

A Flow System Based on a Fluorometer and a Luminometer to Monitor the Correlation of Protein Conformation and Function

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A flow system to monitor the reactions in solutions was constructed, which connected a fluorometer and a luminometer in series. Using a stream through it, the correlation of fluorescence and luminescence was measured. The system was tested with actin. The fluorescence emission gave the signal of polymerization of labeled actin and the luminescence gave that of ATP hydrolysis by actin. The observations showed the correlation between ATP hydrolysis and polymerization. Applications of other proteins to this system will give insights into the characteristics of proteins.

KEY WORDS: Flow; excimer emission; luminescence; actin; ATPase.

INTRODUCTION

A study of correlations of protein conformations and functions is essential for the understanding of protein characteristics. Using commercial apparatuses, a simple and convenient system was devised. The test of this equipment was done with actin. Since actin is a dynamic protein, which plays an essential role in muscle contractions and cell movements, the study of correlation of both factors is important. Actin in a low-salt buffer shows ATPase activity [1,2] and polymerizes on the addition of ATP [3]. Using the flow system, the correlation of actin polymerization and ATP hydrolysis was measured. The example shown here demonstrates the usefulness of the application of this system to other proteins.

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MATERIALS AND METHODS

Chemicals

Actin in G buffer (0.1 mM ATP, 0.1 mM CaCl₂, 2 mM Tris-HCl, pH 8.0, and 1 mM NaN₃) was prepared as described previously [4] and labeled with the fluorescent probe, *N*-(1-pyrene)iodoacetamide (P-29; Molecular Probes) [5]. The labeling ratio of actin (PIA-actin) was 0.8. The stock actin (0.13 mg/ml) in G buffer was diluted 10 times with Ca buffer (0.1 mM CaCl₂, 2 mM glycine, pH 8.0 adjusted with Tris, and 1 mM NaN₃) just before the observations. Luminescence was observed by adding luciferase-luciferin (L0633; Sigma), which was diluted with LF buffer [50 mM glycine, 1 mM Tris, 5 mM MgSO₄, 0.55 mM EDTA, 1 mM (w/v) sodium azide, pH 7.6], or glycine diluent (F-3766; Sigma). ATP was purchased from Boehringer Mannheim (102 202).

Instrumentation

Fluorescence was measured with an Hitachi F-2000 fluorometer using a semimicrocell (0.4 × 0.4 cm, 0.7-

ml volume). Luminescence was monitored with a luminometer using a UV monitor (Bio-Mini UV Monitor AC-5200; ATTO), in which the UV lamp was turned off. The flow cell in the monitor had a volume of 60 μl and a path length of 0.5 cm. The fluorometer and luminometer were connected in series with tubes of 0.19-mm inner diameter. The solutions were pumped with an Ismatec SA peristaltic pump (Mini-S; 20 rpm). A block diagram of the system is shown in Fig. 1. Fluorescence of PIA-actin (0.6 ml) was measured through cell A. ATP was added to PIA-actin and mixed by inversion of the cell twice. The solution was pumped out from cell A through a Dramon capillary at a flow rate of 60 $\mu\text{l}/\text{min}$. Reservoir B was filled with 0.6 ml of LF buffer and 10 μl of luciferase-luciferin mixture. Equal amounts of solution from A and B were mixed at C (three-way connector; Pharmacia) and led to the luminometer. There, the amount of ATP in actin solution was measured. Before the run of sample solutions, buffers from cell A and reservoir B were pumped through the lines for about 10 min to arrive in a steady stream. Observations were performed in an air-conditioned room at 23°C.

RESULTS AND DISCUSSION

PIA-actin in a low-salt buffer showed monomer emission peaks at 386 and 405 nm and a broad peak of excimer emission at 480 nm (Fig. 2). Previously we have shown that the excimer decrease induced by dilution was caused by polymerization of PIA-actin [4,5]. Later, we showed that this polymerization was caused by a rise in the ATP concentration to a molar excess over actin [3]. Figure 2 shows the changes in the fluorescence spectra

