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Mobile segments in rabbit skeletal muscle F-actin detected by ^{1}H nuclear magnetic resonance spectroscopy

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Abstract Polymerization of actin by increasing the ionic strength leads to a quenching of almost all ^{1}H NMR signals. Surprisingly, distinct signals with relatively small line widths can still be observed in actin filaments (F-actin) indicating the existence of mobile, NMR visible residues in the macromolecular structure. The intensity of the F-actin spectrum is much reduced if one replaces Mg^{2+} with Ca^{2+}, and a moderate reduction of the signal intensity can also be obtained by increasing the ionic strength. These results can be explained in a two-state model of the actin protomers with a M- (mobile) state and a I- (immobile) state in equilibrium. In the M-state a number of residues in the actin protomer are mobile and give rise to observable NMR signals. This equilibrium is shifted towards the I-state specifically by replacing Mg^{2+} with Ca^{2+}-ions and unspecifically by addition of monovalent ions such as K^+. The binding of phalloidin to its high-affinity site in the filaments does not influence the equilibrium between M- and I-state. Phalloidin itself is completely immobilized in F-actin, its exchange with the solvent being slow on the NMR time scale.

Key words: Actin; Mobility; ^{1}H NMR; Muscle

1. Introduction

In both its monomeric (G) and in its polymerized (F) form actin binds one adenine nucleotide molecule and one divalent metal ion per protomer at a high-affinity binding site. In vivo this metal ion is most probably Mg^{2+}, while in vitro, as a consequence of the widely used method of actin isolation and purification, it is usually Ca^{2+} [1–3]. In the crystal structure of the actin-DNaseI complex [4], two domains of the protein can be distinguished with the nucleotide–metal complex located in the cleft between these domains. The nature of this complex significantly influences the biochemical and biophysical properties of actin. The type of divalent metal ion as well as the type of the nucleotide bound strongly influences the nucleation and actin polymerization, the rate of ATP hydrolysis depends on the metal ion present in the active center of actin (for a recent review see [3]). However, little is known about structural features of actin that are responsible for these phenomena.

In addition to X-ray crystallography, X-ray fiber diffraction and electron microscopy spectroscopic methods such as NMR can help to elucidate structural differences. A number of NMR studies on actin structure and dynamics in its G- and F-form have been published [5–21]. The conformational transitions of G-actin upon Mg^{2+}/Ca^{2+} exchange were studied by NMR spectroscopy [9]. ^{1}H NMR spectra of Ca-G-actin and Mg-G-actin revealed differences in the aromatic as well as in the aliphatic region of the spectrum. Whereas the ^{1}H NMR spectra of G-actin published so far are rather similar, there are large variations between the NMR spectra of F-actin. According to Highsmith and Jardetzky [7] all resonance lines of F-actin are broadened beyond detection as is expected for a rigid, high molecular weight complex. However, other groups have reposted ^{1}H NMR spectra of F-actin which display intense resonance lines which are very similar to those observed for G-actin [15,20]. Surprisingly, these major differences have never been studied in detail, and it is not clear, whether they reflect different physiological states of F-actin or are simply due to denaturation of actin. Studies of F-actin by electron microscopy suggest the existence of various states of F-actin which differ in the overall flexibility of the filaments [22–23] and which depend on the type of divalent ions bound to the high-(or intermediate-)affinity binding site(s). It is conceivable that the differences observed in ^{1}H NMR spectroscopy of F-actin are due to differences in the internal mobility of the filaments and that they are related in some way to the different states of flexibility observed by electron microscopy.

In the following we will present the results of ^{1}H NMR studies of actin in its G- and F-forms with either Mg^{2+} or Ca^{2+} occupying the high-affinity binding site, with the aim of showing whether there are different states of actin which can be observed by ^{1}H NMR of the protein and, if so what is their structural and dynamic basis.

