Coupling of Folding and Binding of Thymosin β4 upon Interaction with Monomeric Actin Monitored by Nuclear Magnetic Resonance*

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Thymosin β4 is a major actin-sequestering protein, yet the structural basis for its biological function is still unknown. This study provides insight regarding the way this 43-amino acid peptide, mostly unstructured in solution, binds to monomeric actin and prevents its assembly in filaments. We show here that the whole backbone of thymosin β4 is highly affected upon binding to G-actin. The assignment of all amide protons and nitrogens of thymosin in the bound state, obtained using a combination of NMR experiments and selective labelings, shows that thymosin folds completely upon binding and displays a central extended region flanked by two N- and C-terminal helices. The cleavage of actin by subtilisin in the DNase I binding loop does not modify the structure of thymosin β4 in the complex, showing that the backbone of the peptide is not in close proximity to segment 42–47 of actin. The combination of our NMR results and previously published mutation and cross-link data allows a better characterization of the binding mode of thymosins on G-actin.

Cell locomotion or changes in cell shape are mediated by the rapid polymerization of actin in response to signaling. The source for this massive increase in F-actin is provided by a large reservoir of unassembled, “sequestered” actin, which is kept monomeric by interaction with G-actin-binding proteins (1). Thymosin β4 (Tβ4), first identified as the most abundant (300 μM) actin-sequestering protein in human blood platelets (2, 3) and neutrophils (4), was eventually shown, together with variants of the β-thymosin family, to bind G-actin in all vertebrates (5–8) and some invertebrates (9). In contrast to profilin, another G-actin-binding protein, which can either sequester actin when filament barbed ends are blocked by capping proteins or participate in barbed end assembly when barbed ends are unoccupied (10), Tβ4 acts as a simple sequestering protein that prevents G-actin association to both ends of actin filaments. It reacts passively to the changes in critical concentration for actin assembly while amplifying them. Tβ4 essentially binds G-actin with a high specificity for the ATP-bound form (11), an affinity in the 106 M−1 range (3, 11), and relatively slow association-dissociation kinetics that are indicative of a structural change of the complex after rapid equilibrium binding (12). Finally, in binding to G-actin, Tβ4 slows down metal ion/nucleotide dissociation (13, 14). This protein is composed of a single WH2 domain, which has recently been identified in 37 other proteins belonging to various organisms, and was shown to be an evolutionarily conserved actin-monomer binding motif (15). Interestingly, different WH2 domains were found to perform different functions in the regulation of actin monomeric concentration and actin assembly processes. Whereas β-thymosins inhibit the assembly of actin filaments, the other WH2 domains characterized thus far display a profiling-like activity, promoting the assembly of actin filaments at the barbed end (16–20). A fine structural and dynamic characterization of WH2 domain interfaces with actin is therefore essential to understand the functional variability of WH2 domains.

Whereas the structure and interface with actin of other G-actin binding proteins such as DNase I (21), gelsolin segment-1 (22), and profilin (23) are well known from crystallographic studies, Tβ4 and, more generally, WH2 domains remain elusive. NMR studies have shown that Tβ4 was mostly unstructured in aqueous solution, except for a short helical region (residues 5–16) at low temperature (24). Mutagenesis studies indicated that the α-helical structure of this region was required for binding to actin (25–27). Biochemical results based mainly on chemical cross-linking indicated that Tβ4 bound G-actin in an extended conformation, with the N-terminal helical region (residues 1–19) making contacts with residues in subdomain 1 of actin, whereas the C-terminal region could be cross-linked to His46 and Glu111 of actin in subdomain 2 (12, 28, 29). According to these results, the ability of Tβ4 to interfere with actin-actin contacts involved in filament assembly at both the barbed and pointed ends of the actin monomer was thought to account for its inhibition of polymerization via a simple steric mechanism and also for its competition with profilin and DNase I for binding to G-actin. Recent biochemical and thermodynamic studies of the binding of Tβ4 to CaATP-actin and MgATP-actin, based mainly on fluorescence quenching of 5(6)-acetamido-5(6)-ethylaminonaphthalene-1-sulfonate conjugated to Cys374 of actin upon Tβ4 binding and on tritium exchange, also suggested that this small protein changed the conformation and structural dynamics of actin monomers (12).

