Single molecular assay of individual ATP turnover by a myosin-GFP fusion protein expressed in vitro

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Received 28 February 1997; revised version received 19 March 1997

Abstract Fusion proteins of a truncated mutant of myosin subfragment-1 (S1dC) and green fluorescent protein (GFP) were expressed in vitro by T7 RNA polymerase and rabbit reticulocyte lysate. Single S1dC-GFP fusion proteins were clearly seen and their individual ATP turnovers were directly monitored using low background total internal reflection fluorescence microscopy (LBTIRFM), recently developed by our laboratory. LBTIRFM using GFP as a fluorescent tag allowed us to assay functions of single protein molecules expressed in vitro. Thus, the results suggested that this method may be particularly useful to analyze functions of proteins that cannot be produced in an active form and/or in large quantities in conventional heterologous expression systems.

Key words: Myosin subfragment-1; Green fluorescent protein; Single molecule assay; ATPase; In vitro expression

1. Introduction

We have recently demonstrated that images of single fluorescent dye molecules in aqueous solution can be directly observed by low background total internal reflection fluorescence microscopy (LBTIRFM) [1]. This technique enabled us to observe individual ATP turnovers [1,2] and movement of individual motor proteins [3] at the level of single molecules. To be visualized by LBTIRFM, individual biomolecules must be covalently attached to fluorescent dyes, which has been previously accomplished by the introduction of a highly reactive cysteine residue for specific labeling [3,4]. However, it is not always possible to specifically label the cysteine residue that has been introduced into a protein. To resolve this limitation, we have developed a chimeric green fluorescence protein (GFP). GFPs have recently attracted great interest since their intrinsic chromophores emit green fluorescence and have been used as reporters of gene expression [5]. Recently, there have been many reports of GFP mutants [6]. One of these mutants, S65T, has a higher extinction coefficient (39.2×10^3 M^{-1} cm^{-1} at 490 nm) and forms a fluorophore by oxidation more rapidly (30 min) than the native one [7]. In many cases, proteins fused with GFP at either the N- or C-terminus retain their normal biological activities, as well as maintaining the fluorescent properties of native GFP [5,8,9].

In this report, we show that fluorescence images of individual fusion proteins of GFP can be clearly observed at a full video rate by LBTIRFM. This technique enabled us to detect individual protein molecules without attaching exogenous dyes. Furthermore, fusion proteins of GFP can be easily obtained using an in vitro expression system of reticulocyte lysate. In conventional recombinant protein production systems such as those using *Escherichia coli* or baculovirus, expressed proteins sometimes accumulate intracellularly in an insoluble form which diminishes their function. On the contrary, the in vitro expression system overcomes the problem of the formation of such insoluble inclusion bodies.

We have combined techniques of GFP tagging, in vitro synthesis of recombinant proteins, and LBTIRFM to study skeletal muscle myosin at the single molecular level. Myosin is a motor protein that converts chemical energy driven by ATP hydrolysis to mechanical work and induces muscle contraction. A truncated mutant of myosin subfragment-1 (S1dC) of *Dictyostelium discoideum* myosin II has ATPase activity and functions as a force-generating machine [4]. However, detailed characterization of skeletal S1dC has not been performed yet mainly because S1dC expressed in either *E. coli* or baculovirus accumulated in inclusion bodies which could not be solubilized. Thus, we selected S1dC as the model protein to demonstrate the usefulness of the method developed in this study. A chimeric protein of chicken S1dC and GFP was expressed in vitro and ATP turnovers by S1dC were successfully visualized. This approach is applicable to the study of various kinds of proteins at the level of a single molecule.

2. Materials and methods

2.1. Synthesis of complementary DNA of adult chicken S1dC

Polyadenylated mRNAs were isolated from 1 g of 14-day-old chicken breast muscle tissue using polyATract mRNA isolation system (Promega, WI, USA). The cDNA of adult chicken myosin subfragment-1 (S1) was synthesized from 1 μg of the mRNA by reverse transcription-polymerase chain reaction [10] and a 5′-AmpliFINDERTM RACE kit (Clontech Laboratories, CA, USA). The chicken adult S1 cDNA fragments obtained were subcloned and sequenced using a Sequencing High chemiluminescent DNA sequencing Kit (Toyobo, Japan). The length of the coding region of S1, from the initiation (ATG) to the C-terminus of S1 (AAG: 844-Lys), was 2532 base pairs. The amino acid sequence derived from the cDNA sequence of S1 was completely identical to that determined from the protein by the Edman method [11]. The C-terminus of chicken adult S1dC was determined as 782-Asp by comparing the amino acid homology between adult chicken myosin heavy chain [11] and *D. dis-*

