. Folding of globular proteins

We have recently summarized the main principles of globular protein folding (Povarova et al., 2010). One of the fundamental laws of physics states that an isolated polypeptide chain must attain the conformation that corresponds to the free energy minimum, F :

$$F = H - TS, \quad (1)$$

where H is the enthalpy, the value of which is determined by all the interactions between the atoms of a polypeptide chain, as well as interactions with solvent molecules, and S is the entropy, a measure of the number of conformations, N , which determines the realization of a given protein state: $S = R \ln N$ (in this formula, R is the universal gas constant and T is the absolute temperature). In these terms, protein folding is the process of the attainment by a polypeptide chain of a thermodynamically stable state that corresponds to the global free energy minimum. It is important to remember, however, that alongside the single global minimum, the system can possess a series of local minima. The presence of such local free energy minima reflects the existence of intermediate states between the native (N) and unfolded (U) conformations. The state corresponding to a local minimum can also be a final state of the system if it is separated from other, deeper free energy minima by a high-energy barrier(s).

What is the nature of the state that corresponds to the free energy minimum for a given protein molecule? Will this state be native (biologically active)? To answer these questions, it is necessary to remember another fundamental law of nature - the biological law of evolutionary selection. An amino acid sequence must be composed in such a way that the protein becomes functionally active (native) after reaching the state corresponding to its free energy minimum. Thus, "nonsense" amino acid sequences that are nonfunctional in the state corresponding to the free energy minimum will be rejected by evolutionary selection. However, this theory implies that, if such so-called "nonsense" amino acid sequences are detected, then they likely serve some purpose, though this purpose may be unknown.

The unfolded state is entropically favorable because it represents a dynamic ensemble of a large number of conformations that originate from the rotational isomerization of the main chain. In contrast, any compact state imposes significant restrictions on the conformational freedom of the polypeptide chain and is therefore entropically unfavorable. The capacity of

a given polypeptide chain to attain a compact state is determined by its ability to form intramolecular contacts that compensate for the free energy increase that is caused by the decrease in the entropy component. The compactness of a structure formed by a polypeptide chain is determined by its amino acid composition and sequence. Therefore, depending on the peculiarities of their compositions and sequences, newly synthesized amino acid chains would adopt globular or partially or completely disordered structures. The structures formed by a polypeptide chain in water are significantly different from that of the Gaussian coil. This difference arises because water is a poor solvent for a polypeptide chain, not only due to the existence of numerous hydrophobic amino acid residues but also because water is a poor solvent for the protein backbone. In fact, despite the absence of hydrophobic residues, polar polypeptides (polyglutamine and glycine-serine block copolypeptides) prefer ensembles of collapsed structures in aqueous milieu (Crick et al., 2006; Tran et al., 2008). Furthermore, residual secondary structure is repeatedly found in unfolded globular proteins, even in concentrated solutions of strong denaturants, such as 8 M urea or 6 M guanidine hydrochloride (GdnHCl), which are much better solvents for polypeptide chains than water (Shortle & Ackerman, 2001; Shortle, 2002). Upon being unfolded, many globular proteins can refold into the same native structure, which means that all of the information necessary for a given polypeptide chain to fold into a unique tertiary structure is encoded in its amino acid sequence (Anfinsen et al., 1961). Furthermore, amino acid sequences must also bear information about the pathways of their formation, because otherwise, the random optimization of a polypeptide chain of hundreds amino acids will take billion of years, as was shown by Levintal.

Another special feature of the polypeptide chain of each globular protein is that it proves the existence of a free energy barrier between the native and denatured states (Finkelstein & Ptitsyn, 2002). This circumstance is vital for the correct functioning of globular proteins because only the existence of a free energy barrier between the native and denatured states maintains the conformation of molecules of globular proteins in the native state. This precise circumstance provides the possibility of crystal formation by globular proteins in their native states.

In medias res, protein folding can be regarded as a realization of the second part of the genetic code because the amino acid sequence contains information on the functional 3D structure of the protein. The first part of the genetic code is predominantly solved, but the study of the problem of protein folding is far from completion.

The models of protein folding have changed several times with the progress of experimental studies. According to the current view, protein folding is achieved via various pathways that are determined by the protein's energy landscape. The energy landscape model not only elucidates the mechanisms of globular protein folding but also explains the nature of the so called intrinsically disordered proteins (see Section 5), describes the formation of their supramolecular complexes, and delineates the formation of potentially pathogenic oligomers, amorphous aggregates, amyloid, and amyloid-like fibrils.

4. Actin folding–unfolding

The first investigation of actin folding and unfolding was performed by Lehrer and Kerwar (Lehrer & Kerwar, 1972). In this work, it was shown that the release of calcium ion by EDTA or EGTA treatment leads to the transformation of G-actin into an inactivated form in which

the protein molecule loses its capability to polymerize. For many years, it was generally accepted that actin unfolds via a single intermediate state called inactivated actin (West et al., 1967; Lehrer & Kerwar, 1972; Contaxis et al., 1977; Tatumashvili & Privalov, 1984; Bertazzon et al., 1990; Le Bihan & Gicquaud, 1993; Turoverov et al., 1999a). The investigations of actin unfolding and refolding that we commenced in 1998-1999 led to an essential revision of the concept of inactivated actin (see section 4.1) and of our understanding of the transformations of actin induced by different concentrations of various agents (see section 4.2). In the conclusion of this section, data regarding actin folding *in vivo* will be presented (section 4.3).

4.1 Inactivated actin

The release of calcium ions by EDTA or EGTA treatment does not lead to actin unfolding but renders the protein inactive, i.e., a state in which the molecule loses its ability to polymerize (Lehrer & Kerwar, 1972; Turoverov et al., 1999a). Actin in this state was named inactivated actin (I). Inactivated actin may also be obtained by heat denaturation, exposure to moderate urea or GdnHCl concentrations, dialysis with 8 M urea or 6 M GdnHCl, or spontaneously during storage (Kuznetsova et al., 1988). On the basis of these data, inactivated actin (I) was considered to be an on-pathway intermediate between the native (N) and completely unfolded (U) states.