Our study constitutes the first essential step for the under-
standing of the molecular basis of G-actin sequestration by thymosin β4. A number of specificities of the biological system under study render its structural analysis especially challenging for both crystallography and NMR. The tendency of G-actin to self-assemble, even at low ionic strength, at concentrations greater than 50–70 µM, the high flexibility of Tβ4, and the limited stability of the Tβ4-G-actin complex at high concentrations have hampered crystallization of the complex thus far. These difficulties, combined with the large size of the complex (47 kDa), preclude 1H, 13N, 15C NMR spectroscopy studies of the Tβ4-actin complex that require long acquisition periods. Here we show that the assignment of the whole 15N-1H2 correlation spectra of Tβ4 bound to monomeric CaATP-actin and MgATP-actin is successfully performed using an alternative strategy based on appropriately chosen specific labels. Our results demonstrate that the whole Tβ4 backbone is tightly bound to monomeric actin and that the structures of the peptide bound to CaATP-actin, MgATP-actin, and subtilin-cleaved actin are identical. The secondary structure of actin-bound thymosin β4 is obtained, and novel information is also provided about its fast dynamics using 1H-15N heteronuclear NOE experiments. A hypothetical model of the complex is proposed, based on biochemical data and our NMR results.

**EXPERIMENTAL PROCEDURES**

**Protein Production**

Actin—Actin was purified from rabbit muscle acetone powder (30) and isolated in the CaATP-G-actin form by chromatography through Sephades G-200 (31) in G buffer (5 mM Tris-Cl, pH 7.8, 0.1 mM CaCl2, 0.2 mM diethiothreitol, 0.2 mM ATP, 0.01% NaN3). Subtilisin-cleaved actin was obtained as described previously (24, 25). Briefly, CaATP-G-actin (25–50 µM) in G buffer was incubated with subtilisin (Sigma) at a 1:1500 (w/w) ratio to actin at 20 °C for 45 min. The reaction was stopped by addition of 1 mM phenylmethylsulfonyl fluoride. SDS-PAGE showed that ~95% of the 42-kDa actin polypeptide had been converted into a 37-kDa fragment, with no further degradation. Pyrenyl-labeled actin was prepared as described previously (32).

Thymosin β4 and Thymosin β10—Recombinant thymosins β4 and β10 were bacterially expressed in Escherichia coli using pET3d vectors kindly provided by Dr. Helen Lu Yin (University of Texas Southwestern Medical Center). Bacteria (BL21(DE3) strain) were grown at 28 °C in diverse media depending on the labeling: (a) [U-15N]Tβ4 and [U, 13C]-[15N]Tβ10, minimal (M9) medium containing 1 g/liter [15N]NH4Cl and supplemented with 5 mg/liter thiamine, 0.2% glucose, 1 mM MgSO4, and 0.1 mM CaCl2; (b) [15N]Tβ4, minimal (M9) medium containing 1 g/liter [15N]NH4Cl and 2 g/liter glucose and supplemented with 5 mg/liter thiamine, 1 mM MgSO4, and 0.1 mM CaCl2; and (c) [15N]-labeled A. Watersgate sequence was used in all experiments to achieve water signal suppression (36).

**NMR Samples and Experiments**

NMR Samples—Thymosin NMR samples consisted of [15N]-labeled thymosins at a concentration of 1–2 mM in a 4 mM phosphate buffer (pH 6.9) containing 10 µM ATP, 20 mM CaCl2, 10% D2O, and 0.015% sodium azide. NMR samples of complexes between actin and thymosins were prepared by mixing 20 µl of 47 µM actin with thymosins taken from a 2 mM stock solution up to a 1:1 molar ratio, occasionally with a small excess of thymosins. The proteins were then concentrated up to 350–500 µM complex using a Centriprep 30 device (Amicon) and centrifuged at 3000 g for 45 min. The complexes were stored in 5 mM Tris-Cl, 0.1 mM CaCl2, 0.2 mM ATP, 0.2 mM diethiothreitol, 5% D2O, and 0.015% sodium azide. The final pH value was 6.9.

NMR Experiments—NMR experiments were performed on Bruker Avance 600 MHz and 800 MHz spectrometers equipped with 5-mm triple resonance gradient probes. Quadrature phase detection in the indirectly detected dimension was obtained via States-TCP1 mode (35). A Watergate sequence was used in all experiments to achieve water signal suppression (36).

Assignment of 1H, 15N, and 13C resonances of Tβ4 free in solution was obtained using standard procedures from two-dimensional 1H-15N NOEYS and total correlation spectroscopy experiments, three-dimensional 1H-1H NOEYS-HSQC, and three-dimensional 1H-15N total correlation spectroscopy-HSQC experiments and HNCA, HN(CO)CA, HCNO, HN(CA)CO, CBCANH, and CBCA(CO)NH experiments. Mixing time for NOEYS experiments was set to 120 ms. Two mixing times of 50 and 80 ms were used for total correlation spectroscopy experiments.

