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The N-Terminal Sequence (5–20) of Thymosin β4 Binds to Monomeric Actin in an α-Helical Conformation

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The relationship between the conformation of a peptide in solution and its interaction capacity is generally unclear. Trifluoroethanol (TFE), which stabilizes alpha-helical conformations, can be used to induce definite folding in synthetic peptides. The N-terminal part of thymosin β4, including the 5–20 sequence, is implicated in binding to monomeric actin. The corresponding peptide was synthesized and its conformation studied by CD. The peptide is unstructured in solution, and becomes folded at medium TFE concentrations, below 30%. In contrast, TFE does not significantly modify the conformation of monomeric actin which conserves its intrinsic properties, such as gelsolin interaction and DNase-I inactivation. We report here that the apparent affinity of the synthetic peptide to monomeric actin is increased by an order of magnitude in the presence of TFE, which implies that the peptide adopts a folded conformation needed for accurate interaction.

Many actin binding proteins (ABP) are known to modulate cellular actin polymorphism (1), namely thymosin β4, a small 4.5 kDa protein belonging to the family of sequestering proteins (2). Their activities involve stabilization of a large pool of cellular G-actin in conditions under which actin is known to polymerize (2,3). The N-terminal part of thymosin has been recently implicated in the G-actin binding site (4–6). In addition, we previously demonstrated that this interface included a conserved VEKFD sequence that is found in several ABPs such as gelsolin family (7). Folding predictions and structural studies demonstrated that the thymosin β4 sequence, including the constant sequence, had a high propency to adopt a helical conformation (6–8). Some alcohols, particularly trifluoroethanol (TFE), can stabilize unstable configurations of such peptides. TFE efficiency depends directly on the nature of the primary structure of the peptide. There is a relationship between the TFE-induced helical structure and the helical tendency, predicted on the basis of the primary structure (9). This relationship has been namely verified with thymosin derived peptides (6–8).

The aim of this paper was to correlate actin binding efficiency of the thymosin β4 synthetic peptide and its functional folding which involved evaluation of the TFE contribution in the structure of native actin. This latter investigation afforded some new information on folding of the actin molecule.

MATERIALS AND METHODS

Rabbit skeletal actin was prepared as described in (10). Actin was specifically dansylated at cysteine 374 with 10 molar excess of N-iodoacetyl-N-(5-sulfonaphthyl)ethylenediamine (1,5-I-AEDANS) (11). The synthetic peptide derived from the thymosin β4 sequence was obtained by solid-phase synthesis with a Milligen Pepsynthetizer TM 9050 (Milligen Bioresearch Division, Warford hertz., UK) as previously described (7). Gelsolin from bovine serum was prepared as in (12). Antiserum to gelsolin was elicited in rabbits as described by Soua *et al.* (12). Anti-IgG antibodies labeled with alkaline phosphatase were purchased from Biosys (Compiègne, France). DNase-I was purchased from Boehringer.

Fluorescence experiments were performed using a Perkin Elmer Luminescence Spectrometer LS 50. Polarization of

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fluorescence was carried out as described by Houmeida *et al.* (13). The exitation and emission wavelengths and the precise experimental conditions are detailed in the figure legends.

Direct ELISA was previously reported in details (14). Microtiterplate wells were coated with actin (0.3 μg/ml). The coated plates were saturated with gelatin (0.5%) and gelatin hydrolyzate (3%) in 140 mM NaCl, 50 mM Tris buffer (pH 7.8). Gelsolin dilutions were carried out in the same buffer containing 4 mM $CaCl_2$ and incubated with coated actin in the presence or absence of 17% TFE. Binding was revealed using alkaline-phosphatase-labeled anti-IgG. Nonspecific absorption was determined for each sample, using uncoated wells. Each assay was carried out in triplicate.

Circular dichroism spectra were obtained using a Jobin Yvon Mark V dichrograph and 0.1 cm pathlength quartz cells. Data were collected within the 190–260 nm wavelength range. Four scans of each sample were accumulated. % helix was calculated from the ellipticity at 220 nm.

DNase-I activity was determined in the presence of various amounts of actin as determined by Lazarides and Lindberg (15). DNA (50 μg/ml) digestion was monitored at 260 nm in 20 mM Tris, 0.2 mM $CaCl_2$, 20 μM $MgCl_2$, buffer (pH 7.5).

RESULTS

Structure of the Thymosin β4 Synthetic Peptide and G-Actin in the Presence of TFE

TFE is known to promote a stable secondary structure of the polypeptide chain in peptides and numerous proteins. Folding of a synthetic peptide derived from the N-terminal of thymosin β4 (sequence 5–20) (Fig. 1A) in the presence of TFE was monitored by CD experiments. As shown in a previous paper (7), this peptide essentially presents a random-coil arrangement in aqueous solution, but has a high propency to adopt a helical structure in the presence of TFE. Fig. 1A shows the increased helical content determined at 220 nm versus TFE concentrations. Maximum folding was observed at around 30% TFE concentrations.