All equilibrium experiments appeared to support this model. The spectrum of the intrinsic fluorescence of inactivated actin has its maximum at wavelength 340 nm, which is intermediate between that of the native ($\lambda_{\max} = 325$ nm) and completely unfolded protein ($\lambda_{\max} = 350$ nm). The fluorescence red shift can be caused by the polar environment formed by intrinsic residues of the protein (Turoverov et al., 1999a). It has been shown that the microenvironment of tryptophan residues is rather rigid (Turoverov et al., 1999b).

The secondary structure of inactivated actin is substantially distorted. Changes in the far UV CD spectrum caused by protein inactivation are consistent with the partial transformation of α -helices either into a disordered conformation or into the β -structure. The appearance of β -structural elements during denaturation has been described for a number of proteins and has been correlated with protein association or aggregation (Fink, 1998). It was shown that the far UV CD spectra of inactivated actin generated by different denaturing agents are practically identical. Moreover, protein inactivation was accompanied by a considerable increase of the fluorescence anisotropy value ($r = 0.09 \pm 0.01$, 0.17 ± 0.02 and 0.07 ± 0.01 for native, inactivated and unfolded actin, respectively), reflecting a considerable decrease in the internal mobility of the tryptophan residues in the inactivated actin (Kuznetsova et al., 1988).

A Perrin plot ($1/r$ versus T/η dependence, where T and η are temperature and viscosity, respectively) shows that inactivated actin is characterized by the independence of $1/r$ from T/η . This result is consistent with the assumption that inactivation is accompanied by the association of partially folded actin molecules in large particles. The rotational relaxation time of these particles is much greater than the Trp fluorescence lifetime. Interestingly, the Perrin plot measured for F-actin, which is known to be a long, rigid filament comprised of

numerous actin molecules, also has no visible dependence of $1/r$ on T/η . However, the intercepts of the Perrin plot on the Y axis ($1/r_0'$ value) for G- and F-actin practically coincide and exceed that for the inactivated protein (8.3 ± 0.2 and 6.1 ± 0.2 , respectively). This fact indicates that the amplitude of high-frequency intramolecular mobility or the rotational relaxation time of inactivated actin, or both, are much lower than the corresponding values of native actin (Kuznetsova & Turoverov, 1983). Thus, it was shown that the interior of denatured (inactivated) actin has considerable mobility limitation.

The fact that the inactivated actin represents a specific aggregate and that its properties do not depend on the method of its generation was proven by gel-filtration and sedimentation experiments (Kuznetsova et al., 1999). The apparent molecular masses (M^{app}) of native and inactivated actins were determined using a gel-filtration column that was calibrated by a set of 20 native globular proteins with known molecular masses ranging from 5.78 to 660 kDa. The M^{app} for native and inactivated actin was determined as 40 ± 2 and 710 ± 20 kDa, respectively. For the determination of the real molecular mass (M^{true}), the Stokes radii (R_s) of native and inactivated actins were evaluated. These values for native and inactivated actins were determined to be 28 ± 2 and 61 ± 4 Å. Using these values and sedimentation coefficients, the M^{true} values for native and inactivated actin were determined to be 40.5 and 712.6 kDa, respectively (Kuznetsova et al., 1999). One can see that the value calculated for the native actin is consistent with the molecular mass estimated from its amino acid sequence (42.05 kDa). It can also be seen that, within the limits of experimental error, the values of the true molecular mass, calculated for native and inactivated actin from R_s , coincide with the apparent molecular mass values that were measured by gel-filtration.

It should be emphasized that this observation is very important because the use of a size-exclusion column calibrated with a set of native proteins with known M values allows one, in principle, to estimate the molecular mass for the protein of interest. This value should be considered to be an apparent one, as it will be correct only for native globular proteins. In contrast, the R_s value determined chromatographically reflects the real hydrodynamic dimensions of the given protein under the conditions studied, regardless of its conformational state (of course in the case if there is no interaction between the protein and column matrix). It is known that the sedimentation constant is a parameter that can be measured directly. Thus, two quantities that have been used for the calculation of M^{true} are independent of the model. The fact that M^{app} coincides with M^{true} is consistent with the conclusion that inactivated actin has hydrodynamic dimensions typical of the native globular protein with a molecular mass of ~700 kDa, which means that the protein in this form has overall native-like packing density. On the basis of the above experiments, it was concluded that inactivated actin represents an ordered aggregate: a supramolecular, monodisperse complex of 14-16 monomers of partially unfolded actin (Kuznetsova et al., 1999).

The interaction of inactivated actin with the hydrophobic fluorescent probe 1-anilinonaphthalene-8-sulfonic acid (ANS) is particularly interesting. High affinity to ANS is a well-known characteristic of many partially folded protein conformations (Fink, 1995; Ptitsyn, 1995). The formation of a complex between ANS and a protein molecule leads to a considerable increase in the dye fluorescence intensity. It has been established that ANS fluorescence is minimal in a solution with native and completely unfolded actin, whereas the inactivation of the protein is accompanied by a considerable increase in this parameter (Turoverov et al., 1999a).

In view of the finding that inactivated actin represents an ordered aggregate, the dependence of ANS interaction with actin on the concentrations of GdnHCl and urea was studied (Povarova et al., 2010). As can be expected, the intensity of ANS fluorescence weakly depends on the urea concentration, up to the concentration at which the supramolecular complexes are destroyed. However, the dependence of ANS fluorescence on the concentration of GdnHCl was found to follow a curve with maximum in a narrow range of small concentrations of the denaturant. Furthermore, the maximum of light scattering (or even precipitation at high protein concentration) was observed in the same range of GdnHCl concentrations. These findings indicate that inactivated actin forms large aggregates in this narrow range of GdnHCl concentrations and that the affinity of ANS molecules for these aggregates is very high. ANS incorporates into the hydrophobic pockets between the molecules that form the aggregates, resulting in the dramatic increase in its fluorescence intensity.

