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The effect of pyrene labelling on the thermal stability of actin filaments

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Abstract

The ability of actin to form filaments is fundamental to its biological function and often characterised by various methods in vitro. One of the most frequently used methods capitalises on the observation that the fluorescence emission of a pyrene label on the Cys-374 residue of actin is enhanced by a factor of ∼20 during polymerisation. This method inherently involves the chemical modification of actin monomers with pyrene. It was reported earlier that the pyrene labelling of actin monomers has only small effect on the polymerisation and depolymerisation rates of actin, indicating that the method is suitable to characterise the effect of actin-binding proteins or peptides on the polymerisation kinetics.

In our present work we tested the effect of the pyrene labelling on the thermal denaturation of actin filaments by using the method of differential scanning calorimetry (DSC). By recording the heat denaturation profiles of unlabelled and pyrene labelled actin filaments we observed that pyrene labelling shifted the melting point (T_m) of actin filaments from 66 to 68 °C. A similar effect was detected in the presence of equimolar concentration of phalloidin where the T_m shifted from 79 to 82 °C. We concluded that the observed pyrene labelling induced differences of the thermal denaturation of actin filaments were small. The DSC results, therefore, confirmed that the methods based on the measurements of pyrene intensity during actin polymerisation are suitable to characterise the polymerisation kinetics of actin under in vitro conditions.

Keywords: Actin; Protein conformation; Pyrene; Phalloidin; DSC

1. Introduction

Actin is one of the most abundant proteins in eukaryotes and plays essential roles in many cellular processes. The actin cytoskeleton maintains the mechanical stability of cells by providing the framework for a number of cell functions including cell motility [1], endocytosis [2], phagocytosis [3], protrusion of lamellipodia [4], the formation and function of focal contacts [5], and the interaction of pathogens with the host cell [6]. The dynamic behaviour of the actin cytoskeleton is attributed in vivo to the ability of actin filaments to rapidly elongate or depolymerise in response to extrinsic or intrinsic cues [7]. The rapid changes of the actin cytoskeleton require regulatory cellular factors [8].

One of the approaches to understand the details of the mechanisms underlying the complex regulation of the actin cytoskeleton in cells is to design and describe in vitro model systems, which resembles the biological function of actin. Amongst the many in vitro techniques fluorescence spectroscopy proved to be a powerful tool to study the conformational and dynamic properties of proteins. Actin monomers and filaments were described by these methods in a number of studies. The effect of polymerisation [9], bivalent cations [10–13], nucleotides [14,15], myosin [16,17] and pH [18] was described by using spectroscopic methods. These fluorescence spectroscopic methods require the application of a fluorophore with appropriate spectral properties to provide the conditions for either selective excitation or emission, which enables one to analyse the spectroscopic signal selectively. The selectivity, in many cases, is difficult to achieve by using intrinsic flu-

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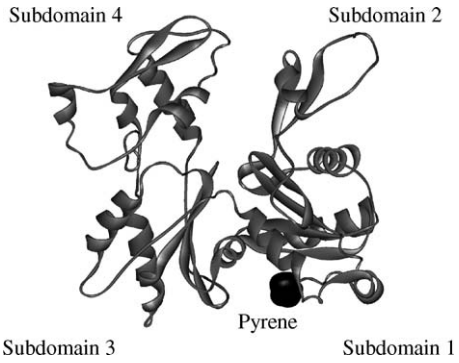

Fig. 1. The ribbon representation of the atomic model of actin monomer (Protein Data Bank file: 1ATN). The pyrene probe is attached to Cys-374 in actin. In the atomic model the position of the Cys-374 was not determined, therefore to indicate the approximate position of the attachment point of the probe the figure shows the neighbouring Arg-373 residue (with a black surface). For better orientation the four subdomains of the actin monomer are also labelled.

orophores, which makes the application of external labels inevitable.

