CIRCULAR POLARIZATION OF THE FLUORESCENCE OF ACTIN-BOUND ϵATP

Effects of binding DNase I

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1. Introduction

The actins of muscle and various non-muscle sources have been shown to resemble each other closely (reviewed [1,2]). In particular, the work in [3] suggests that all actins will polymerize at an appropriate ionic strength. In non-muscle cells especially, it is through this ability to polymerize and to depolymerize in a reversible manner that actin is able to perform most of its intracellular functions. Several proteins which can affect the polymerization–depolymerization equilibrium of actin have been identified (reviewed [2]) Among these is the enzyme deoxyribonuclease I (DNase I, EC 3.1.4.5). DNase I interacts with either monomeric or polymeric actin to shift the equilibrium strongly to the monomeric G-actin form [4,5]. Conversely, actin is a naturally occurring inhibitor of DNase I [6]

We report here the results of studies employing a fluorescent ATP analog, ϵATP, bound to the nucleotide binding site of G-actin. ϵATP in the past has proven to be a useful probe of actin structure. Changes in the steady state fluorescence and in the fluorescence lifetime of ϵATP on binding to G-actin and on subsequent polymerization have provided information about the nucleotide binding site [7–9] and about the polymerization process [7,10,11]. The binding of the heavy meromyosin fragment of myosin to ϵATP–F-actin revealed that actin undergoes cooperative conformational changes during the process [12–14].

To study the mode of binding of ϵATP to G-actin and subsequently to investigate the binding of DNase I, we measured the circular polarization of the luminescence (CPL) of ϵATP free in solution, bound to G-actin and bound into the G-actin DNase I complex. CPL provides information about the molecular conformation of a fluorescent chromophore in the excited, emitting state in a manner analogous to that in which circular dichroism (CD) tells us about the ground, absorbing state. In complex systems, such as proteins, CPL may have an advantage over CD in that it is specific for fluorescent chromophores. Therefore, the resultant CPL spectrum will be simpler than the CD spectrum and the assignment of spectral contributions to specific chromophores will be less difficult. Reviews by Steinberg et al. provide information about CPL theory and instrumentation [15,16] and about practical applications of CPL [17,18]

2. Materials and methods

Actin was prepared from rabbit skeletal muscle powder by the method in [19]. The actin was used immediately as G-actin or converted to F-actin by the addition of KCl to 50 mM and stored at 4°C in the

Abbreviations DNase I, pancreatic deoxyribonuclease I (EC 3 1.4 5), ϵATP, 1,N^6-ethenoadenosine 5′-triphosphate, CPL, circular polarization of luminescence, CD, circular dichroism, ac, alternating current, dc, direct current

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presence of 1 mM DTT and 0.2% NaN_3. The maximum storage time as F-actin of actin employed in our experiments was 1 week Pancreatic DNase I (EC 3.1.4.5) was purchased from the Sigma Chemical Company (DN 100). Phenylmethyl sulfonyl-fluoride at 3 mM was added to solutions of DNase I to block possible contaminating tryptic or chymotryptic protease activity. Solution concentrations of actin and DNase I were estimated spectrophotometrically on the basis of extinction coefficients reported [4] for actin (1.1 ml mg^{-1} cm^{-1}) and for DNase I (1.23 ml mg^{-1} cm^{-1}). Molecular weights of 43 000 for actin [20] and 31 000 for DNase I [6] were employed in calculations.

ϵATP was purchased from the Sigma Chemical Company. ϵATP–G-actin was prepared by dialysis of G-actin solutions (3–5 mg/ml) against two changes of 50 vol. 2 mM Tris–HCl, 0.2 mM ϵATP, 0.2 mM $CaCl_2$, 0.2 mM DTT, pH 7.5 ('ϵATP buffer'). Excess ϵATP was removed by passage of the protein through a column of Biogel P-2 (Bio-Rad Labs.), eluting with 2 mM Tris–HCl, 0.2 mM $CaCl_2$, pH 7.5. The resultant ϵATP–G-actin was used immediately. In such preparations, the ϵATP to G-actin molar ratio was estimated using essentially the method in [22] for the determination of protein-bound nucleotide. The absorbance of a solution of ϵATP–G-actin was measured at 265 nm and at 280 nm before and after immersion into a boiling water bath for 2 min, cooling on ice and subsequent millipore filtration to remove precipitated protein. The ϵATP content was calculated from the A_{265} of the final filtrate using an extinction coefficient of 5700 M^{-1} cm^{-1} [23], and the actin concentration was estimated from the difference between the intial and final A_{280} values.