2. Materials and methods

2.1. Actin preparation

Actin from rabbit skeletal muscle was prepared from acetone powder as described by Pardee and Spudich [2]. In an additional step actin was polymerized with 100 mM KCl in the presence of 0.4 mM EDTA. After centrifugation it was depolymerized in the G-buffer (2 mM Tris, 0.1 mM $CaCl_2$, 0.2 mM ATP, 0.002% NaN_3, pH 7.8). The purity of actin was checked with SDS-Page. The exchange of the Ca^{2+} ion with Mg^{2+} at the high-affinity binding site was performed by dialysing the sample against a modified G-buffer (0.1 mM $MgCl_2$ instead of $CaCl_2$, 0.2 mM EGTA) for 20 h. In order to exclude preparation artifacts, all critical experiments were also performed with actin prepared using two modified preparation methods. In the first method the polymerization step in the presence of EDTA was omitted. In the second method an additional gel-filtration step on a Fractagel TSK HW 55 (Merck, Darmstadt) column (3 × 120 cm) in the G-buffer was performed. The principal experimental results obtained with all three preparation methods were identical however, the polymerization step in the presence of EDTA led to the removal of some low-molecular weight impurities contained in low concentations in actin samples.

2.2. NMR spectroscopy

^{1}H NMR spectra were recorded on a Bruker AMX-500 NMR spectrometer operating at 500 MHz. The water signal was suppressed by

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Abreviations: G-actin, globular actin; F-actin, filamentous actin.

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selective presaturation. Typically, in one-dimensional NMR spectroscopy 512 free induction decays were accumulated with a repetition time of 5 s including the water suppression pulse of 1 s. Under these conditions, the actin signal was fully relaxed since the T_1 relaxation times estimated for G- and and the NMR visible resonances of F-actin were in the range from 400 ms to 1 s. However, a relaxation time of the same magnitude (1 s) is also expected for the extremly broadened resonances of F-actin, since in proteins the T_1 relaxation times of individual resonances become equalized by spin diffusion and T_1 relaxation is dominated by the internal motion of the methyl groups (see e.g. [24]). Typical acquisition parameters were: pulse length 9.4 μs (corresponding to a pulse angle of 80 degree), spectral width 6,024 Hz, delay before acquisition 118.4 μs,16 k data points. Typical processing parameters (used also for the spectra depicted in this paper) were: 1 Hz additional line broadening by exponential multiplication of the free induction decay, data size after Fourier transformation 16 k, base line correction by a third order polynomial fit.

All spectra are referenced relative to 4,4-dimethyl-4-silapentane sodium sulfonate (DSS) used as internal standard. The G-actin spectrum contains two dominant singlet resonances at 2.048 ppm and 2.066 ppm which do not shift under our experimental conditions. In cases where the addition of DSS was not desirable these resonances were used for the calibration of the spectra. Where not indicated otherwise, the measurements were performed at 278K.The sample was contained in 5 mm NMR tubes. Usually, actin was polymerized in the NMR tube by the addition of appropriate aliquots of the stock solutions (3 M KCl , 1 M $MgCl_2$ or 1 M $CaCl_2$).

2.3. Viscosity measurements

Polymerization was monitored in a Cannon Capillary Viscosimeter, using a solution of 47.8 μM Ca- or Mg-G-actin in G-buffer and modified G-buffer (see above). The viscosity of the two solutions was determined at room temperature as a function of the salt concentration. In subsequent experiments the KCl concentration was increased stepwise to 30 mM, 100 mM, 200 mM and 300 mM. A delay of 15 min. was included in the experimental protocol before the viscosity was measured. Finally, 10 mM $MgCl_2$ was added to the Mg-actin solution and 10 mM $CaCl_2$ to the Ca-actin solution, and the corresponding viscosities were determined.

3. Results and discussion

3.1. ^1H NMR spectra of F-actin

Polymerization of G-actin by addition of KCl (Fig. 1) leads to a large reduction of the signal intensity. However, a number of signals can still be observed with sufficient signal-to-noise ratio. Besides the signals of the low molecular weight compounds contained in the buffer, a number of broader lines remain, which can be shown to originate from the protein. The spectrum is not simply a G-actin spectrum with reduced intensity, even though it includes the narrow lines already observable in the G-actin spectrum (which probably represent residues with high internal mobility in G-actin). A closer inspection shows that the situation is more complicated. The lines visible in the spectrum of F-actin are only a subset of the narrow lines of G-actin: some of the narrow lines of the G-actin spectrum clearly disappear. Examples are the two sharp singlet resonances at 2.048 ppm and 2.066 ppm in the G-actin spectrum, of which only the line at 2.048 ppm is present in the F-actin spectrum, or the sharp singlet lines around 7.6 ppm (probably from histidine residues) which disappear completely in the F-actin spectrum.