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Abbreviations: S1dC, a truncated mutant of myosin subfragment-1 that lacks the myosin light chain binding site; S1, myosin subfragment-1; GFP, green fluorescent protein; LBTIRFM, low background total internal reflection fluorescence microscopy; Cy3-ATP, 3′(2′)-*O*-[*N*-[2-[(Cy3)amino]ethyl]carbamoyl]-ATP

PII S0014-5793(97)00359-1

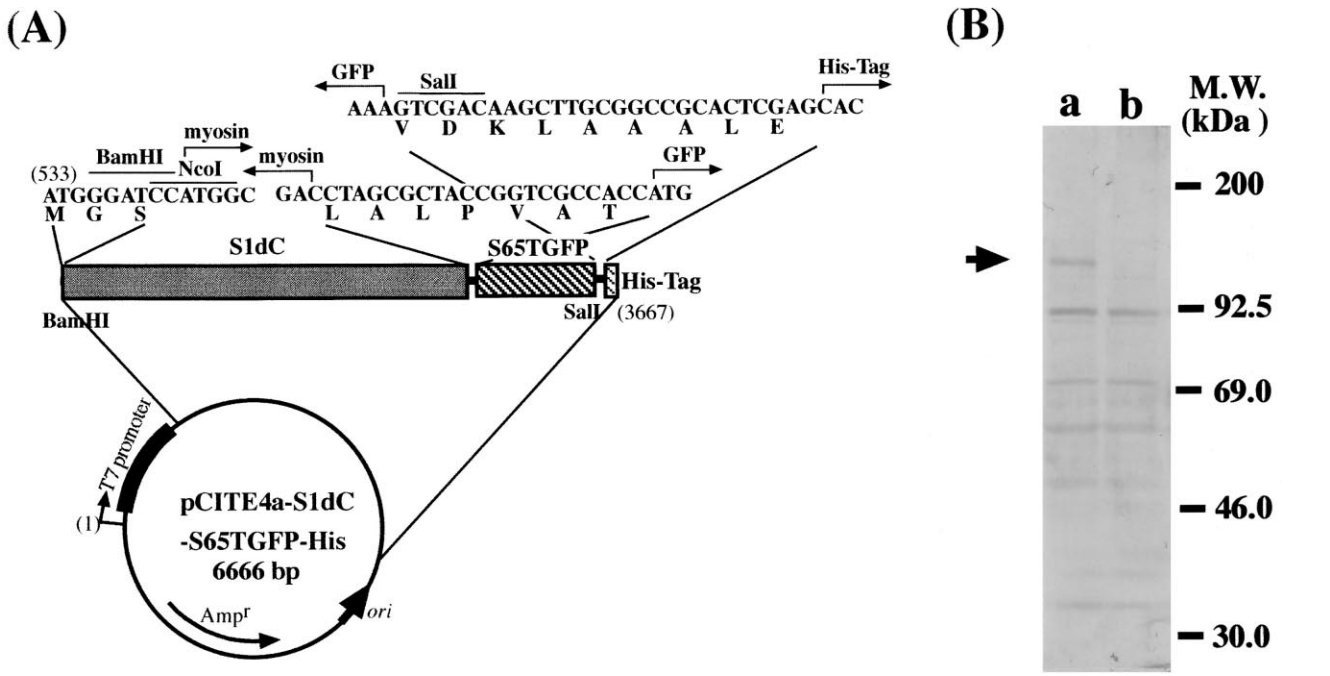

Fig. 1. A: Schematic representation of the recombinant protein of S1dC-GFP and its in vitro expression plasmid. See details in the text. B: Immunoblot tests of the expression of S1dC-GFP by using an antibody against GFP. The expression plasmid containing S1dC-GFP (lane a) and β-galactosidase (lane b) were added to an in vitro expression system of rabbit reticulocyte lysate. Approximately 5 μl of the lysate was loaded in each lane.

coideum myosin II [4,12]. cDNA of chicken adult S1dC was obtained by polymerase chain reaction using cDNA of chicken adult S1 as a template.