In addition, the potential effect of TFE upon the G-actin conformation was also investigated. Fig. 1B shows the far UV CD spectra of actin in the presence or absence of 20% TFE. The two spectra are similar with a minimum near 220 nm and a high positive peak below 200 nm, characteristic of a helical conformation. This folding concerned about 30% of the protein, in agreement with reference (16). At TFE concentrations over 20%, some aggregates appeared, experiments were therefore generally performed in 17% TFE. In contrast, in the presence of EDTA to chelate divalent cations, the CD of actin is characterized by a decrease in its intensity (of about 20%), which could be caused by the opening of some α-helix, although the secondary structure is largely conserved (17).

Effect of TFE on the Spectral and Functional Properties of G-Actin

Tryptophan 340 and 356 are located in subdomain 1 of actin, in a hydrophobic environment (18). The intrinsic emission fluorescence spectrum is centred near 330 nm, while that of tryptophan

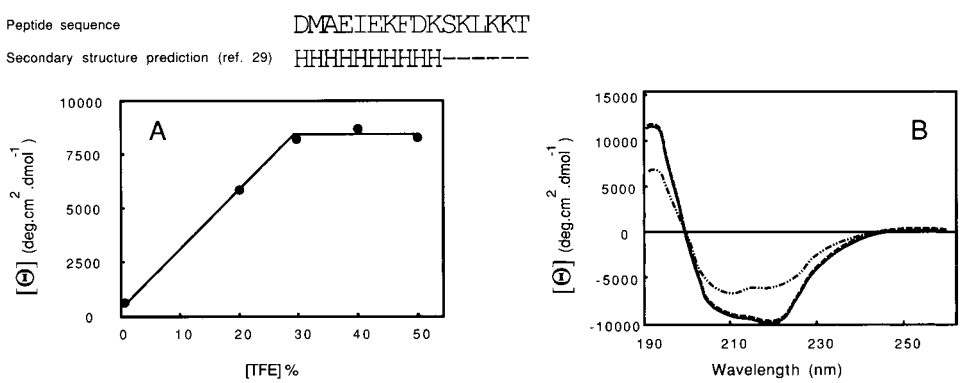

FIG. 1. CD spectra of the synthetic peptide derived from thymosin β4 and G-actin sequences. A, Effect of TFE in the CD spectra of the synthetic peptide. Ellipticity is plotted versus TFE concentrations. B, Far UV CD spectra of G-actin in 2 mM Tris, 0.02 mM ATP, 0.1 mM $CaCl_2$ buffer (pH 7.5) (——), in 17% TFE (— — —) or in 1 mM EDTA (▪ ▪ — — — ▪ ▪ — — — ▪ ▪ — — —).

chromophore in solution is near 360 nm (Fig. 2A). In the presence of 20% TFE, which does not alter the secondary structure of actin, we only observed a very slight shift of about 3 nm (Fig. 2A) to a longer wavelength compared to that of native actin. However, no significant spectral shift was observed up to 14% TFE (Fig. 2A). For comparison, in the same figure the perturbation of N-acetyl-tryptophan-ethylester by TFE is shown.

The stability of actin subdomains was also illustrated by quenching experiments. Iodine quenching of trytophans was similar in the presence or absence of TFE (Fig. 2B). In addition, intrinsic fluorescence polarization of actin was not significantly modified when TFE was added to the medium. These last three approaches thus demonstrated that the hydrophobic environment of tryptophans, as well as the folding of actin subdomain 1, were preserved in the presence of TFE.

In contrast, the C-terminal extremity of actin, particularly the environment of Cys 374 which is located outside subdomain 1, would be more affected by TFE. In fact, as shown in Fig. 3, the fluorescence of dansylated cysteine 374 was perturbed by TFE. A decrease in fluorescence associated with a shift towards a longer wavelength than native actin was observed. Closer values for wavelengths corresponding to dansylated cysteine 374 and free dansylated mercaptoethanol used as a model were obtained in the presence of TFE than in its absence (Fig. 3), suggesting some changes in the micro-environment of cysteine 374.

Two functional properties of G-actin were then studied, inhibition of DNAse I activity and gelsolin interaction.

DNase-I-actin interaction leads to inactivation of DNase-I activity (15). The inhibitory capacity was lost when EDTA treated (19) or unfolded (data not shown) actins were used. TFE did not modify DNase-1 activity. Therefore, a possible effect of TFE in the presence of actin was tested. As shown in Fig. 4, DNase-I inhibition by actin efficient in the presence of TFE.