The remaining signal is very unlikely to originate from an additional protein in the actin preparation since the results obtained are very reproducible and do not depend on the exact mode of actin preparation, e.g. an additional gel filtration step does not change the NMR spectrum of the sample under polymerization conditions. In addition, because of its rather high intensity in the NMR spectrum such a contamination of the sample should easily be seen in the gel electrophoresis. The observed spectrum is different to the spectra expected for a completely denatured (random-coil) actin.

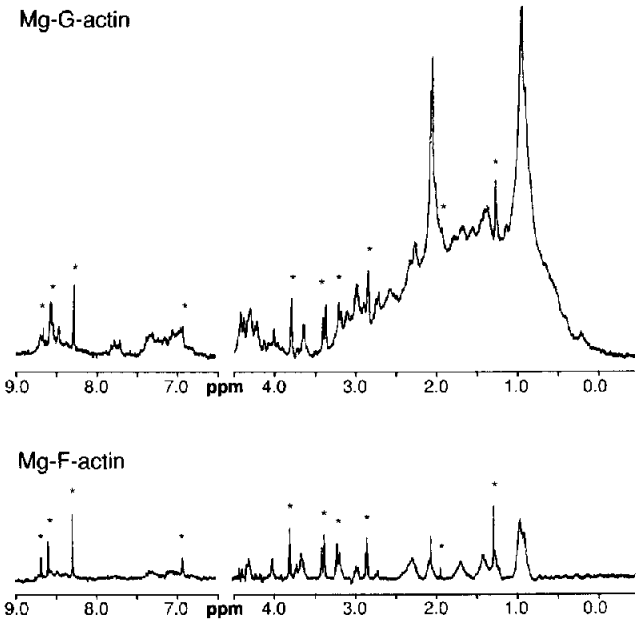

Fig. 1. ^1H NMR spectra of G-actin and F-actin. (Top) 75 μM Mg-G-actin in 0.2 mM ATP, 0.1 mM $MgCl_2$, 0.2 mM EGTA, 1 mM Na_2HPO_4; pH 8.0; 90% H_2O/10% D_2O. (Bottom) after recording of the Mg-G-actin spectrum the protein was polymerized in the NMR tube by the addition of 5 μl of a 3 M KCl solution (end concentration 30 mM). Temperature 278 K; the experimental conditions (including the number of scans) and the data processing were identical for the two spectra, and the top and the bottom spectrum are scaled identically, so the intensities can be compared directly. Signals from low molecular weight compounds are labeled by stars.

The experiments show that the polymerization of actin leads to a drop of intensity and a change of typical spectral features. Surprisingly, after polymerisation certain individual resonances with relatively small line widths are still visible. F-actin has a molecular mass of more than 10,000 kDa, and the expected large rotational correlation time should lead to a wide line spectrum with dipolar line widths in the kHz range. With the experimental setup (high-resolution NMR spectrometer, large dead time of the preamplifier, first sampled data point after > 100 μs) this unstructured broad signal should barely be distinguishable from base line artifacts. The only likely explanation is that there are two different processes which are responsible for the experimental observations: a quenching of the strong G-actin signal and most of the F-actin signal by the polymerization of the protein, and the persistence of some weak NMR signals from highly mobile regions in the supramolecular state.

3.2. Ion dependence of the actin polymerization

Fig. 2 shows the aliphatic region of two representative spectra of F-actin. The upper trace shows the spectrum of Mg-actin polymerized by the addition of 10 mM $MgCl_2$, the lower trace the spectrum of Ca-actin polymerized by the addition of 10 mM $CaCl_2$. If one neglects differences caused by the sharp signals