2.2. Preparation of the recombinant fusion protein of S1dC and GFP

The gene of S65T, a variant of the green fluorescent protein from *Aequorea victoria*, was obtained from the pS65T-C1 plasmid (Clontech Laboratories). The cDNA of S1dC (*Nco*I-*Avr*II fragment, 2351 bp) and S65T (*Nhe*I-*Sal*I fragment, 736 bp) were constructed in tandem into an in vitro expression vector of pCITE-4a (Novagen, WI, USA), which lacked S-tag sequence (Fig. 1A). The structure of the expression plasmid for the S1dC-S65T (henceforth named 'S1dC-GFP') fusion protein is shown in Fig. 1A. Seven amino acids, Leu-Ala-Leu-Pro-Val-Ala-Thr, were inserted between S1dC and S65T, and additional amino acids, Val-Asp-Lys-Leu-Ala-Ala-Ala-Leu-Glu-His-His-His-His-His-His (His-tag), were added at the C-terminus for facilitating the purification.

The in vitro expression plasmid (Fig. 1A) of the S1dC-GFP fusion protein was transcribed by a STP2 T7 RNA Transcription mix (Novagen) for 30 min at 30°C, immediately followed by the translation in a STP2 Translation Mix (Novagen), which contains optimized rabbit reticulocyte lysate, for 120 min at 30°C and then stored on ice.

To observe ATP turnovers by S1dC-GFP, we purified S1dC-GFP from other ATP binding proteins in the in vitro expression system on Ni-chelating resins (His Bind Resin, Novagen). 100 μl of the lysate was diluted with 300 μl of a solution containing 0.15 M NaCl, 20 mM imidazole, and 20 mM PIPES (pH 6.8). 15 μl of precipitated Ni-chelating resins were added to the solution and incubated for 20 min on ice. The resins were collected by centrifugation and washed with a solution containing 80 mM imidazole. The S1dC-GFP bound to the resin was eluted by a solution containing 0.15 M NaCl, 0.15 M imidazole, and 20 mM PIPES (pH 6.8).

2.3. Immunoblotting

SDS gel electrophoresis was carried out using a 7% polyacrylamide gel. Proteins were transferred onto a nitrocellulose sheet and reacted with a primary rabbit polyclonal antibody against GFP (Clontech Laboratories), followed by a secondary antibody of the biotinylated anti-rabbit IgG. The protein band of GFP was visualized by enzymatic reaction of biotinylated horseradish peroxidase, which attached to the antibody via a streptavidin, using diaminobenzine and nickel chloride as a substrate (Vector Laboratories, CA, USA).

2.4. Preparation of Cy3-ATP

3′(2′)-*O*-[*N*-[2-[(Cy3)amino]ethyl]carbamoyl]-ATP (Cy3-ATP) [2] was prepared by coupling Cy3.29-OSu (Amersham Life Science, PA, USA) with 3′(2′)-*O*-[(aminoethyl)carbamoyl]-ATP [13,14] and purified by fast protein liquid chromatography.

2.5. Microscopy

The total internal reflection optics were installed on the inverted microscope (Diaphot TMD300, Nikon, Tokyo, Japan) as described previously [1]. A beam of an Ar laser (model 2013-150ML, Uniphase, CA, USA) was obtained by passing through an interference filter (488 nm for GFP and 514.5 nm for Cy3, Melles Griot, Tokyo, Japan). The beam was incident on a quartz microscope slide through a quartz prism. The gap between the microscope slide and the prism was filled with non-fluorescent pure glycerol. The incident angle at the quartz slide-to-solution was 75° to the normal (the critical angle, 65.5°). The beam was focused by a lens to be 40×160 μm at the specimen plane. Dichroic mirrors (separation wavelength of 505 nm for GFP and 530 nm for Cy3, Sigma Koki, Saitama, Japan) and emission filters (530DF30 for GFP and 590DF90 for Cy3, Omega Optical, VT, USA) were used to reject scattering light and background luminescence. A 100× objective (NCF Fluor 100×, numerical aperture 1.3, Nikon) was used. Images were captured by a SIT camera (C2400-08, Hamamatsu Photonics, Hamamatsu, Japan) coupled to an image intensifier (VS4-1845, Video Scope International, VA, USA) and recorded on video tape through a digital image processor (Argus 10, Hamamatsu Photonics). Fluorescence intensities were quantified from the video tape recorder using a computer image processor (Avio Excel, Nippon Avionics, Tokyo, Japan).