The protein aggregation was explained by interactions between the GdnHCl cations (GuH^+) and the side-chain $\text{C}=\text{O}$ groups of the glutamic acid, glutamine, aspartic acid and asparagine residues of the actin molecule. The possibility of such interactions has been shown earlier (Anufrieva et al., 1994; Mason et al., 2004). In actin, the number of negatively charged groups from glutamic and aspartic acids (OD2 - 22 groups and OE2 - 28 groups) is greater than the number of positively charged groups from lysine (NZ - 18 groups), arginine (NH1 - 18 groups) and histidine (NE2 - 9 groups). Therefore, the actin molecule is negatively charged (pI 5.07) at a neutral pH. With an increase in the number of GuH^+ ions bound to inactivated actin, the number of positively charged groups increases, and at a certain concentration of GdnHCl (0.2 - 0.3 M), the initially negatively charged molecules become neutral, which leads to their aggregation. Upon further increases in the GdnHCl concentration, the number of positively charged groups on the surfaces of the protein molecules will exceed the number of negatively charged groups. Therefore, the conditions will no longer be favorable for aggregation. This is the reason for the abrupt decrease in light-scattering intensity. The less-abrupt decrease in the intensity of ANS fluorescence in comparison with light scattering as the GdnHCl concentration increases can be explained by the higher affinity of the negatively charged ANS molecules for inactivated actin when it is positively charged, though aggregates are already destroyed (Povarova et al., 2010). Due to the complex process of actin denaturation and the dependence of the transition rates upon the GdnHCl concentration (Kuznetsova et al., 2002; Turoverov et al., 2002), the maximum of ANS fluorescence intensity shifts to a lower concentration of GdnHCl as the incubation time increases. Thus, after 24 h of incubation, the maxima of light scattering and of ANS fluorescence intensity are recorded at practically the same concentrations of GdnHCl as for initially inactivated actin.

Hydrophobic interactions apparently play a significant role in both the initial formation of inactivated actin and the formation of inactivated actin aggregates in the presence of low concentrations of GdnHCl. As mentioned above, due to the existence of hydrophobic pockets in inactivated actin, the ANS fluorescence intensity is 20 times greater in the presence of inactivated actin than in the presence of native actin. Inactivated actin already has hydrophobic clusters on its surface, but molecules of inactivated actin do not “stick together” because the negative charges on their surfaces prevent them from doing so. At low concentrations of GdnHCl, the aggregation of inactivated actin leads to a significant increase in the number of hydrophobic pockets and, consequently, to an increase in the

number of bound ANS molecules, that is detected by an increase in ANS fluorescence. Protein aggregation in the presence of low concentrations of GdnHCl is particularly pronounced for actin because large supramolecular complexes of inactivated actin (Kuznetsova et al., 2002; Turoverov et al., 2002) are involved in the aggregation.

Thus, the study of the characteristics of inactivated actin allowed us to make important conclusions regarding the properties of the molten globule state and of ANS fluorescence. It was shown that low concentrations of GdnHCl can cause the aggregation of proteins in a partially folded state and that the fluorescent dye ANS binds with these aggregates rather than with hydrophobic clusters on the surfaces of proteins in the molten globule state. This finding explains why an increase in ANS fluorescence intensity is often recorded during the process of protein denaturation by GdnHCl, but not by urea. Therefore, what was previously believed to be the molten globule state in the pathway of protein denaturation by GdnHCl in reality, for certain proteins, represents the aggregation of partially folded molecules (Povarova et al., 2010).

4.2 Intermediate states in the pathway of actin unfolding

To clarify the process of actin unfolding, the GdnHCl-induced changes in the intensity of intrinsic fluorescence, the parameter $A = I_{320}/I_{365}$, fluorescence anisotropy and CD were studied (Kuznetsova et al., 2002; Turoverov et al., 2002). The minima in the kinetic dependencies of fluorescence intensity in the range of 1.0 - 2.0 M GdnHCl indicate that the transition from the native to the inactivated state occurs via some essentially unfolded intermediate state of actin. Based on these experimental data, a kinetic scheme for actin unfolding was proposed:



where k_i are the rate constants of the corresponding processes and U^* is an essentially unfolded kinetic intermediate, the fluorescence properties of which are similar to those of the completely unfolded state but the secondary structure is much more ordered. The GdnHCl dependencies of the rate constants, k_1 , k_2 and k_3 , illustrate the conditions in which the essentially unfolded intermediate state can be recorded. At 1.0 - 2.0 M GdnHCl, the value of the rate constant for the transition from native to essentially unfolded actin exceeds that for the following step of inactivated actin formation. This imbalance leads to the accumulation of essentially unfolded macromolecules early in the unfolding process, which in turn causes the minima in the time dependencies of tryptophan fluorescence intensity, parameter A , intrinsic fluorescence spectrum position, and tryptophan fluorescence anisotropy. To examine the properties of the newly identified kinetic intermediate U^* , the predecessor of inactivated actin, and to elucidate the roles of inactivated actin and its kinetic predecessor in the processes of actin folding and unfolding, a parametric representation of the kinetic dependencies of the tryptophan fluorescence intensity changes recorded at two wavelengths was studied (Figure 4A). The use of a parametric relationship between two independent extensive characteristics of the system allowed us to determine whether protein unfolding is a two-state transition or if an intermediate state(s) is formed: if the parametric relationship between any two extensive characteristics is linear, then protein unfolding follows the model "all-or-none"; in contrast, a break in the line indicates the existence of an intermediate state (Kuznetsova et al., 2004).