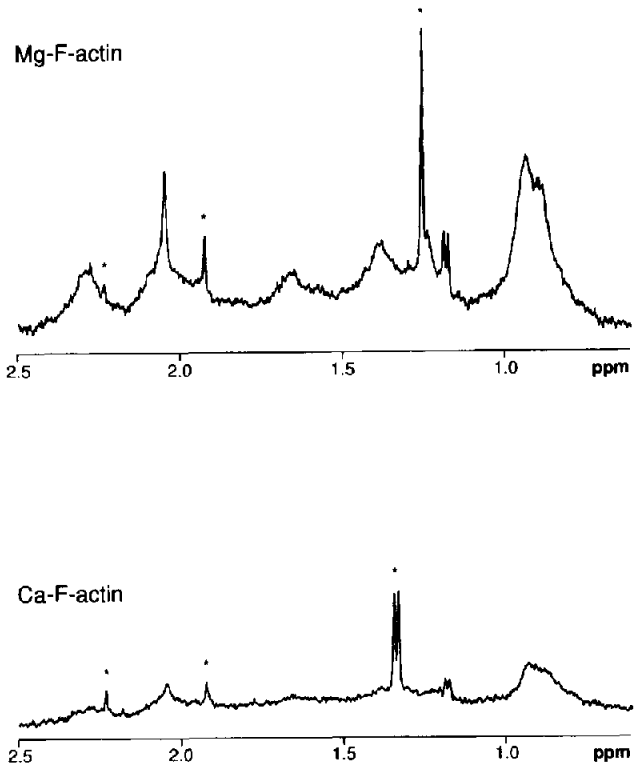

Fig. 2. ^1H NMR spectra of Mg- and Ca-F-actin. Part of the ^1H NMR spectra of Mg-actin (top) and Ca-actin (bottom) polymerized in the NMR tube by the addition of $CaCl_2$ or $MgCl_2$ (end concentration 10 mM), respectively. Signals from low molecular weight compounds are labeled by stars. Before polymerization the Mg-actin sample contained 79 μM Mg-actin in 0.2 mM ATP, 0.1 mM $MgCl_2$, 0.2 mM EGTA, 2 mM Tris-HCl, pH 8.1, 90% H_2O/10% D_2O. The Ca-actin sample contained 82 μM Ca-G-actin dissolved in 0.2 mM ATP, 0.1 mM $CaCl_2$, 2 mM Tris-HCl, pH 7.8, 90% H_2O/10% D_2O. Temperature 278 K, identical parameters were used for recording, processing, and representation of the spectra. Note that in the spectra shown Mg-G-actin was prepared from Ca-G-actin by dialysis, which explains the observation of different signals from low molecular weight impurities. As expected, when identical preparation procedures for Mg- and Ca-actin were used, the differences in the protein signals remained but the impuritiy signals were superimposable.

from low molecular weight compounds in the buffer, the signals from the protein are very similar (concerning their line positions, their relative intensities, and their line widths) but the absolute intensities of resonances of Ca-F-actin are much smaller than that of Mg-actin. This is not only true for the spectral region depicted in Fig. 2 but also for all parts of the NMR spectra of F-actin which are free of artifacts or signals from small molecules.

Similar results are obtained when Mg-actin or Ca-actin is polymerized by addition of KCl (Fig. 3): on polymerization of actin the signal of G-actin disappears but a weak F-actin signal remains. As far as can be judged from comparison of the spectra of F-actin, the basic features of the spectra are again independent of the type and concentration of the ions used, but the absolute intensity of the signals is strongly dependent on these factors. This can be most clearly demonstrated in the signals of the F-actin spectrum around 0.9 ppm depicted in Fig. 3. The general spectral shape in this region does not change after increasing the salt concentrations but the line intensities decrease. This decrease in intensity is not due to a gradual broadening of the resonance lines: after appropriate scaling of the spectra they are completely superimposable and the line width (homogeneous and inhomogeneous) is unchanged. At the same KCl concentration the NMR signal of Mg-F-actin is much more intense than that of Ca-F-actin. Even at 300 mM KCl and 10 mM $MgCl_2$ the signal from Mg-F-actin is more intense than the signal of Ca-F-actin at 30 mM KCl after correction for differences in protein concentrations (Fig. 4). This effect was observed repeatedly with actin from different preparations and is highly reproducible; in particular, the relative reduction of the signals does not depend critically on the actin concentrations in the concentration range between 60 μM to 200 μM. The decrease of intensity was quantified by integration of the spectra in the range from 0.6 ppm to 1.1 ppm. A plot of the relative integrals in this spectral range as a function of the ionic conditions (Fig. 4) shows that the reduction of the signal amplitude after polymerization with 30 mM KCl is much more pronounced in Ca-actin than in Mg-actin. Even a ten-fold increase in the KCl concentration cannot induce such a reduction of the signal in Mg-actin.