The arsenal of external labels useful for studies with actin has become large in the last decades. One of these labels, the pyrene probe, deserved special attention. The derivative of pyrene (*N*-(1-pyrene)iodoacetamide) binds specifically to the Cys-374 residue of actin under appropriate labelling conditions (Fig. 1). The fluorescence emission of the actin filament bound pyrene proved to be an excellent signal to study the interaction of myosin and myosin fragments with actin [19–28]. It was also shown that the fluorescence intensity of pyrene increases during the polymerisation of actin by a factor of ∼20. The advantages of the spectral sensitivity to these protein processes were applied in many previous studies [28–33].

In general, the application of external probes to label a protein can cause modifications in the conformation of the protein, which can result in an altered biological function and in alterations in its interaction with other proteins (e.g., [34]). In the case of pyrene labelling of actin it was shown by time dependent fluorescence spectroscopic experiments that the binding of pyrene to actin had very little effect on the kinetics of actin polymerisation [29] and on the kinetic steps involved in the interaction between actin and myosin fragments [19].

Apart from fluorescence spectroscopic assay many other methods can also detect the conformational changes of proteins. One of these methods is calorimetry which provides detailed information regarding the thermal denaturation of proteins. A number of previous studies showed that calorimetry is sensitive to the ligand induced changes in proteins [35–47]. It is expected, that substantial modification of the protein structure by the binding of fluorophores, would have influence on their thermal stability, and thus calorimetric methods could sense the modifications in the stability of actin caused by the attachment of external probes, such as pyrene. As the effect of pyrene labelling on the thermal stability of actin filaments was not measured before, in the present work our aim was to test whether the covalently attached pyrene group modifies the thermal behaviour of actin filaments.

Differential scanning calorimetry (DSC) experiments were carried out to characterise the temperature dependence of heat absorption of unlabelled actin filaments and pyrene labelled actin filaments as well. In previous studies it was shown that toxic hexapeptides, such as phalloidin, could stabilise actin filaments [15,48,49]. To test whether the pyrene labelling modified the interaction between phalloidin and actin the DSC experiments were repeated in the presence of phalloidin concentrations suitable to saturate the binding sites on the actin filaments. The calorimetric data showed that although the application of pyrene labels slightly modified the thermal stability of actin filaments in either the presence or absence of toxin. Our observations corroborate the previous conclusions that pyrene labelling of actin does not modify the conformation of the protein substantially, and the methods based on the application of pyrene actin adequately reflects the biological properties of this abundant protein.

2. Materials and methods

2.1. Chemicals

KCl, $MgCl_2$, $CaCl_2$, Tris, EGTA, NaN_3, ATP, β-mercaptoethanol, phalloidin was obtained from Sigma Chem. Col (St. Louis, MO, USA). *N*-(1-pyrene)iodoacetamid were purchased from Molecular Probes (Eugene, OR, USA).

2.2. Actin preparation and labelling

Actin was prepared from rabbit skeletal muscle [50,51] and labelled with *N*-(1-pyrene)iodoacetamide on the Cys-374 as described previously [19]. Actin concentration and labelling ratios were determined by measuring the absorption spectra of samples with a Shimadzu UV-2100 spectrophotometer. In the calculations the extinction coefficient was taken as $1.11\,ml\,cm^{-1}\,mg^{-1}$ at 280 nm [52] for monomer actin with a relative molecular mass of 42,300 Da. For the pyrene probe the coefficient of $2.2 \times 10^4\,M^{-1}\,cm^{-1}$ was used at 344 nm. The absorption of the probe at 280 nm was taken into account in the calculation of the actin concentration. The labelling ratio was determined by calculating the ratio of pyrene concentration to the actin concentration. The labelling ratio was 0.95 ± 0.1 in the presented experiments.

The experiments were carried out with actin filaments in which the high affinity cation-binding site was saturated with magnesium. The preparation of the samples started with either unlabelled or pyrene labelled Ca–G–actin in buffer A. Buffer A was composed of 4 mM Tris/HCl, pH 8.0, 0.2 mM ATP, 0.005% NaN_3, and 0.5 mM 2-mercaptoethanol. The actin monomer bound calcium was replaced with magnesium by adding 100 μM $MgCl_2$ and 200 μM EGTA (final concentrations) to Ca–G–actin and incubating the solution for