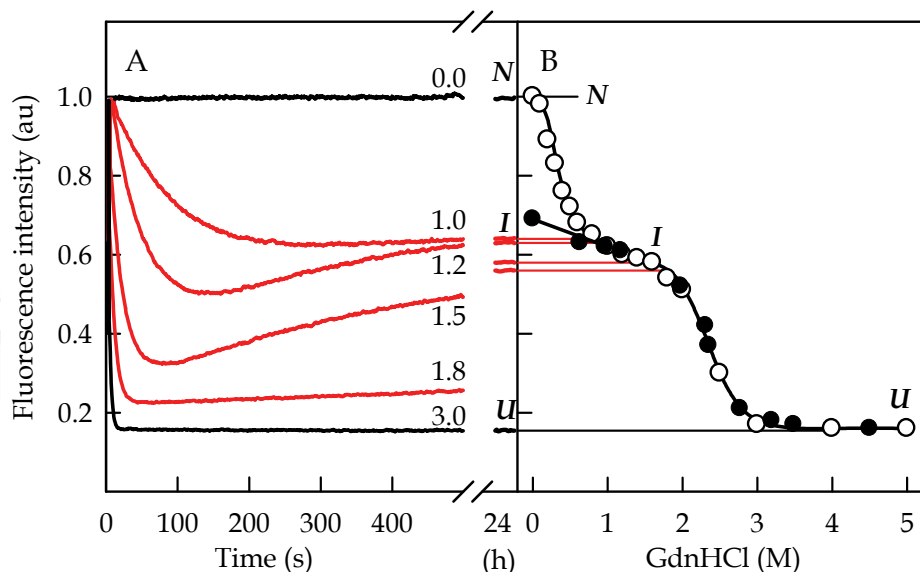


Fig. 3. Actin denaturation monitored by the change of intrinsic fluorescence intensity at 320 nm. A. The kinetics of actin denaturation induced by GdnHCl. The values on the curves indicate the concentration of GdnHCl (M). B. The fluorescence intensity of actin recorded after 24 h of incubation in the listed concentrations of GdnHCl. The open and closed symbols correspond to the unfolding and refolding experiments, respectively. In the refolding experiment, the appropriate concentrations of GdnHCl were obtained by the dilution of the actin solution in 5 M GdnHCl. $\lambda_{ex} = 297$ nm, $\lambda_{em} = 320$ nm. N, I and U indicate the ranges of the predominant content of native, inactivated and unfolded actin, respectively, after 24 h of incubation of actin in solution with appropriate concentrations of GdnHCl. The values on the kinetic curves indicate the concentration of GdnHCl (M).

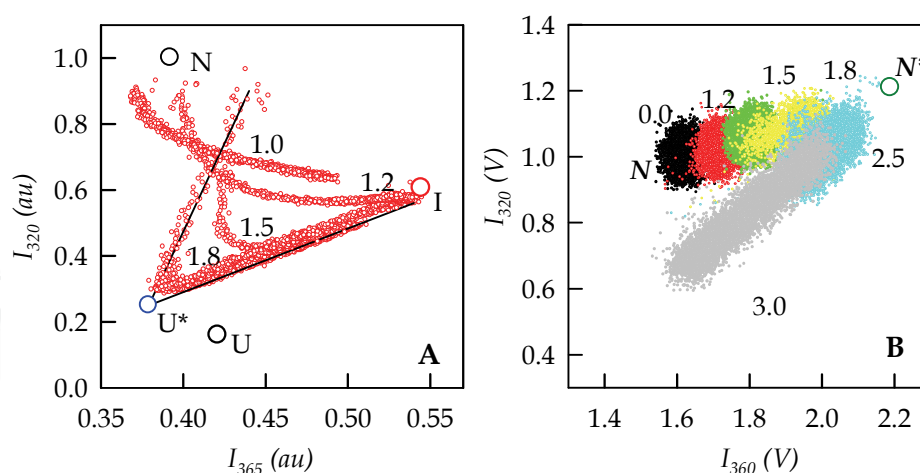


Fig. 4. Actin denaturation induced by GdnHCl. A. Parametric dependencies of fluorescence intensity at 320 and at 365 nm; the parameter is the time from the beginning of denaturation. The averaging time of signal is 0.6 s. The values on the curves indicate the concentration of GdnHCl (M). $\lambda_{ex} = 297$ nm. B. Parametric dependencies of fluorescence intensity at 320 and at 360 nm; the parameter is the time from the beginning of denaturation. The averaging time of signal is 0.1 ms. The values on the curves indicate the concentration of GdnHCl (M). $\lambda_{ex} = 297$ nm.

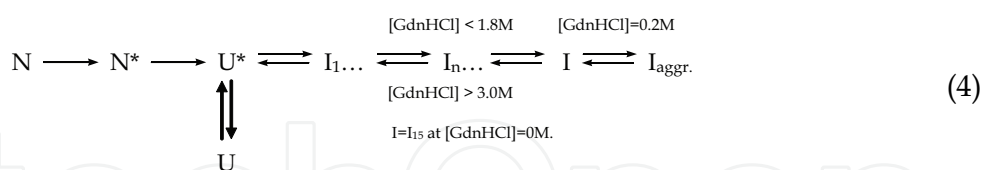
The fluorescence intensities recorded at the wavelengths 320 and 365 nm were used as independent extensive characteristics, and the time from the moment of protein solution mixture with a GdnHCl solution of the appropriate concentration was taken as a parameter. Figure 4A shows that these curves consist of two branches that are most pronounced for a concentration of 1.8 M. One branch corresponds to the $N \rightarrow U^*$ transition and the other to the $U^* \rightarrow I$ transition. This panel also shows that the fluorescence properties of the kinetic intermediate U^* differ from those of completely unfolded actin (U) in 4-6 M GdnHCl. The kinetic intermediate has more blue fluorescence spectrum in comparison with the completely unfolded state of actin (Kuznetsova et al., 2002; Turoverov & Kuznetsova, 2003; Povarova et al., 2005; Povarova et al., 2007). Actin in this state, in contrast to the completely unfolded state, was shown to preserve its secondary structure. Furthermore, the parametric relationships between I_{320} and I_{365} were found to originate not from one point that corresponds to the native state of actin in the absence of GdnHCl but from distinct points (Figure 4A). This observation indicates that the change of the solution leads to rapid changes of the fluorescence properties of actin. The existence of one more intermediate states was proven in our experiments (Povarova et al., 2005) and in the work of Altschuler et al. (Altschuler et al., 2005) by stopped flow (Figure 4B). The total obtained data allowed us to propose a new kinetic pathway for actin unfolding and refolding induced by GdnHCl. In this scheme, the state N^* precedes the transformation of native actin into the essentially unfolded state (U^*). However, this state is far from a complete understanding and characterization. At the same time, the formation of an essentially unfolded state (U^*) preceding the completely unfolded (U) or inactivated actin (I) has been proven. In the processes of folding and unfolding, the essentially unfolded state (U^*) is an on-pathway intermediate, whereas inactivated actin (I) is an off-pathway associate, the appearance of which competes with the transition to the native state.