In the viscosity measurements done in parallel to the NMR studies (Fig. 4), the opposite effect can be observed. At 30 mM KCl the viscosity increases more rapidly for Mg-actin. However, at high salt concentrations the viscosity of the Ca-actin and Mg-actin solution is almost identical. Therefore, the changes in macroscopic viscosity are not responsible for (or directly related to) the reduction of the NMR signals. These data make it very unlikely that the observed spectrum results from non-polymerizing, denatured actin, since the signal intensity did not decrease in proportion to the increase in viscosity. Such a proportionality would be expected if the reduction of the signal intensity was caused by an increase in the rotational correlation time of a denatured actin monomer. In contrast, the

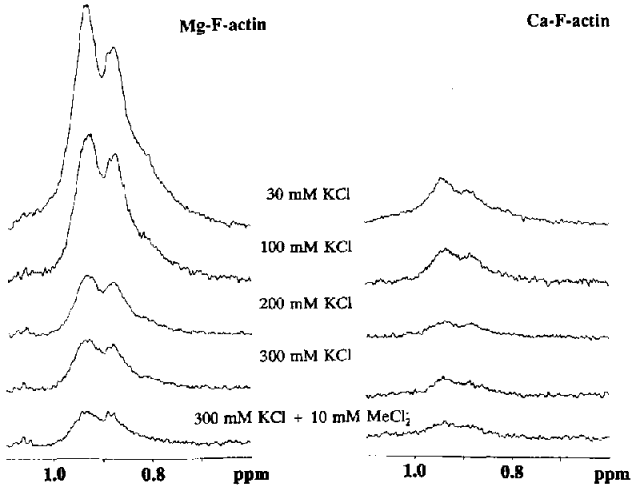

Fig. 3. Polymerization of actin with KCl. The samples contained 120 μM 72 μM Mg-actin (left) or Ca-actin (right) in the buffers described in Fig. 3. Actin was polymerized in the NMR tube by the addition of the appropriate amount of salts. $MeCl_2$ means $MgCl_2$ in the case of Mg-actin and $CaCl_2$ for Ca-actin. Temperature 278 K, identical parameters were used for recording, processing, and representation of the spectra.