Chemical shifts were referred to sodium 2,2-dimethyl-2-silapentane5 sulonate, CS5s (37–39) were calculated with NMRVIEW software (40) using the “Wright(urea)” reference with sequence correction-dependent factors (39).

Pulse sequences used to determine 15N R1, R2(N2), R2(N1), and heteronuclear 1H-15N NOE values were similar to those described previously (41–43). R1(N2) experiments were performed with 10 relaxation delays [0.008, 0.020, 0.040, 0.200, 0.600, 2.000, and 2.400 s] for R1(N2) experiments, a set of 10 experiments was acquired, with relaxation delays of 0.008, 0.040, 0.080, 0.136, 0.200, 0.304, 0.480, 0.640, and 0.800 s. In the two sets of experiments, the points corresponding to different relaxation delays were acquired in an interleaved manner to avoid any bias that could arise from progressive shim drifting. For all experiments, the 15N saturation was set to 17 ppm in the F1 dimension (15N) and 7.6 ppm in the F2 dimension. Spectral widths were 23 ppm in the F1 (15N) dimension and 3.5 ppm in the F2 (1H) dimension. Cross-peak intensities were determined from peak heights using the SPARKY peak picking routine (Goddard, T. D., and Kneller, D. G. SPARKY 3 Software, University of California, San Francisco, CA). The relaxation spectrum R2(N1) was obtained from nonlinear fits to mono-exponential functions using the Levenburg-Marquardt algorithm (45). The quality of the fits and error estimations were obtained using established Monte Carlo procedures, with an experimental gaussian error set to 4 times the rms base plane...
noise level and using 500 synthetic data for each NH vector. 1H, 15N NOE enhancements were obtained as the ratio of the peak heights in the spectra recorded with and without saturation of protons during the relaxation delay.

For 15N-H HSQC experiments carried on Tβ4-actin complex, carrier frequencies were set at 117 ppm in the F1 dimension (1H,N) and 7.6 ppm in the F2 dimension. Spectral widths were 28 ppm in the F1 (1H,N) dimension and 5 ppm in the F2 (15N) dimension. The three-dimensional 1H, 15N NOESY-HSQC experiment used for the assignment of Tβ4 bound to actin was performed with a mixing time equal to 40 ms.

**Measurements of the Binding of Tβ4 to G-actin and Subtilisin-cleaved Actin**

The affinity of Tβ4 for actin or subtilisin-cleaved actin (subactin) was derived from the increase in actin tryptophane fluorescence (λexc, 295 nm; λem, 350 nm) linked to the binding of Tβ4 (46). Experiments were carried out in a Spex spectrofluorimeter at 20 °C, using 2 μM CaATP-G-actin or subtilisin-cleaved actin in G buffer containing only 20 μM ATP to minimize the screen effect.

The relative increase in fluorescence, \((F - F_0)/(F_{\text{max}} - F_0)\), was expressed as function of the molar fraction of actin in complex with Tβ4, \((\text{T}A)/\text{W}A\), according to the following equation:

\[
\frac{(F - F_0)}{(F_{\text{max}} - F_0)} = \frac{\langle A \rangle_0 + \langle T \rangle_0 \cdot K_T - \sqrt{\langle A \rangle_0 \cdot \langle T \rangle_0 + \langle A \rangle_0 \cdot K_T + 4\langle A \rangle_0 \cdot K_T}}{2K_T}
\]

(Eq. 1)

Alternatively, the affinity of Tβ4 for subtilisin-cleaved actin could be derived from its sequestering activity, by measuring the amount of G-actin or subtilisin-cleaved actin in G buffer containing only 20 μM associated to its binding to actin (\(\text{CT}_{\text{der}}\)) derived from critical concentration plots. The value of \(\text{CT}_{\text{der}}\) was derived from the increase in actin tryptophane fluorescence (\(\langle T \rangle\)).

\[
\frac{(F - F_0)}{(F_{\text{max}} - F_0)} = C_w - C_w \cdot K_T
\]

(Eq. 2)

in which \([T]A\) is the concentration of Tβ4-actin complex; \(C_w\) and \(C_w\) are the mass concentrations of F-actin in the absence and presence of Tβ4, respectively; \(C_w\) is the critical concentration for F-actin assembly; and \(K_T\) is the equilibrium dissociation constant of the Tβ4-actin complex.