The 1:1 ϵATP–G-actin . DNase I complex was formed by mixing appropriate amounts of a DNase I solution and a solution of G-actin which had been dialysed against the ϵATP buffer, dialysing the mixture overnight against the ϵATP buffer and eluting the complex through a Biogel P-2 column with 2 0 mM Tris–HCl, 0.2 mM CaCl, pH 7.5. The complex formed was analysed by Sephadex G-100 (Pharmacia Fine Chemicals) gel filtration chromatography. The column had been calibrated for molecular weight estimation using DNase I (mol. wt 31 000), G-actin (mol. wt 42 000), bovine serum albumin (mol. wt 68 000) and the subfragment 1 portion of a tryptic digest of myosin (mol. wt 115 000). The resultant ATP–G-actin DNase I complex was used immediately.

CPL measurements were performed on an instrument constructed in the Chemical Physics Department, detailed [24]. ϵATP-containing solutions were excited at 313 nm using a 200 W high pressure mercury arc (Osram, HBO 200 W/2) and passing the light through a Bausch and Lomb high intensity monochromator. To exclude stray light from the emission monochromator, a Schott glass UG 11 band-pass filter was inserted into the excitation beam and a 1.0 cm pathlength 0.05 M solution of sodium nitrite, transmitting light only above 380 nm, was placed into the fluorescence beam. The spectral resolution of the excitation beam was 30 nm and of the emission beam was 15 nm. The circularly-polarized component of the ϵATP fluorescence was selectively modulated with an elasto-optic light modulator (Morvue M-FS3, Hinds International, Inc.) and the resultant ac signal from the photomultiplier was amplified using a lock-in amplifier (Ithaco, Dynatrac 291A). Photomultiplier signals, ac and dc, were monitored over the wavelength interval from 385–450 nm. CPL is expressed by the anisotropy factor for emission, $g_{em}=\Delta f/(f/2)$, where f is the total fluorescence intensity and Δf is the intensity of the circularly-polarized component of the fluorescence (assigned a plus sign for left-handed circular polarization). Measurements were performed at room temperature (~22°C).

Absorption measurements were performed with a Zeiss PMQ II spectrophotometer Fluorescence intensities were measured as the dc signal from the photomultiplier tube of the CPL instrument employed in these studies

3. Results and discussion

Biogel P-2 gel filtration effectively removed free ϵATP from ϵATP–G-actin solutions. The molar ratio of ϵATP to G-actin in such preparations was close to unity, 0.94–1.03 in our preparations.

The formation of a 1.1 ϵATP–G-actin · DNase I complex of mol. wt 70 000 was confirmed by the elution of a single peak from a Sephadex G-100 column onto which an equimolar mixture of ϵATP–G-actin and DNase I had been applied

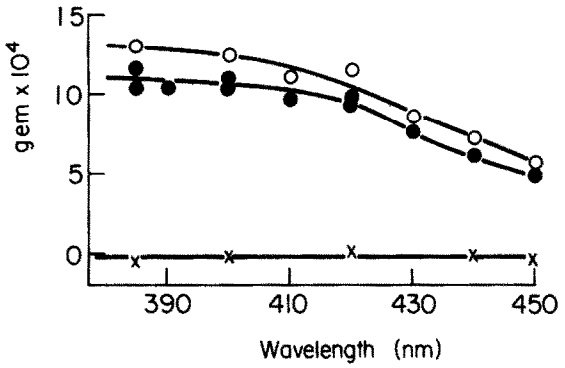

Fig.1. CPL spectra in 2 0 mM Tris–HCl, 0 2 mM $CaCl_2$, pH 7.5 (X–X–X) 1 0 X 10^{-5} M ϵATP, (•–•–•) 10^{-5} M ϵATP–G-actin, (o–o–o) 10^{-5} M ϵATP–G-actin DNase I

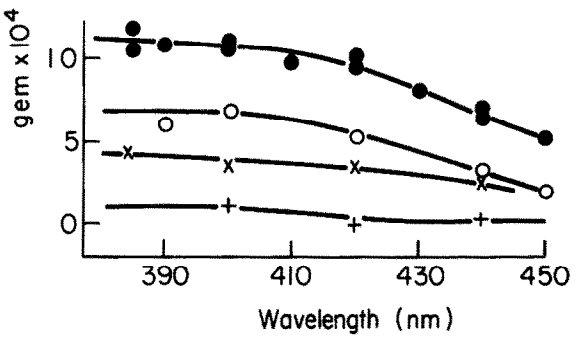

Fig.2 Loss of CPL signal of ϵATP–G-actin on storage at 4°C (•–•–•) freshly prepared as in Fig 2, (o–o–o) 1 day of storage, (X–X–X) 2 days of storage, (+–+–+) 4 days of storage

There was negligible CPL in the fluorescence of ϵATP free in solution (fig.1). However, when bound to the nucleotide-binding site of G-actin, the fluorescence of ϵATP exhibited a significant CPL signal across the 385–450 nm wavelength interval (fig.1). The CPL signal observed suggests that ϵATP is bound to G-actin in an optically asymmetric manner. A similar induced CPL phenomenon has been reported in studies of the binding of another enthino-adenine derivative, ϵNAD, to the NAD^+ binding sites of several dehydrogenase enzymes [25,26].