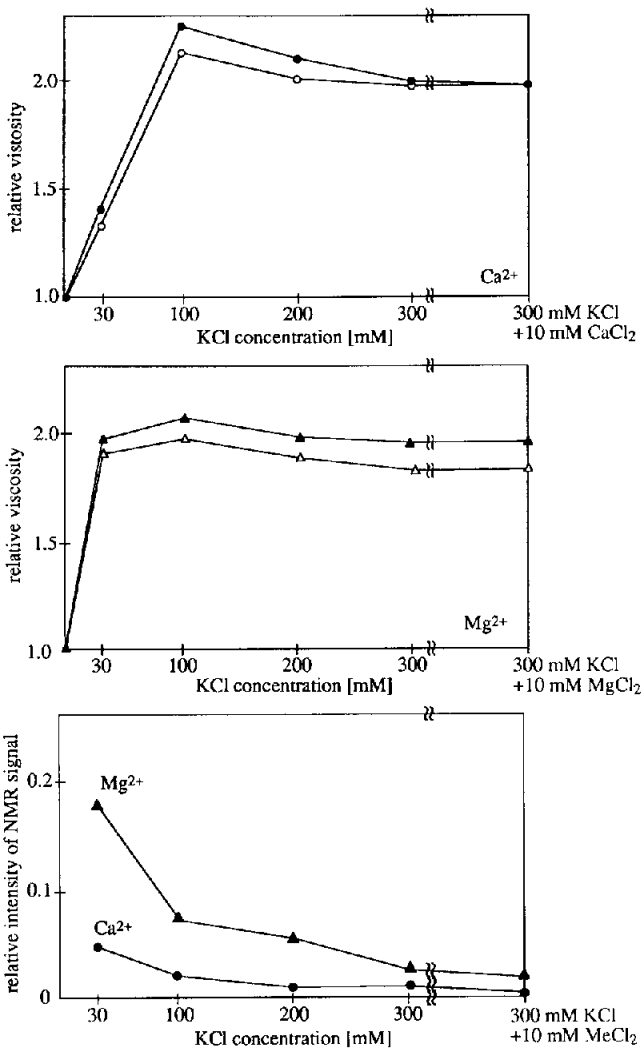

Fig. 4. Viscosity and Intensity changes of the ^{1}H NMR signal of actin as function of the ionic strength. Measurements were performed with the same protein used in Fig. 3. The relative viscosity η is plotted in dependence on the ionic conditions. The relative viscosity of the corresponding G-actin solution is set to 1. (○, ●) Ca-actin, (△, ▲) Mg-actin. Filled and open symbols represent two different measurements (top and middle). The integrals from the methyl ranges (between 0.6 ppm and 1.1 ppm) in Mg-F-actin (△) and Ca-F-actin (○) relative to the corresponding integrals in G-actin are plotted as function of the ionic conditions (bottom).

decrease in intensity shows a different characteristic, it continues to decrease with the concentration of cations even after the viscosity has reached its maximum.

3.3. *Polymerization of actin in the presence of phalloidin*

Ca- and Mg-actin were polymerized in the presence of phalloidin at various concentrations. As long as the phalloidin concentration was smaller than the actin concentration no phalloidin signal could be observed. This indicates that phalloidin is tightly bound to F-actin and completely immobilized. At relative concentrations higher than 1:1 the signal from free phalloidin is visible in accordance with the known stoichiometry of binding. Phalloidin is known to stabilize the actin filaments and to promote the polymerization of actin. However, the phalloidin does not significantly influence the NMR signals of F-actin. Polymerization of actin by monovalent ions (30 mM KCl) in the presence of phalloidin does not change the typical features of the F-actin spectrum. The characteristic reduction of the signal intensity is independent of the presence of phalloidin and the differences observed for Mg- and Ca-actin are completely conserved.

3.4. *Comparison with data from other authors*

As mentioned in the introduction, the F-actin spectra published in the literature vary greatly. This is not surprising when we remember that the signal intensity depends strongly on the polymerization conditions. In the F-actin spectra reported by Highsmith et al. [6–7], all resonance lines are broadened beyond detection. Taking into account the polymerization conditions (e.g. 5 mM $MgCl_2$, 50 mM KCl) this is not surprising since the signal should be rather weak. With the instrumentation available at that time such a weak signal was probably not detectable above the noise level. The F-actin spectra published by Prince et al. [15] and Trayer et al. [20] are characterized by a high signal intensity and spectral features which are in some respects intermediate between our F-actin and G-actin spectra. Qualitatively, they could be explained as a superposition of the two spectra, that is, they could be caused by an incomplete polymerization of the sample. We have obtained similar results for preparations where a part of the actin was denatured. Such a partial denaturation could result from the freeze-drying of the protein, which appears to affect actin's properties.

3.5. *A simple model for the ionic dependence of the internal mobility*

If we accept that the signals we see originate from mobile domains of actin in a supramolecular assembly (F-actin), we have to explain first is the gradual decrease of the signal intensity with increasing salt concentration and its dependence on the type of divalent ions present. In the standard model of actin polymerization (see e.g. [25–26]), polymerization is essentially all-or-none; under polymerization conditions essentially only two forms of actin coexist, G-actin and F-actin, while the concentration of small polymers such as the supposed trimers necessary for nucleation is minute. This is also in line with our observations on fluorine labeled actin [26] where only G-actin and large polymers could be detected. When one assumes this standard model our observations demonstrate that different states of actin protomers exist in actin fibers.

Since the type of the spectrum and the corresponding line widths are essentially independent of the degree of signal reduction, the simplest explanation would be the existence of two states of actin protomers in F-actin. In one state, some parts of the molecule would be rather mobile (M-state) and hence NMR visible, in the other state these domains of the actin protomers would be completely immobilized (I-state) and invisible in the high-resolution NMR spectrum. A change of signal intensity would then correspond to a shift of the equilibrium between the two states induced by the experimental conditions. In Ca -actin a larger number of the protomers would be in the I-state than in Mg-actin.