As was mentioned above, protein folding is associated with a decrease in entropy that leads to an increase of free energy transfer, which must be compensated by the formation of contacts. In this scenario, two competing processes are possible: formation of intramolecular contacts and formation of intermolecular contacts. In the first case, native protein appears, and in the second, amyloid fibrils, amorphous aggregates and associates such as inactivated actin appear. Investigation of the characteristics of such states and the factors that influence their appearance is not only of fundamental value but also important for medicine (in connection with the so called conformational diseases) and for biotechnology (in connection with the accumulation of insoluble aggregate forms of various proteins in inclusion bodies). The reversibility of the actin denaturation state in early works (Contaxis et al., 1977; Tatanashvili & Privalov, 1984; De La Cruz & Pollard, 1995) was not reproduced by us (Kuznetsova et al., 2002; Turoverov et al., 2002; Turoverov & Kuznetsova, 2003; Povarova et al., 2005). The Ca^{++} ion probably plays a crucial role in the irreversibility of actin denaturation: the native structure became energetically profitable only in the presence of the Ca^{++} ion, and otherwise, the structure is stabilized by intramolecular contacts, which leads to the formation of inactivated actin. Thus, the presence of chaperones is likely to be an essential requirement for actin folding.

The study of actin unfolding in different concentrations of GdnHCl allowed us to conclude that actin unfolding by 4 M GdnHCl also proceeds via the intermediate states N^* and U^* . The rate constants of the processes $N \longrightarrow N^*$, $N^* \longrightarrow U^*$ and $U^* \longrightarrow U$ increase with the GdnHCl concentration. The rate constants of these processes are very large in 4 M

GdnHCl, which is why it appears that actin unfolding by 4 M GdnHCl is a two-state transition, $N \longrightarrow U$. The Ca^{++} ion plays a stabilizing role in the structure of actin as ligands do for other proteins. Thus, Ca^{++} ions in actin can be regarded as triggers, the removal of which launches the cascade of conformational changes, i.e., the appearance of the kinetic intermediates N^* and then U^* . The dissociation of Ca^{++} in low concentrations of GdnHCl is a slow process (Kuznetsova et al., 2002). Actin molecules that transfer to the U^* state are rapidly associated with the formation of inactivated actin (I) because the rate constant for the $U^* \longrightarrow I$ process is large in the absence of a denaturant. Due to this effect, the transition of native actin to the inactivated state occurs even spontaneously, in the absence of denaturant, during actin sample storage (Kuznetsova et al., 1988). However, the same process caused by EDTA is very rapid: the rate constant of the process $N \longrightarrow N^*$ is very large, and in the absence of GdnHCl, the rate constant of the process $U^* \longrightarrow I$ is also very large. This effect explains why it appear that the EDTA-induced unfolding of actin is a two-state process, $N \longrightarrow I$; in reality, however, it is a multi-state process in which the process $N \longrightarrow N^* \longrightarrow U^* \longrightarrow I$ occurs. Experiments performed on stopped-flow device confirm the existence of the kinetic intermediate N^* . In a temperature denaturation experiment, we also appear to record a two-state transition, $N \longrightarrow I$. However, in reality, as is the case in actin unfolding by EDTA, a multi-state process occurs: $N \longrightarrow N^* \longrightarrow U^* \longrightarrow I$, though in the case of temperature denaturation, the rate constants of the first transitions ($N \longrightarrow N^* \longrightarrow U^*$) are low, while the transition $U^* \longrightarrow I$ in the absence of denaturant is very fast.

Thus, the investigation of actin unfolding and refolding, as well as the data we obtained for certain other proteins, allowed us to conclude that, in the unfolding pathway, the order and number of the partially folded denatured states are independent of the denaturing agent. Overall, the obtained data allowed us to propose a new kinetic pathway for actin unfolding and refolding induced by GdnHCl:



In this scheme, the transition state N^* precedes the transformation of native actin into the essentially unfolded state (U^*). However, this state is far from representing a complete understanding and characterization of the underlying process. The formation of an essentially unfolded state (U^*) preceding the completely unfolded (U) or inactivated actin (I) is proven. In the processes of folding and unfolding, the essentially unfolded state (U^*) is an on-pathway intermediate, whereas inactivated actin (I) is an off-pathway associate, the appearance of which competes with the transition to the native state.

4.3 Actin folding *in vivo*

As was mentioned above, the conclusions regarding the reversible unfolding of actin that had been reported in earlier works (Kasai et al., 1965; Contaxis et al., 1977; Tatumashvili &

Privalov, 1984; De La Cruz & Pollard, 1995) were not reproduced (Kuznetsova et al., 2002; Turoverov et al., 2002; Turoverov & Kuznetsova, 2003; Povarova et al., 2005). Thus, the presence of chaperones is likely to be an essential requirement for actin folding.