The values of \(C_w\) for actin and subtilisin-cleaved actin were independently derived from critical concentration plots. The value of \(K_T\) was derived from the slope \(C_w/(C_w + K_T)\) of the lines describing the linear decrease of \(C_w\) with \([T]A\).

**RESULTS**

**Thymosin β4 Is Mainly Unfolded in Solution at 25 °C, Except for a Small Helical Content in Its N-terminal Region**

The assignment of the proton, nitrogen, and carbon resonances of Tβ4 free in solution were obtained using standard procedures. The assignments of the proton resonances of Tβ4 in solution at 2 °C were in agreement with those reported previously (24). The HSQC spectrum of Tβ4 in solution is typical of an unfolded protein, with a very narrow range of amide proton resonances (Fig. 1A). However, structural and dynamic data clearly show the partial formation of secondary structures in the N-terminal and C-terminal segments of the peptide. At 2 °C, the presence of NH-NH(i, i + 1), Ho-Ho(i, i + 2), Ho-NH(i, i + 3), Ho-NH(i, i + 4), and Ho-Hβ(i, i + 3) NOE correlations for all residues along segment 5–16 indicates that this part of the protein folds as a α-helix. This conclusion is corroborated by the CSI of Ho and Ca nuclei (Fig. 1B) (37–39). The concomitant presence of strong Ho-NH(i, i + 1) correlations in this region, however, shows that the helix form is in fast exchange with an extended strand conformation. No NOEs characteristic of the existence of secondary structures were found in any other region of the protein. However, the small negative Ho and positive Ca shifts relative to the random coil shifts observed for residues 31–37 suggest that this segment has a weak tendency to fold into an α-helix. The tendency of segment 31–37 to form a helix at 2 °C in water is also in agreement with the folding of this segment.
into an α-helix in trifluoroethanol (52). The α-helical fold of the N-terminal part of Tβ4 is anticipated from the analysis of primary sequence. The N-terminal sequence Pro^4-Asp^5-Met^6-Ala^7-Glu^8-Ile^9 indeed corresponds to an N-capping box motif: h-(D,N,T,S)-N1-N2-(E,Q)-h, where N stands for any amino acid, and h is a hydrophobic residue (53–55). This motif is known to initiate the first turns and the propagation of α-helices in the C-terminal direction and to strongly stabilize their N-terminal extremity. In contrast with data obtained at 2 °C, no NOE correlation indicative of any secondary structure was found at 25 °C.

The 15N relaxation data obtained on Tβ4 free in solution at 2 °C and 25 °C reinforces the conclusion obtained from structural data. The low values of heteronuclear NOEs, which are all below 0.5 at 2 °C and below 0 at 25 °C, provide clear evidence of the high flexibility of Tβ4 in aqueous solution (Fig. 2). In a fully structured, globular protein of this size, these values are expected to lie around 0.7 at 2 °C and 0.4 at 25 °C. Heteronuclear NOEs found here are thus compatible with a globally unfolded protein. The spectral densities for the N-H vectors of the backbone at the three frequencies 0, ωN (60 MHz), and around ωN (540 MHz) were calculated from R2(N) and R2(Nx,Ny) relaxation time constants and heteronuclear 1H,15N NOEs measured at 2 °C and 25 °C using a reduced matrix approach (56, 57) (data not shown). Both absolute and relative values of these spectral densities at the three frequencies again emphasize the internal flexibility along the whole polypeptide chain. However, their profiles along the sequence are not completely homogeneous. At both temperatures, higher values of J(0) compensated by lower values of (Δωx) in the segment 10–16 demonstrate a restriction of internal flexibility in this fragment typical of a nascent helix. This restriction of motions in the hundred of picoseconds time scale can be also seen in the C-terminal segment 31–36 of the protein, although to a smaller degree.

Thymosin β4 Forms a Tight 1:1 Complex with Monomeric CaATP-actin at 25 °C—Fig. 3A shows the HSQC spectra recorded at 25 °C for a sample containing equimolar amounts of [U,15N]Tβ4 and unlabeled CaATP-actin (see “Experimental Procedures”). A completely new set of 46 peaks of the 47 peaks expected for Tβ4 is obtained. All the resonances are spread out as compared with the free form. The large dispersion of amide proton resonances in the bound form is typical of a fully structured protein. The large spreading of all resonances of Tβ4 testifies that at 25 °C, Tβ4 forms a tight complex with G-actin, in which the environment of all residues of the peptide is drastically modified, either by folding or by making contacts with G-actin.