Since the concentrations of divalent ions used are rather low, it is likely that the high-affinity binding site in the active center [28–30] is mainly responsible for the different mobilities in Ca-F-actin and Mg-F-actin. However, the 3 to 5 binding sites with intermediate affinities for divalent ions (K_a between 0.018

mM^{-1} and 6 mM^{-1}) [26,31–34] could also be responsible for the observed effects. The effect is already pronounced at Ca^{2+} concentrations of 0.1 mM; thus, the corresponding sites should have binding constants > 10^4 M^{-1}, which has not been found by all of the previous authors. In addition, the intermediate -affinity binding sites are reported to have a similar affinity for Ca^{2+} and Mg^{2+} ions. If merely the occupation of these sites (and not the type of ion at the high-affinity site) were responsible for the equilibrium between I- and M-states, one would expect that the polymerization of Ca-actin with 10 mM $CaCl_2$ or Mg-actin with 10 mM $MgCl_2$ would lead to the same intensities of the NMR spectra of F-actin. Since this is not the case one can conclude that the reduced mobility is at least partly dependent on the type of ion occupying the high-affinity site. Since an increase in ionic strength using monovalent ions likewise shifts the equilibrium towards the I-state, a second factor must influence the equilibrium. This could be the occupation of the not completely occupied intermediate-affinity sites for divalent ions or the binding to additional, unspecific low-affinity binding sites.

The assignment of the NMR-visible, mobile residues cannot be done by NMR spectroscopy since the obtainable signal-to-noise ratio is not high enough. However, on the basis of the F-actin structure published recently [35–36], one can exclude the regions which form close contacts between the actin protomers as canditates for high mobility. Since the presence of phalloidin has no influence on the mobility of the NMR visible residues, the residues in contact with phalloidin are most probably not part of this domain. These contacts probably immobilize also the adjacent structures, so that the amino acids of the whole subdomains 3 and 4 which form the inner parts of the filament are not likely candidates for the observed mobility. Subdomains 1 and 2 form the outer part of the filament and are therefore possible canditates for high mobility in F-actin. Normal mode studies of G-actin [37] show a high mobility of subdomain 2. Within subdomain 2 the fragment of a sequence from residue 32 to 52 is of the highest mobility. In the F-actin model this loop interacts with the neighboring protomer; however the model was based on data from Ca-actin and could therefore represent the I-state whereas in the M-state this loop could be freely mobile. Another candidate is the N-teminus of actin, which is exposed to the solvent in the F-actin model. The spectrum of F-actin contains a singlet resonance at 2.05 ppm which could originate from the N-terminal acetyl group (but also from a methionine methyl group elsewhere in the structure).

Orlova and Egelman [22–23] have described changes of the average flexibility of F-actin polymerized in the presence of ATP depending on the divalent ion in the active-center. They found that Ca^{2+} actin filaments were more rigid than Mg^{2+} filaments. Since we observe also a higher mobility in Mg-actin it is tempting to assume that the two observations reflect the same process. The rigidity and flexibility described by Orlova and Egelman [22–23] mainly are a property of the whole actin filament, although large parts of subdomain 2 of actin were not visible in the reconstruction of Mg-F-actin. The data were interpreted as indicating an enhanced mobility of this domain. However, one should realize that the supposed dynamic property is only derived from static electron micrographs. Our M- and I-states correspond to a true dynamical state (in the nanosecond time-scale) of actin protomers. Although the two methods measure in principal two different properties, the two properties could be related if immobilisation of the M-domain were the cause of the rigidity of the filaments. However, the two properties are not strongly correlated, since for example the rigidity of Mg-actin in electron microscopy inreases with the addition of phalloidin, which has no influence on the NMR observations.

3.6. Conclusion

^1H NMR spectroscopy reveals that actin protomers in F-actin can exist in two different dynamic states, the M- and the I-state. The populations of these states in F-actin depend on two factors; the type of divalent ion in the active center, and the occupation of unspecific binding sites by mono- or divalent ions. The presence of Ca^{2+} and high ionic strength shift the equilibrium from the high mobility state (M-state) to the state with low internal mobilty (I-state). Unfortunately, the signal-to-noise ratio obtainable is not sufficient for a sequential assignment of the mobile resonances. But even without knowing the location of this highly mobile region in actin, the data presented are additional evidence that F-actin should not be viewed as a rigid cable. F-actin itself has dynamic properties which may play some role during the cross-bridge cycle during muscle contraction.

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