The CPL spectrum of the ϵATP–G-actin DNase I complex differed only slightly from that of the ϵATP–G-actin alone (fig.1). The similarity both in shape and magnitude of the spectra suggests that the binding of DNase I to G-actin causes minimal alteration of the nucleotide binding site of actin The binding of DNase I is probably at a site removed from that at which ϵATP is bound. This hypothesis is supported by the observation that dialysis of ϵATP–G-actin . DNase I against 2 mM Tris–HCl, 0.5 mM ATP, 0.2 mM $CaCl_2$, 0.2 mM DTT, pH 7.5, released the ϵATP from the protein, as determined by direct measurement of the ϵATP fluorescence associated with the protein after 1 day of dialysis. DNase I does not, therefore, act as a lid to cover the nucleotide binding region.

With time, the CPL signal from ϵATP–G-actin fell to give essentially zero values for g_{em} across the emission band of ϵATP (fig.2) After 1 day of storage at 4°C, the g_e values decayed by about 50% and by 3 days, the values fell to zero This loss of CPL signal reflects the release of ϵATP from G-actin into the symmetric solvent environment. As literature values for the binding constant of ϵATP to G-actin are very high, from 5 X 10^6 M^{-1} [7] to 2.5 X 10^9 M^{-1} [9], the observed release of ϵATP from G-actin does not take place simply to satisfy equilibrium conditions. Denaturation of the actin on standing in solution or the degradative action of contaminating proteolytic enzymes could release the bound ϵATP from its protein environment.

In contrast to the decay rate of CPL spectra of ϵATP–G-actin, that for the ϵATP–G-actin : DNase I I complex was much slower (fig.3). After 3 days

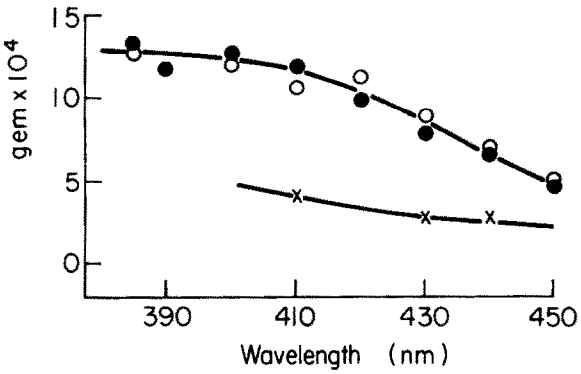

Fig.3 Loss of CPL signal of ϵATP–G-actin DNase I on storage at 4°C. (•–•–•) freshly prepared as in fig 2, (o–o–o) 3 days of storage, (X–X–X) 6 days of storage

storage at 4°C, no detectable change in the CPL spectra of such complexes could be detected. With longer storage times, varying from sample to sample, the g_{em} values did fall to zero. Therefore, the binding of DNase I to ATP–G-actin in some way stabilizes at least the nucleotide binding region of the molecule, and perhaps the whole molecule, against the proteolytic or denaturative forces which acted on the εATP–G-actin alone. The stabilization of protein structure by interprotein interactions is a topic of particular interest in the case of actin. Actin polymers are less susceptible to proteolytic degradation than actin monomers [27]. Also, actin interacts with several cellular proteins [2], the role of some of which may be to protect actin from degradative forces.

Our results suggest that DNase I is a protein which not only can stabilize the G-form of actin with respect to the F-form, but it also can stabilize actin against denaturative forces. Such a dual role, not necessarily played only by DNase I, may be important to the cell, insuring that a maximal fraction of the cell's actin will be in a functional conformation when it is required to polymerize.

An actin-binding protein, profilin, which binds to actin to form a nonpolymerizable complex devoid of nucleotide has been described [28]. Profilin has been suggested [29] to play an intracellular role in stabilizing actin in a nondenatured monomeric state devoid of nucleotide. Our experiments demonstrate that the binding of DNase I to εATP–G-actin does not quench the CPL signal from the εATP (fig.1). Therefore DNase I does not cause nucleotide release when it binds to actin, confirming [5]. DNase I, or proteins similar to it, may act to stabilize cytoplasmic actin in a monomeric state in which the nucleotide binding site is occupied by ATP. In fact, such an actin-containing bound ATP may be the form of preference to a cell. When the actin is required for polymerization, the step of binding cytoplasmic ATP to the G-actin prior to the polymerization process would not be required, reducing the complexity of the overall process. Also, direct polymerization of a nucleotide-bound form of G-actin would prevent a partial depletion of ATP in the form in which it exists in the cytoplasm.

Future CPL investigations of εATP–G-actin should prove helpful in gathering information concerning the role of nucleotide binding and the roles of several muscle and nonmuscle actin-binding proteins. Special interest should be paid to incidences of competition of more than one protein for an overlapping site on the surface of actin, and the effect of this competition on actin polymerization. Such competition could determine the functional form in which actin exists in the cell.

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