A Selective Labeling Strategy Allows the Assignment of Tβ4 Bound to Monomeric CaATP-actin—The assignment of the resonances of Tβ4 bound to monomeric CaATP-actin was carried out at 25 °C. However, the low concentration and stability of the sample, added to the large size of the complex, precluded the use of classical strategies for the assignment. The most sensitive three-dimensional HNCO experiment gave only three detectable peaks, corresponding to the most flexible terminal

**Fig. 1.** Structural results obtained on [U-15N]Tβ4 free in solution at 2 °C. A, two-dimensional 1H,15N correlation spectrum of [U-15N]Tβ4 free in solution at 2 °C. The assignment of 1H,15N correlation peaks is indicated in the spectrum. For the sake of clarity, the central region of the spectrum (gray zone) was reproduced at a greater scale in the bottom right corner of the spectra. B, CSAs as calculated with NMRVIEW software using the Wright(urea) parameters.

**Fig. 2.** Steady-state heteronuclear (1H-15N) NOEs. Steady-state heteronuclear 1H-15N NOEs constitute a probe for fast dynamics of NH bonds in the hundred of picoseconds timescale. Lower values indicate a high degree of flexibility; the maximal value of 0.83 indicates an absence of internal flexibility. □, Tβ4 free in solution at 2 °C; ○, Tβ4 free in solution at 25 °C. ■, Tβ4 bound to G-actin at 25 °C. In the case of Tβ4 free in solution, the uncertainties are lower than the size of the markers.
residues. Use of TROSY versions of these experiments was unsuccessful. The three-dimensional 1H-15N NOESY-HSQC was the only experiment that provided reliable results. Assuming inter-residual and sequential amide/side chain protons proximities, the analysis of HN-HN and HN-H5251 connectivities allowed a possible assignment of the N-terminal segment Met6-Ser15 and the C-terminal fragment Glu 35-Ser43. However, no correlation could be found allowing the assignment of amino acids in the central fragment 16–34. A method based on selective labeling was then considered.

The strategy for selecting Leu, Thr, and Lys as 15N-labeled amino acids is described under “Experimental Procedures.” All the results obtained for selective labeling of Tβ4 were in good agreement with amino acid evolution in the nitrogen cycle of E. coli (33, 34). 15N HSQC spectra of [15N]AA Tβ4 (where AA is Leu, Thr, or Lys) bound to G-actin are shown in Fig. 3B. Resonances obtained for the selectively labeled Tβ4 perfectly superimposed those obtained for the uniformly labeled protein.

Finally, the three successful selective labelings, in conjunction with the 15N NOESY-HSQC experiment, allowed the complete assignment of nitrogen and amide protons of Tβ4 bound to G-actin. All amide protons strips could be assigned by searching similarity between strips, with the additional knowledge of the type of the amino acids that had been selectively labeled. The amino side chain undergoing the larger shift upon binding to G-actin could be assigned to Asn26, suggesting its location at the interface of the complex. Assignment of Hα protons can also be suggested from the cross peaks between amide and Hα protons in the 15N NOESY-HSQC. When HN-H5251 correlations indicative of a helical folding were present, the most intense HN-Hα correlation was considered to be intr Residual. In the other cases, the most intense HN-Hα correlation was considered to be sequential (HN(i)-Hα(i-1)).

Besides this strategy, an HSQC spectrum of the complex between Tβ10 and monomeric CaATP-actin was performed. A large spreading of the resonances upon binding was observed, as for Tβ4, demonstrating the formation of an analogous tight complex of Tβ10 with actin. The central segment 15KLKK-TETQEKN is strictly conserved between Tβ4 and Tβ10. The absence of chemical shift variations in the HSQC spectra of the two thymosins for the peaks previously assigned to 18LKK-TETQEK in the Tβ4-CaATP-actin complex confirmed the assignment of these 9 residues (data not shown).

Tβ4 Bound to G-actin Is Composed of an Extended Central Segment Flanked by Two α-Helices, and Its Dynamic Behavior Demonstrates the Binding of the Whole Peptide’s Backbone to G-actin—The very large variations of chemical shifts observed for amide nitrogens and protons along the whole backbone of free and actin-bound Tβ4 demonstrate that the polypeptide chain undergoes a complete reorganization upon binding (Fig. 3C). The CSI calculated on 15N, 13N, and Hα of Tβ4, together with the analysis of 15N-1H and 15N-Hα NOE connectivities,
converges on the existence of two α-helices, Asp5-Leu17 and Lys8-Ala19, linked by an extended fragment, Lys8-Asn26 (Fig. 3D).