2.6. Imaging of single S1dC-GFP molecules and their individual Cy3-ATP turnovers

Single S1dC-GFP molecules could be visualized without purification. The reticulocyte lysate which contained S1dC-GFP was diluted 100-fold with a solution containing 25 mM KCl, 3 mM $MgCl_2$, and 20 mM HEPES (pH 7.8) and observed by LBTIRFM. Only when ATP turnovers by S1dC-GFP were observed, S1dC-GFP was purified with Ni-chelating resins to remove other ATP binding proteins. The purified S1dC-GFP was diluted 3-fold with a volume of solution containing 0.1 M NaCl, 10 mM Tris (pH 8.4), and saturated casein (Nacalai Tesque, Kyoto, Japan). The specimen was placed onto a quartz slide and covered with a coverslip. Two opposite sides of the

coverslip were sealed with polyester sheets of 25 μm thickness. The solution was exchanged by drawing solution from one unsealed side of the coverslip with a piece of filter paper. After a single wash, the solution was exchanged with a solution containing 30 nM Cy3-ATP, 25 mM KCl, 3 mM $MgCl_2$, 20 mM HEPES (pH 7.8). To reduce photobleaching, 4.5 mg/ml glucose, 36 μg/ml catalase, 216 μg/ml glucose oxidase, and 0.5% 2-mercaptoethanol were added as previously described [15]. The polyester sheets were removed and the coverslip was sealed with enamel. The S1dC-GFP molecules adsorbed onto the quartz slide surface were visualized with LBTIRFM using an Ar laser (488 nm), and their positions were marked. Cy3-ATP was visualized by LBTIRFM using an Ar laser (514.5 nm) and its turnover events on S1dC-GFP were detected by directly observing association-(hydrolysis)-dissociation of Cy3-ATP corresponding to the position of S1dC-GFP molecules on the surface. Experiments were performed at 25°C.

3. Results and discussion

3.1. Expression of S1dC-GFP in vitro

The fusion protein was obtained in the in vitro expression system only 2.5 h after the addition of the plasmid. The expression of S1dC-GFP was confirmed by the immunoblot analysis using polyclonal antibody against GFP. A specific protein band with an apparent molecular mass of 120 kDa was detected only in the lysate to which the expression plasmid of S1dC-GFP had been added (lane a in Fig. 1B). This band was observed only when the expression plasmid of S1dC-GFP was added to the in vitro expression system (Fig. 1B) and the apparent molecular mass was in good agreement with the calculated one (117 796 Da). Thus, the results indicate that S1dC was synthesized in the in vitro expression system as expected. The concentration of the S1dC-GFP was estimated to be 0.1–1 nM from the band intensity on the immunoblot. The concentration of 1 nM was consistent with the number of S1dC-GFP molecules attached to a quartz slide and visualized by LBTIRFM as later described.