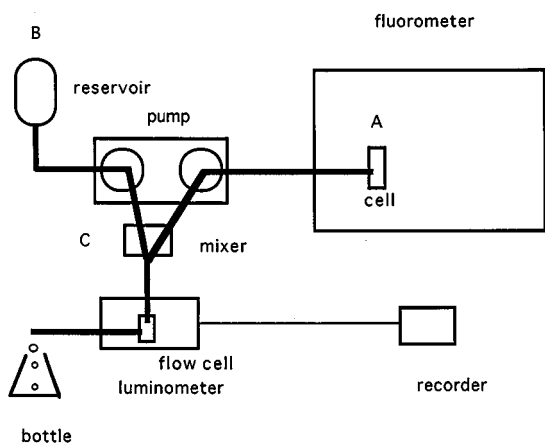


Fig. 1. Block diagram of the flow system. The thick line shows the routes of solutions. Actin solution in A and luciferase-luciferin mixture in B were conducted to C and mixed there.

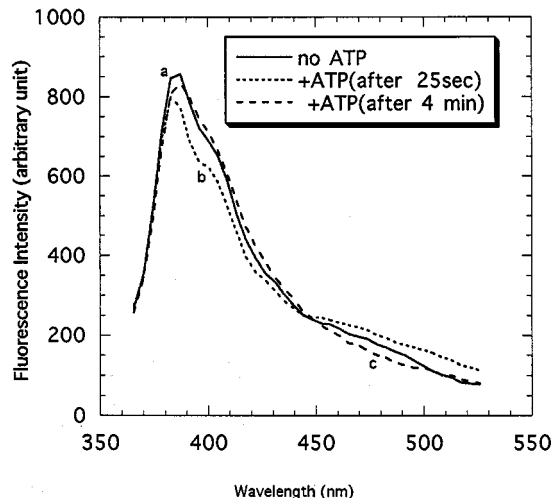


Fig. 2. Fluorescence spectra of 13 $\mu\text{g}/\text{ml}$ PIA-actin diluted with Ca buffer. Observation without the addition of ATP (a). Observations at 25 s (b) and at 4 min (c) after the addition of ATP to a concentration of 0.1 mM . Excitation at 344 nm.

induced by ATP. As ATP (0.1 mM) was added to a 13 $\mu\text{g}/\text{ml}$ solution of PIA-actin; the monomer emission at 405 nm (I_{405}) decreased quickly, then within about 2 min, it began to increase. In contrast, the excimer emission at 480 nm (I_{480}) changed in reverse, i.e., it first increased, then decreased. Thus the relative ratio of excimer emission (I_{480}/I_{405}) first increased, then decreased gradually. As shown previously [4,5], the excimer decrease is related

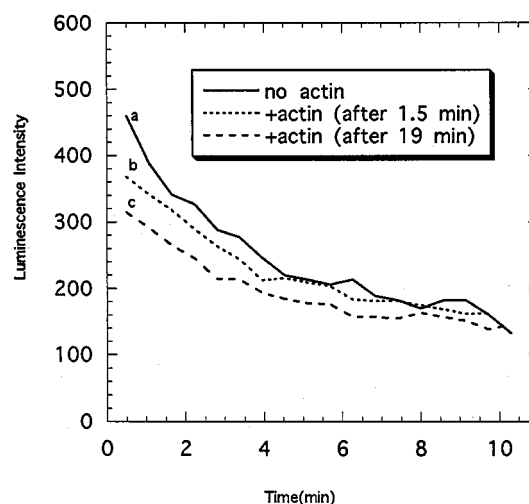


Fig. 3. ATP hydrolysis of actin observed by luminescence. (a) Without PIA-actin. PIA-actin (13 $\mu\text{g}/\text{ml}$) was incubated at 23°C with the addition of 0.1 mM ATP. Five microliters of luciferase-luciferin mixture was added to an aliquot of actin (0.3 ml) at 1.5 min (b) and at 19 min (c). The luminescences were observed through the fluorometer without turning on the excitation lamp.