Heteronuclear 1H-15N NOEs could be measured on Tβ4 bound to G-actin at 25°C. The histogram of resulting NOEs is shifted from a value centered around −0.2 for free Tβ4 to 0.7 for bound Tβ4 (Fig. 2B). Due to the low concentration of the complex and the restricted recording time imposed by the limited stability of the sample, a low signal to noise ratio and therefore imprecise values of heteronuclear NOEs were obtained. However, the large positive shift of NOE values upon Tβ4 binding to actin clearly indicates that Tβ4 adopts the correlation time of a fully bound peptide. The smaller values of heteronuclear NOEs in the center of the sequence indicate that the corresponding N-H bonds of Tβ4 in the complex retain some internal flexibility in the hundred of picoseconds range (Fig. 2A).

The Structures of Tβ4 Bound to CaATP-actin and MgATP-actin Are Identical—De La Cruz et al. (12) recently observed significant differences in the binding characteristics of Tβ4 to CaATP-actin and MgATP-actin. The 15N-1H HSQC spectra of Tβ4 bound to CaATP-actin or MgATP-actin are identical (data not shown). Amide nitrogen and proton chemical shifts are very bound to CaATP-actin or MgATP-actin are identical (data not shown). However, the large positive shift of NOE values upon Tβ4 binding to actin clearly indicates that Tβ4 adopts the correlation time of a fully bound peptide. The smaller values of heteronuclear NOEs in the center of the sequence indicate that the corresponding N-H bonds of Tβ4 in the complex retain some internal flexibility in the hundred of picoseconds range (Fig. 2A).

Cleavage of G-actin by Subtilisin Does Not Alter the Tβ4-actin Complex—Tβ4 binding to subtilisin-cleaved actin (subactin) was linked to a 9% increase in the actin tryptophane fluorescence, as compared with an 11.6% increase upon binding to unmodified G-actin (Fig. 4A). Binding constants of 1.5 ± 0.2 and 2.7 ± 0.4 μM were found for the complexes of Tβ4 with CaATP-bound actin and subactin, respectively. The actin- and subactin-sequestering activities of Tβ4 were compared under different ionic conditions. Within low ionic strength polymerization buffer (1 mM MgCl2 and no KCl), the critical concentrations for filament assembly were 0.35 μM for actin and 1.7 μM for subactin (Fig. 4B, inset). The slopes of the linear decrease in F-actin versus the total concentration of Tβ4 yielded values of 0.4 μM for Kf and 1 μM for Kβ (Fig. 4B, a). These values refer to binding of Tβ4 to the MgATP-bound form of G-actin. At physiological ionic strength (1 mM MgCl2 and 0.1 M KCl), the critical concentrations were 0.1 and 0.5 μM for actin and subactin, respectively (Fig. 4B, a, inset). When increasing amounts of Tβ4 were added to a 16 μM unmodified F-actin solution in Mg/KCl polymerization buffer, the decrease in F-actin was not a linear function of Tβ4 concentration, as observed previously (11), which reflected the interaction of Tβ4 with F-actin at high concentration (Fig. 4B, b). From the linear portion of the curve at low Tβ4 concentration, a Kf value of 1.2 μM was derived. In contrast, the concentration of subtilisin-cleaved F-actin decreased perfectly linearly with increasing Tβ4, demonstrating that when loop 38–52 is cleaved, Tβ4 no longer interacts with F-actin at high ionic strength (Fig. 4B, b). A Kβ value of 2.7 μM was derived from these experiments.

Nucleotide exchange is known to be strongly inhibited on G-actin upon Tβ4 binding (13). We find that this property is conserved for subtilisin-cleaved actin. The rate of nucleotide exchange was 1.6-fold higher on subtilisin-cleaved actin than on unmodified actin. Binding of Tβ4 to subactin lowered the exchange rate by 100-fold, as for unmodified actin. The Tβ4 concentration dependence of the observed first order rate constant was consistent with a Kf value of 1.5 μM for binding of Tβ4 to subactin (data not shown).

In conclusion, cleavage of loop 38–52 on G-actin, which leads to higher critical concentrations for actin filament assembly, causes only a 2-fold decrease in the affinity of Tβ4 and does not affect the functional properties of Tβ4, except for the loss in ability of Tβ4 to weakly interact with subtilisin-cleaved F-actin. The 15N-1H HSQC spectrum of Tβ4-subactin at 0.5 mM at 25°C was fully superimposable over the one obtained with unmodified actin (data not shown), except for slightly narrower linewidths in the case of subactin, due to the lower concentration of polymerized actin. NMR data provide information on the localization of Tβ4 on actin. The structural integrity of actin is known to be conserved after subtilisin cleavage of segment Val51-Met47 in the DNase I binding loop, which is locked up in the three-dimensional structure of G-actin by a β-sheet itself stabilized by an α-helix (Ref. 58 and this study). The lack of difference in the chemical shifts between Tβ4 bound to actin and subactin demonstrates that all amide nitrogens and protons of Tβ4 are in the same environment in both complexes and that they are thus not likely to be in the vicinity of the cleaved segment Val51-Met47 in subactin.