Chaperones constitute a broad family of proteins with various molecular masses, structures and functions. Two classes of ATP-dependent chaperones, Hsp70 and the chaperonins, are known to play crucial roles in the folding of nascent, non-native polypeptides into their native, functional states inside eukaryotic cells. The Hsp70 chaperones, with the assistance of the co-chaperones of the DnaJ/Hsp40 family, interact with the small hydrophobic clusters of the newly synthesized polypeptide chain (Feldman & Frydman, 2000). These interactions are not specific because hydrophobic clusters are present in almost every partially folded polypeptide chain. The major role of Hsp70 is likely in preventing undesirable interactions that might result in the aggregation of the newly synthesized polypeptide chain with other molecules. For many proteins, interactions with Hsp70 are sufficient for correct folding. However, the folding of multidomain proteins requires the participation of other helpers. For example, the correct folding of actin relies on its interaction with prefoldin (PFD), which participates in the translocation of the partially folded actin to the CCT chaperonin. CCT consists of two stacked toroids, each of which contains eight three-domain proteins. The equatorial domains are responsible for the intertoroid interactions and for the interaction with ATP, whereas the apical domains mediate the interaction with the substrate and provide for the passage of the substrate to the central cavity. The folding of actin is a complex, multi-stage, ATP-dependent process controlled by CCT (Neiryneck et al., 2006; Altschuler & Willison, 2008). The indispensable participation of PFD and CCT in actin folding is likely the reason that recombinant actin cannot be expressed in *E. coli* (Frankel et al., 1990) but can be expressed in yeast (Karlsson, 1988; Verkhusha et al., 2003). The refolding of EDTA-denatured actin in the presence of CCT *in vitro* was observed by Altschuler et al. (2005).

It is important to remember that, despite the crucial roles of chaperones in the folding of globular proteins *in vivo*, chaperones do not carry the structural information that is necessary for a newly synthesized polypeptide chain to fold into a unique, rigid and native globular structure. It is very likely that interactions with chaperones and other proteins are even more important for proteins the native states of which are partially or completely disordered. Such interactions would definitely protect these proteins from aggregation and proteolysis. Thus, the amino acid sequence of actin is such that it cannot fold into a compact state without chaperones. This phenomenon would be difficult to explain if, at the turn of the century, the so-called ID proteins had not been predicted.

5. Intrinsically disordered proteins

For a very long time, a protein's function was believed to depend on its prior folding into a unique three-dimensional structure. At the same time, over many decades, numerous proteins have been found to be either wholly disordered or to contain lengthy disordered segments, yet to carry out function. These proteins were typically considered to be outliers and were mostly ignored. However, at the turn of the century, it became clear that proteins without tightly folded 3D structures can also perform vital biological functions (Dunker et al., 2002; Daughdrill et al., 2005; Dyson & Wright, 2005; Dunker et al., 2008). The major structural characteristics of intrinsically disordered (ID) proteins include the inability to

form crystals, low circular dichroism signals in the near- and far-UV regions, a large hydrodynamic dimension, and high proteolytic sensitivity (Uversky, 2011). It has now been established that the inability of ID proteins to form rigid globular structures is linked to the peculiarities of their amino acid sequences. One of the reasons for native disorder to exist is encoded in the overall hydrophobicity and net charge of a given polypeptide chain. The smaller the content of hydrophobic residues and the higher the net charge of a polypeptide chain, the smaller the probability that this chain will fold into a compact globular state. The distinctiveness of the amino acid sequences of ID proteins formed the basis for the development of various computational tools for predicting such proteins (Ferron et al., 2006; Dosztanyi & Tompa, 2008). The application of disorder-predicting algorithms revealed that ID proteins are widely spread in nature. The length of the amino acid sequences that are unable to form ordered structures and the degree of disorder can vary significantly between ID proteins (Uversky, 2011). Because of this great variability, there is no strict boundary between globular and partially disordered proteins.

The atoms in the unstructured parts of a polypeptide chain possess a high degree of mobility, which is why they cannot be detected by X-ray analysis. The majority of globular proteins are enzymes and transporters that are naturally designed to have a strictly determined function. However, even in globular proteins, there is a some structural mobility. The most mobile atoms are the atoms in the active sites of enzymes, or the atoms in loops that might also be functional, participating in interactions with partners. Therefore, globular proteins also require a definite level of mobility for their functioning. Some polypeptide chains cannot fold into compact globular structures by themselves but can form compact structures while interacting with their partners if the free energy of the complex is lower than the free energies of the protein and its partner before their interaction. The potential for partially or completely disordered proteins to form complexes with their partners is the molecular basis of their functions in signaling, recognition and the regulation of different intercellular processes. Although many proteins are involved in such processes, special attention has been paid to the main regulatory proteins, which play key roles in the regulation of these complex processes. Many of these proteins, which are known as hubs and network concentrators and serve as "conductors" of these biological processes, were shown to be disordered (Dunker et al., 2005; Oldfield et al., 2008). Among such disordered hub proteins are α -synuclein, p53, HMG proteins, estrogen receptor α , and many others (Dunker et al., 2008; Uversky, 2008; Olovnikov et al., 2009). Proteome-wide analyses revealed that partially or completely ID proteins are more common in eukaryotes than in prokaryotes or archaea, likely due to the more complex regulation and signaling systems in higher organisms (Uversky, 2011). A striking example of this trend is p53, the function of which is inherent only in multicellular organisms. This protein monitors and coordinates practically all of the intercellular processes (Olovnikov et al., 2009), prioritizing the organism's needs over the interests of different cells: a damaged cell must either accelerate the repair processes or lose the possibility of division, and it may even die as a result of apoptosis (Olovnikov et al., 2009).

The ID proteins in such signalling net play a number of crucial roles in complex regulatory processes and are known as hub proteins or net concentrators. To avoid the risk of being digested and to escape the non-specific aggregation that potentially leads to the formation of oligomers, amorphous aggregates, and amyloid fibrils, disordered proteins should preferentially remain bound to their partners. The pathogenesis of conformational diseases

that are characterized by the formation of amyloids and amyloid-like fibrils is likely to be determined by the failures of the cellular regulatory systems rather than by the formation of proteinaceous deposits and/or by protofibril toxicity. It is evident that more or less actin meets the majority of the characteristics of ID proteins. First of all, like the ID proteins, actin cannot fold into a compact state without chaperones. Second, like many ID proteins, actin interacts with an enormous number of partners (Domingues and Holmes, 2011).