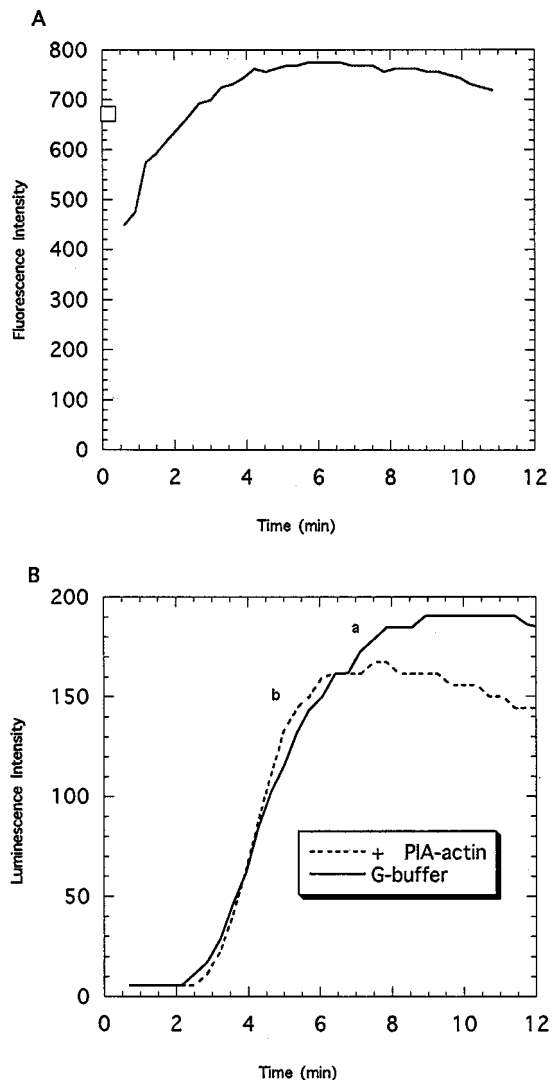


Fig. 4. Correlation between ATP hydrolysis and polymerization. PIA-actin (0.13 mg/ml) in Ca buffer was applied to the flow system. The solutions were flowed after mixing ATP to a concentration of 0.1 mM. (A) Time course of fluorescence emission (I_{405}). The open square shows I_{405} before the addition of ATP. (B) Time courses of luminescences in the absence and presence of PIA-actin. G- buffer (a) and PIA-actin (b) were diluted 10 times with Ca buffer.

to polymerization of PIA-actin. The next question is whether or not this ATP-induced polymerization is related to ATP hydrolysis. Thus, using luciferase–luciferin mixtures, the ATP hydrolysis of PIA-actin was measured at successive time points, removing an aliquot from the

reservoir. As shown in Fig. 3, the luminescence decay curves showed the fast and slow phases as reported [6,7]. Luciferase binds ATP at two kinetically distinguishable sites [6]. ATP hydrolysis by actin was observed from the intercept of the fast phase of the time course of light emission. The intercept decreased during the incubation of actin with ATP. Thus actin showed ATPase activity. The decrease in the intercept was higher in the initial few minutes compared with that observed at the subsequent times, which showed the presence of high ATPase activity in the initial few minutes. This corresponds to the initial large increase in the excimer of PIA-actin shown in Fig. 2.

Next, using the flow system, the correlation between ATP hydrolysis and polymerization was examined (Fig. 4). Here, instead of the relative excimer emission, polymerization was monitored by I_{405} (Fig. 4A). As shown in Fig. 4A, a large decrease in I_{405} appeared in the initial few minutes, followed by a gradual increase. The fluorescence changes were compared with those of luminescence (Fig. 4B). Owing to the length of the tube that connects the fluorometer and luminometer, it took about 3 min for the front of the samples to come out. The luminescence of curve b arrived at a steady level at about 6 min, then decreased about 4 min later. As shown in Fig. 4A, I_{405} increased and reached a plateau about 4 min after the mixing of ATP. This rise showed the polymerization of PIA-actin. Thus ATPase could be correlated with polymerization (curve b). In addition, from the difference in the luminescence of the plateau level shown by curves a and b, we see the appearance of high ATPase activity at the initial stage of ATP mixing. Comparing the time courses of the luminescence and fluorescence changes, it is seen that this initial ATP hydrolysis corresponds to the decrease in I_{405} . These changes in fluorescence and luminescence induced by ATP seem to be caused by conformation changes preceding the polymerization.

CONCLUSIONS

A flow system to monitor the correlation of conformation and function was constructed, which allows the measurement of fluorescence and luminescence continuously. To test of this apparatus, actin was used. The continuous monitoring of fluorescence and luminescence made it possible to observe the correlation of actin polymerization and its ATP hydrolysis clearly.