DISCUSSION

Most structural studies of protein-protein complexes address the interaction of two folded partners with defined three-dimensional structures (59–63). However, a number of proteins are physiologically unstructured and fold only upon binding to their biological target (64). Many of these proteins are involved in cell cycle regulation processes in which their unstructured state provides significant advantages such as a fast turnover, relatively low binding constants, and the ability to bind several targets (64, 65). The unfavorable binding entropy linked to the induced folding process requires a precise control over the thermodynamics of the binding. A structural characterization of the proteins, both alone and bound to their target, is crucial to understand how these polypeptides selectively recognize their targets and perform specific functions. Nuclear magnetic resonance is uniquely well suited to provide detailed structural and dynamic information about unstructured proteins and the level of structure of each partner upon binding and thus to describe the local and global thermodynamics governing the binding process of unfolded states (43, 66, 67).

The present work constitutes the first structural study of the interaction between a major actin-sequestering protein, thymosin β4, and unmodified G-actin at concentrations (0.3–0.5 mM) that are physiological.

Strategies for the Assignment of Thymosin β4 Bound to G-actin—Despite the small size of Tβ4, its assignment when bound to G-actin presents major difficulties due to the low stability and large size of the complex. The impossibility of growing recombinant actin at a significant level precludes the opportunity to obtain large quantities of deuterated actin that could drastically reduce the relaxation of the system and allow the use of classical three-dimensional NMR experiments or their TROSY counterparts for the assignment. We show here that this drawback can be partly bypassed by using a selective labeling strategy. In the case of a relatively small polypeptide such as thymosin, the combined use of the three selective labelings of threonines, lysines, and leucines and a three-dimensional 1H-15N NOESY-HSQC experiment was sufficient to obtain the complete assignment of amide protons and nitrogen resonances.
**Fig. 4. Cleavage of G-actin by subtilisin does not alter the thymosin β4–actin complex.** A, fluorescence titration curves of unmodified and subtilisin-cleaved actin by thymosin β4. Tβ4 at the indicated concentrations was added to 2 μM CaATP-G-actin (●) or subtilisin-cleaved actin (○). The percentage increase in tryptophan fluorescence was measured. **Solid lines** are binding curves calculated according to Eq. 1 and using maximal relative increases in fluorescence of 11.6% and 9% for actin and subtilisin-cleaved actin, respectively, and values of 1.5 and 2.7 μM for K2 and K1. B, thymosin β4 sequesters subtilisin-cleaved actin as well as unmodified actin. Tβ4 at the indicated concentrations was added to solutions of 2% pyrenyl-labeled unmodified F-actin (●) or subtilisin-cleaved actin (○). The fluorescence of pyrenyl-actin was measured after a 16-h incubation at room temperature. a, measurements in low ionic strength assembly buffer (1 mM MgCl2 added to G buffer). Actin concentration, 1.5 μM; subtilisin-cleaved actin concentration, 3 μM. Inset, critical concentration plots for actin (● and ○) and subtilisin-cleaved actin (□ and ○) in low ionic strength (● and ○) and physiological ionic strength (● and ○) buffers. Note that the specific fluorescence of subtilisin-cleaved F-actin is 28% lower than that of unmodified F-actin. b, measurements at physiological ionic strength (1 mM MgCl2, 0.1 M KCl). Actin and subtilisin-cleaved actin concentrations = 16 μM.
cleavage of actin by subtilisin does not modify the electronic environment of THβ4 amide protons demonstrates that the backbone of the C-terminal end of the peptide does not make direct contact with segment 42–47 of actin, in agreement with the accessibility of Gly46 of actin in THβ4-actin complex to proteolytic enzymes (12). However, it seems at first sight less compatible with the formation of cross-links between C-terminal residues of THβ4 and His46–Gln48 of G-actin. All results, however, can be accommodated by the high flexibility of the DNase I binding loop that allows the cross-link of Lys38 and Ser40 of THβ4 with actin. Finally, the results are in favor of an induced fit between the C-terminal end of THβ4 and actin, resulting in a locked conformation of the beginning of the DNase I binding loop that is not perturbed by the cleavage of peptide 43–47 by subtilisin.