6. Actin localization and functions

Though it is now evident that actin can be found in any eukaryotic cell, it is most abundant in muscle cells, which consist almost entirely of actomyosin fibrils. Therefore, it is not surprising that the first purification of actin was performed in muscle cells. This historic experiment was conducted in Albert Szent-Gyorgy's laboratory by Bruno F. Straub, who studied muscular contraction (Straub, 1942). Straub showed that the purified protein was responsible for the activation of muscle contraction, for which reason he called it "actin". This work was a great breakthrough that determined the research guidelines for hundreds of scientific laboratories for decades. Today, muscle contraction has been studied in detail. Thousands of papers, reviews and books are devoted to this topic (see, e.g., (Oosawa, 1971)), and there is no use in repeating copy-book maxim again. We merely wish to emphasize that, the more muscle contraction was studied, the more it became evident that actin is not only an activator of muscle contraction but is also the main organizer and director of complex processes involving dozens of proteins other than actin and myosin, which have been given the general name of actin-binding proteins. Troponin and tropomyosin are notable among these proteins. In muscle, actin permanently exists in the form of F-actin. Once polymerized, it is integrated into the muscle and is not depolymerized unless the muscle is damaged. Accordingly, G-actin is required only for muscle generation in the course of muscle growth or repair.

Interestingly, the structure of F-actin was determined by X-ray analysis on the basis of the previously determined structure of the G-actin monomer (Holmes et al., 1990; Lorenz et al., 1993). F-actin was shown to form a single helix consisting of 13 molecules repeating in almost exactly six left-handed turns ((Holmes et al., 1990; Lorenz et al., 1993). Recently, this helix was directly visualized by electron cryomicroscopy (Fujii et al., 2010). At the same time electron microscopy of stained actin fibers showed F-actin to be made of two chains that twist gradually around each other to form a right-handed, two-chained long helix (Hanson & Lowy, 1963; Egelman, 1985; Dominguez & Holmes, 2011). Surprisingly, the image of F-actin as two-chained helix appeared to be so impressive that many researchers even today consider F-actin to be a two-chained helix. Nonetheless, this misconception is not inoffensive carelessness, as in this case, the model of the assembly and disassembly of actin filaments in principle differs from the generally accepted model.

In addition to its role in muscle cells, actin is an essential component of the cytoskeleton of all eukaryotic cells. This protein plays a crucial role in the generation and maintenance of cell morphology and polarity, in endocytosis and intracellular trafficking, and in contractility, motility and cell division. All of these functions are based on the highly ordered assembly and disassembly of actin filaments and the polymerization and depolymerization of F-actin. All of these processes are regulated by numerous ABPs that are, in turn, under the control of specific signaling pathways (dos Remedios et al., 2003;

Maciver, 2004). For these purposes, both F- and G-actin are required. The first crucial aspect of polymerization is nucleation, which refers to the formation of a nucleus of three associated monomers and constitutes the rate-limiting phase of polymerization. The main role in this phase is played by the complex of the Arp2 and Arp3 proteins, which is usually referred to as the Arp2/3 complex (Winder & Ayscough, 2005). The molecules of these ABPs have a similar tertiary structure to actin, such that, when the Arp2/3 complex binds to actin, it is regarded as a nucleus for polymerization. Then, in the processes of further filament growth, the Arp2/3 complex plays the role of a pointed end-capping protein that enhances the rapid growth of the filament from its barbed end. The Arp2/3 complex can also nucleate filament growth from the side of an existing filament. This ability is important for the dendritic branching that is found at the leading edges of motile cells (Pollard & Borisy, 2003). It has also been established that, *in vivo*, certain other proteins that take part in the regulation of filament growth participate in these processes (Paavilainen et al., 2004). The termination of filament growth is regulated by gelsolin and tensin. These proteins bind to the barbed end of the filament and block the addition of new monomers. Gelsolin also is known to participate in the severing of filaments (Burtnick et al., 2004), whereas tropomyosins (a highly conserved family of ABPs) are known to bind along the side of the actin filament to prevent its spontaneous depolymerization and even to protect it from severing by gelsolin. There are several other ABPs that participate in actin filament length determination. These proteins contain domains that allow them to interact with other proteins of the cell signaling networks. This interaction allows the remodeling of the actin cytoskeleton at appropriate times and places within the cell. An example of such an ABP is nebulin, which is an elongated protein with numerous low-affinity actin-binding sites (Winder & Ayscough, 2005).

When an actin filament is disassembled, F-actin must be depolymerized. The best-characterized proteins that drive depolymerization are the actin depolymerizing factor (ADF) and cofilin family members (Winder & Ayscough, 2005). After depolymerization, several highly conserved ABPs intervene in the process of actin turnover. These ABPs bind ADP-actin as it is released from the end of the filament (e.g., ADF/cofilin, twinfilin), facilitating the nucleotide exchange from ADP to ATP (e.g., profilin, CAP) and delivering the actin monomer to the barbed end of a filament to facilitate a new round of polymerization (e.g., profilin, twinfilin, verprolin/WIP, WASP). For rapid filament growth in cells, there must be a sufficiently large amount of ATP-actin ready to polymerize but preserved in the monomer form until an appropriate signal is given. For this purpose, there are special ABPs, the best studied of which are the thymosin family. A special signal triggers the activation of profilin, which leads to the release of thymosin from actin and results in the release of a large amount of ATP-actin that is ready to polymerize (Hertzog et al., 2004). Beyond these examples, F-actin interacts with many ABPs that do not influence its structure and dynamics. These ABPs include myosins that use actin as a track along which to move (Winder & Ayscough, 2005), cytoskeletal linkers (dystrophin, utrophin, vinculin) that interconnect different cytoskeletal elements and membrane anchors (annexins) that interact with both actin and the membrane.