Another important property of actin concerns its interaction with the regulatory protein gelsolin.

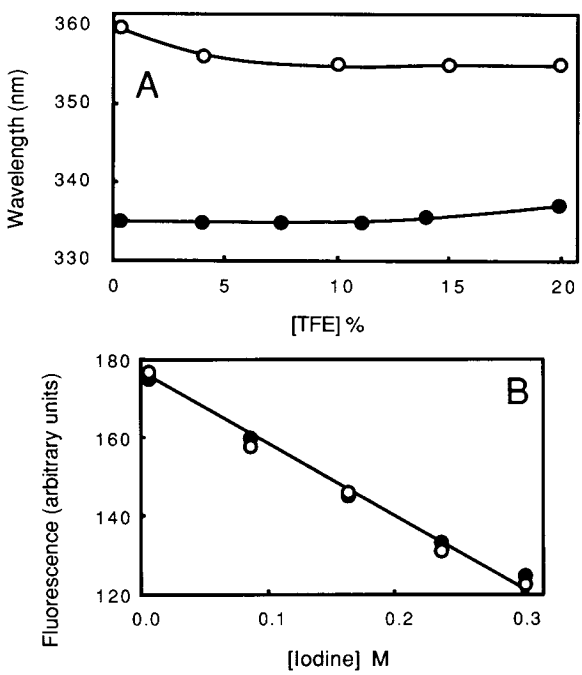

FIG. 2. Fluorescence properties of actin tryptophans. A. Effect of TFE concentrations on the maximum emission wavelength of G-actin at 2 μM (●). The effect on N-acetyl tryptophan-ethyl-ester also is plotted as reference (○). The excitation wavelength was set at 290 nm. B. Quenching of intrinsic fluorescence of G-actin by iodine in the absence (○) or presence (●) of 17% TFE. Excitation and emission wavelength were set at 290 and 350 nm, respectively.

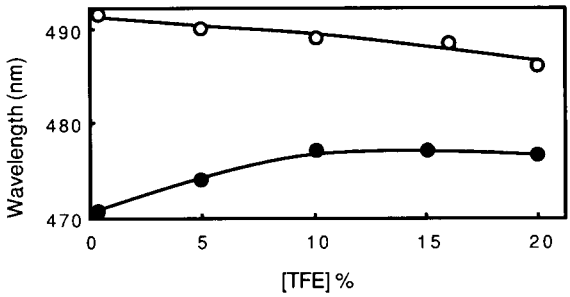

FIG. 3. Effect of TFE on the fluorescence of actin dansylated at Cys 374. The maximum emission wavelength was plotted versus TFE concentrations. (●) actin (0.3 μM), (○) dansylated mercaptoethanol as reference.

In this case, ELISA experiments showed similar gelsolin binding to coated actin with or without TFE in the microtiterplate wells (Fig. 5).

Thymosin Synthetic Peptide Interaction

Since both folding and functional properties were conserved, monomeric actin would be a suitable model to investigate possible relations between the conformational state of a small synthetic peptide and the interaction parameters. In the present paper, this model was applied to the interaction between a peptide derived from the N-terminal part of thymosin β4 (sequence 5–20) and G-actin.

It was deduced from CD measurements that the peptide was unstructured in aqueous medium but could partially adopt a helical conformation in the presence of low concentrations of TFE. Binding experiments were therefore carried out in the absence and presence of this solvent, used at 17% concentration (Fig. 6). In both cases, the peptide bound to actin and the interaction was characterized by enhancement of the actin fluorescence emission spectrum associated with a small red shift of 4 nm (7). Binding of the synthetic peptide with or without TFE in the reaction medium thus induced a change in the environment of tryptophans 340 and 356, which became more hydrophylic. Interestingly, the apparent actin affinity under these conditions, where the synthetic peptide presented a secondary structure, was increased by one order of magnitude (Fig. 6).

DISCUSSION

Synthetic peptides have been widely used to define interfaces between receptors or proteins. We used such an approach in previous papers (20–23) to study interactions between many ABPs and

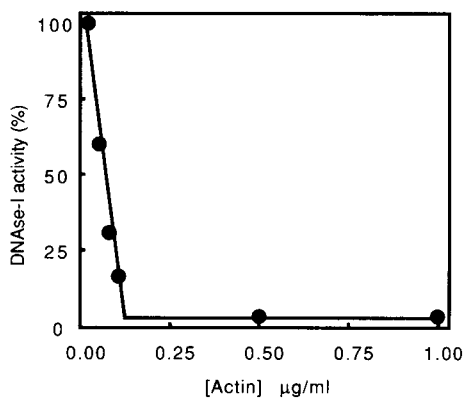

FIG. 4. DNase-I activity inhibition induced by G-actin. DNase-I (0.15 μg/ml) activity monitored at 260 nm, was determined at various actin concentrations (between 0.05 and 1 μg/ml) in the presence of 17% TFE.