Models of the complex were built that are compatible with our NMR results and with the previously mentioned biochemical data (see “Experimental Procedures”). The problem of the docking of two proteins is particularly arduous when one of the proteins undergoes large conformational changes upon binding, as is the case for thymosin here. The use of experimentally derived constraints allowed a delimiting of the interaction surface (from the cross-links and mutagenesis results) and a restriction of thymosin β4-bound conformations (from our NMR results) but was not sufficient to converge toward a unique cluster of complexes. In particular, the lack of orientational restraints for the two helices of THβ4 toward actin leads to a large uncertainty in the position of the extended central segment. However, the models clearly show that thymosin β4 can cross the whole surface of G-actin, with the N-terminal and C-terminal helices making contacts with the pointed and barbed end of G-actin, respectively (Fig. 5).

HSQC spectra unambiguously demonstrate that the structure of THβ4 is the same when bound to CaATP-actin or MgATP-actin. De La Cruz et al. (12) reported significant differences in the thermodynamic and kinetics parameters for the binding of THβ4 to CaATP-actin and MgATP-actin. This suggests that the peptide is able to drive the two different initial states of CaATP-actin and MgATP-actin toward a unique Ca/MgATP-actin-THβ4 bound state.

Implication for the Sequestering Activity of Thymosin β4—Thymosin β4 belongs to the class of WH2 domain proteins that contain an evolutionarily conserved actin monomer-binding motif originally found in β-thymosins. They are all thought to play a role in the regulation of actin dynamics through their binding to actin monomers. The few structural studies of WH2 domains show that they are mostly unfolded alone under physiological conditions. Remarkably, they perform different functions in the regulation of actin polymerization, with some preventing and others promoting actin filament assembly, and the structural basis of these differences has not yet been elucidated.

The extended conformation of THβ4 on G-actin leads to a broad interface, which is compatible with the wide conformational changes undergone by the protein upon binding. The large entropy loss generated by the folding of intrinsically unfolded proteins upon binding to their target is usually compensated by broad interfaces, allowing the formation of numerous hydrogen bonds and the expulsion of a large number of water molecules. In the case of thymosin, the helical propensity of the N-terminal segment also leads to a favored entropy of binding and most probably constitutes the driving force for binding actin. Moreover, it is known that protein-protein binding sites often contain hydrophobic patches. The hydrophobic face of the N-terminal amphiphilic helix of THβ4, consisting of Met6, Ile9, Phe12, and the side chain of Lys16, constitutes a unique favorable recognition hydrophobic patch along the THβ4 primary sequence. This hydrophobic N-terminal surface, which is present in most WH2 domains (15), probably forms a standard interface with actin that suffices to bring a stable association, but not to bring the function specificity of these proteins. The unique sequestering activity of β thymosins among the family of WH2 domains characterized thus far is then very likely due to a higher specificity of the C-terminal segment for actin or to a specific orientation of this C-terminal segment that prevents the nucleotide exchange and impedes actin-actin interaction at the pointed end of the filament. To investigate the specificity of the sequestering function of thymosins, some mutations that could modify this function can be proposed from our NMR results. Two regions can be probed first. The central region containing the so-called “actin binding motif” LKKTET is highly specific of the thymosin family. Residues of this region undergo the largest chemical shift variations upon binding to actin, indicative of a tight interaction in the interaction with actin, most probably through the formation of intermolecular hydrogen bonds. Some of these residues are not strictly conserved in other WH2 domains, especially Thr20 and Asn26, which are replaced by hydrophobic residues (Ala or Val for Thr20 and Ile for Asn26) in a number of other WH2 domains such as the domain 1 of ciboulot, which was recently shown to exhibit a profilin-like function (19). Subtle variations of the interactions in this region could lead to a variation of the orientation of the dynamics of the C-terminal segment of the WH2 domain. The location and stability of this C-terminal segment, which extends to the pointed end of actin in the case of THβ4, are likely to be crucial for the sequestering activity (29).

In this segment, the acidic residue Glu35, which also undergoes a large chemical shift variation upon actin binding, is replaced by non-acidic, hydrophobic residues in a number of other WH2 domains. The analysis of the activity and structural features of mutants suggested from this study should bring additional information on the key interactions that govern the specificity of function of THβ4 and, more generally, actin-binding WH2 domains.

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Coupling of Folding and Binding of Thymosin β4 upon Interaction with Monomeric Actin Monitored by Nuclear Magnetic Resonance
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