Actin in the nucleus was discovered at practically the same time as in the cell (Lane, 1969), but this localization was taken to be an artifact. The focused study of nuclear actin began only recently. Currently, the presence of actin in the nucleus has been unequivocally demonstrated (Vartiainen, 2008). Actin has been shown to be an important regulator of

transcription (Miralles & Visa, 2006), transcription factor activity (Vartiainen et al., 2007), and chromatin remodeling (Zheng et al., 2009). At the same time, nuclear actin is less well studied than the cytoplasmic form. The existence of F-actin in the nucleus was controversial for a long time because it was not recognized by phalloidin fluorescence (Visegrady et al., 2005). However, all of the ABPs that interact with F-actin have been detected in the nucleus (Gonsior et al., 1999), and the actin monomer-sequestering drug Latrunculin has been reported to inhibit several nuclear actin-dependent functions, including the export of RNA and proteins (Hofmann et al., 2001), nuclear envelope assembly (Krauss et al., 2003), transcription (McDonald et al., 2006) and transcription-induced interchromosomal interaction (Nunez et al., 2008). These observations comprised indirect evidence that actin in the nucleus must be in a polymerized form. Several other studies have been published in which the authors try to prove the existence of actin filaments in the nucleus, but the most convincing is a recent microscopy study (McDonald et al., 2006). Approximately 20% of the total nuclear actin pool is in the polymeric state (McDonald et al., 2006). The failure of phalloidin to stain nuclear actin can be explained by its lower concentration relative to that in the cytoplasm (the nucleus contains approximately 1% as much actin as the cytoplasm), its decoration by ABPs (such as ADF/cofilin (McGough et al., 1997)) and possibly by a lower length (Vartiainen, 2008). At the same time, the dendritic actin branches have not been visualized in the nucleus, although Arp2/3 and other components that nucleate these filaments were found in the nucleus. The other unsolved problem is that of actin transport in and out of the nucleus. There is some evidence that actin can cross the nuclear pore complexes in a complex with profilin and exportin-6, although its import mechanism is still unclear (Vartiainen, 2008).

Interestingly, the "functional" form of actin differs in the muscle, the cytoplasm and the nucleus. In the muscle, once they are generated, filaments are not disassembled and new filaments appear only during muscle growth or reparation; therefore, the main functional form is F-actin. In non-muscle cytoplasm, although the cytoskeleton is composed of actin fibrils, it can be assembled and disassembled. Cell motility is also based on actin filament polymerization and depolymerization. Therefore, a sufficiently large amount of actin monomers must be stored in the cytoplasm to support the effective function of actin. In the nucleus, for the first time, actin monomers play a significant role by regulating SFR (serum response factor) activity. The actin monomer pool is involved in controlling the expression of many proteins that are themselves components of the actin cytoskeleton (Miralles & Visa, 2006).

7. Conclusion: Actin as a partially intrinsically disordered hub protein

Overall, actin has many characteristics that are typical of ID proteins. *In vitro*, its unfolding is irreversible, i.e., the information contained in its polypeptide chain is not enough for "regular" folding, or the intramolecular contacts that appear upon folding are not enough for the polypeptide to fold itself without chaperones and to maintain the folded native state without fastening it with Ca^{++} ions. Actin always exists in complexes: while folding, it successively interacts with the chaperone Hsp 70, then with PFD and finally with the chaperonin CCT, which provides for correct folding and Ca^{++} and ATP incorporation; fibrillar actin is formed by the self-association of G-actin molecules; in the cytoplasm or nucleus, actin is in complex with ABPs; and, in particular, the G-actin pool exists preserved in complex with profilin. Interestingly, inactivated actin is also a monodisperse complex

(not an amorphous aggregate) that, possibly, has some functional role. Actin not only cannot fold without chaperons but also cannot form a compact structure without its ligands, the Ca^{++} ion and ATP. Actin has several binding sites and can interact with an enormous number of partners. While interacting with numerous ABPs, actin acts as a hub protein, as is typical for ID proteins. Many of the ABPs themselves are ID proteins of the signaling system and interact with other hub proteins. Actin is ubiquitous. It is one of the main components of the system of muscle contraction, it forms the cytoskeleton, it is found in the cell nucleus in which, except for the motility and scaffold functions, actin acts as a regulatory protein that participates in the processes of transcription and chromatin remodeling. The analysis of the amino acid sequence of actin with the use of the PONDR® program (Obradovic et al., 2005; Uversky, 2011) reveals that actin contains segments of polypeptide chain that are prone to be disordered (Figure 5).

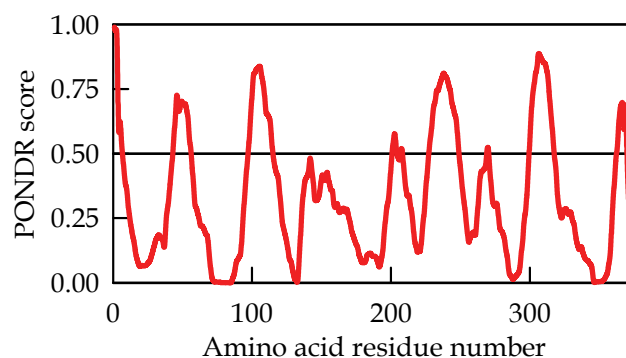


Fig. 5. PONDR score for actin. The PONDR® VLXT protein disorder predictor was used (Obradovic et al., 2005). The portions of the polypeptide chain for which the PONDR score > 0.5 are prone to form disordered fragments of polypeptide chains.

It appears that currently, the "actin" scientific community is not acquainted with, or may be acquainted with but not interested in, the most recent achievements in the field of protein structure and folding, while those researchers who study protein folding, ID protein folding in particular, do not seem to consider what an interesting object for their investigation actin can be. We will be pleased if this publication could help groups of researchers from diverse fields collaborate to join their efforts in the study of actin. Recently, V.N. Uversky published a work with the intriguing title "ID proteins from A to Z" (Uversky, 2011). We believe that this card file will not be full if, in the first cell (letter A), there is not a section entitled "Actin as an ID protein".

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9. References

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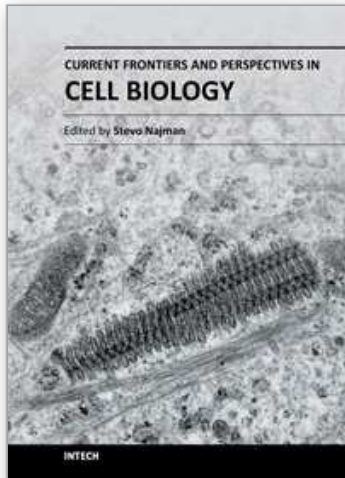
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Phone: +385 (51) 770 447
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