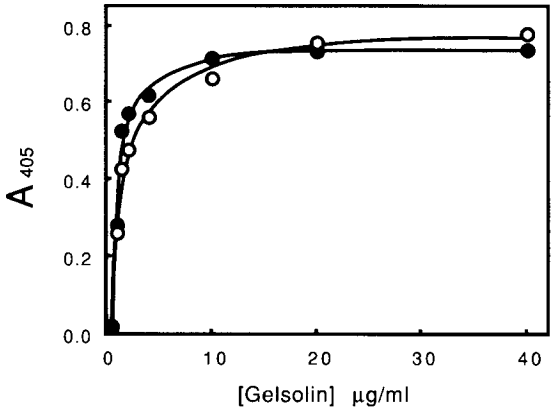

FIG. 5. Interaction of gelsolin with coated actin followed by ELISA. A405 was plotted versus gelsolin concentrations with (○) or without (●) 17% TFE in the reaction mixture.

actin. However, the precise structure of the peptide during their interaction is generally not well understood. In fact, although some synthetic peptides show a definite folding in solution, namely in the case of transducine (24), they usually present no secondary structure at all. In the present paper, we thus present a model based on a small synthetic peptide derived from the thymosin β4 sequence which can be easily folded in the presence of TFE, and thus has an increased binding capacity. TFE is known to enhance and stabilize the helical secondary structure of small peptides. This potentiality is mainly dependent upon the peptide sequence. For instance the case of a synthetic peptide derived from the N-terminal part of actin (sequence 1–28) (25) is significant. Since secondary structure prediction (26) and folding in the native protein (18), revealed a random coil or beta structure and loops, it can not be easily helix structured by TFE. High concentrations of TFE of up to 80% are necessary. In contrast, according to secondary structure prediction, the peptide used in the present study is easily folded with TFE concentrations below 30% (Fig. 1).

The effect of TFE on the structure of native proteins was studied more recently and is more complex. In many cases, TFE induced significant enhancement of the helical structure and destabilized the tertiary structure of native proteins (27, 28). This behaviour has often been interpreted during the folding process by formation of a molten globule intermediate (27). In such cases, the α-helix content obtained by predictive methods is higher than found in the native state. In the present case of actin, predictive methods (26, 29) give about 30–40% helix content compared to about 32% by CD (16) or 40% with a tridimensional model (18). We demonstrated that TFE did

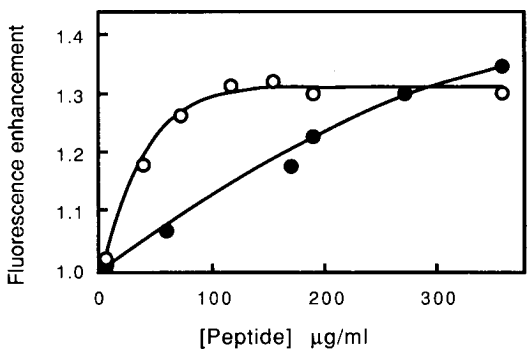

FIG. 6. Interaction of the thymosin β4 peptide with actin monitored by fluorescence. Enhancement of intrinsic actin fluorescence was monitored versus peptide concentrations. Actin (0.5 μM) in the absence (●) or presence (○) of 17% TFE was mixed with increasing amounts of synthetic peptide (0–360 μg/ml). The areas of the emission spectra were recorded.

not modify the secondary structure and, as shown by the lack of change in the micro-environment of buried tryptophan residues, the tertiary structure seemed not significantly altered, as the protein retained the functional properties tested (DNase-I inhibition, gelsolin binding). Thus, our results substantiated the conclusion of Kuznetsova *et al.* (17), which excluded, according to CD results the presence of an intermediary state such as that occurring in the presence of chelating agents in a molten globule structure.

The apparent stability of the actin structure in the presence of TFE finally prompted us to test the importance of the thymosin conformation on its binding. Fluorescence experiments were thus carried out in solution to check the potential effect of the conformational state on peptide binding. They demonstrated the propensity of the thymosin β4 peptide to interact with G-actin. The apparent affinity observed in aqueous medium (7) was enhanced by an order of magnitude in the presence of TFE (Fig. 6). This is important as it demonstrates that the peptide adopts a folded conformation in the alcohol solvent required for the G-actin-thymosin β4 interface (6), and therefore for the inhibitory activity of the entire molecule against actin polymerization.

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