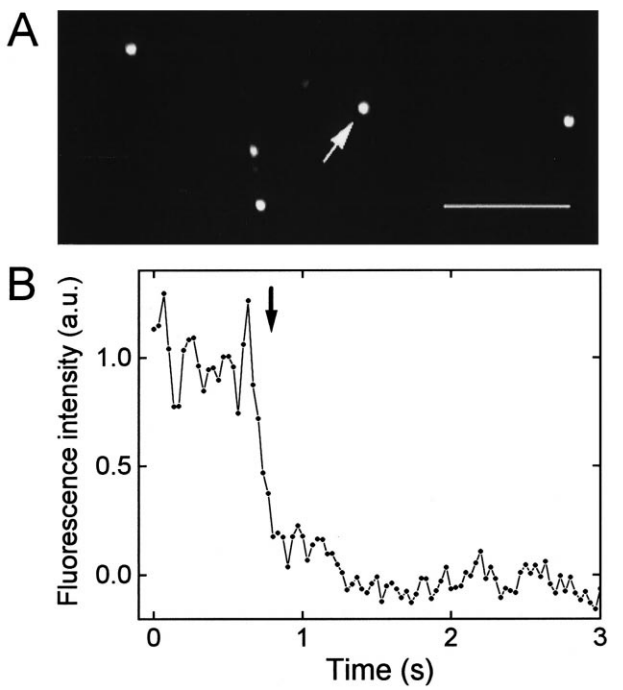

Fig. 2. Imaging of single GFP molecules by LBTIRFM. A: A fluorescence micrograph of single S1dC-GFP molecules. Images were integrated over 16 video frames, i.e. 0.53 s. Power of the Ar laser (488 nm) was 20 mW. The arrow indicates the typical fluorescence spot due to the single S1dC-GFP molecules, whose photobleaching process was analyzed in B. Scale bar, 5 μm. B: Quantized photobleaching of the S1dC-GFP shown by the arrow in A. The fluorescence intensity of the spot was analyzed at video rate (every 33 ms). a.u., arbitrary units.

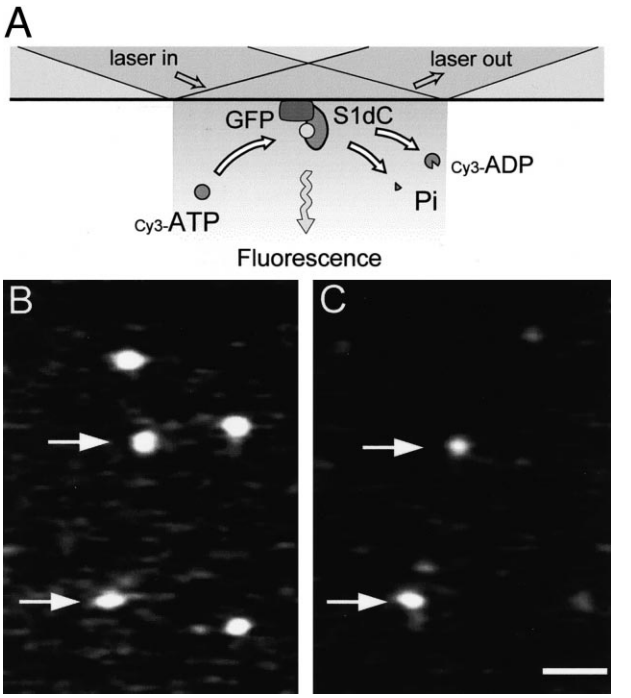

Fig. 3. Visualization of individual ATP turnovers by single S1dC-GFP molecules. A: A schematic drawing of the principles used to measure individual ATP turnovers by single S1dC-GFP molecules. S1dC-GFP molecules were adsorbed onto a quartz slide surface. They were visualized with a 488 nm line from an Ar laser and their positions were marked. Cy3-ATP molecules were visualized with the 514.5 nm line from an Ar laser. The ATP turnover events were detected by directly observing association-(hydrolysis)-dissociation of Cy3-ATP. See details in the text. B: A fluorescence micrograph of single S1dC-GFP molecules bound to the quartz surface. C: A fluorescence micrograph of Cy3-nucleotides attached to S1dC-GFP molecules indicated by the arrow in B. Experiments were performed in a solution containing 30 nM Cy3-ATP, 25 mM KCl, 3 mM $MgCl_2$, 20 mM HEPES (pH 7.8), 0.5% 2-mercaptoethanol, and an oxygen scavenger system [15] at 25°C. Scale bar, 1 μm.

3.2. Visualization of single GFP molecules

The reticulocyte lysate containing S1dC-GFP was diluted 100-fold with a solution containing 25 mM KCl, 3 mM $MgCl_2$, and 20 mM HEPES (pH 7.8) without purification. The specimen was placed on a quartz slide and examined by LBTIRFM. As shown by Fig. 2A, fluorescence spots of individual S1dC-GFP molecules could be clearly observed. Fig. 2B shows the change in the intensity of the fluorescence spot shown by the arrow in Fig. 2A, recorded at video rate. Quantized photobleaching of GFP occurred at the time indicated by the arrow, which provided evidence that the fluorescence spot shown by the arrow in Fig. 2A corresponded to a single GFP molecule. The lifetime of the fluorescence from GFP was several seconds (photobleaching occurred within 1 s in the case shown in Fig. 2B). This was too short to trace the movement of the molecules, but long enough to identify the molecules bound to the glass surface.

This method of imaging single GFP molecules not only improves the detection limit of the gene expression, but also provides a simple procedure for the detection of gene expres-

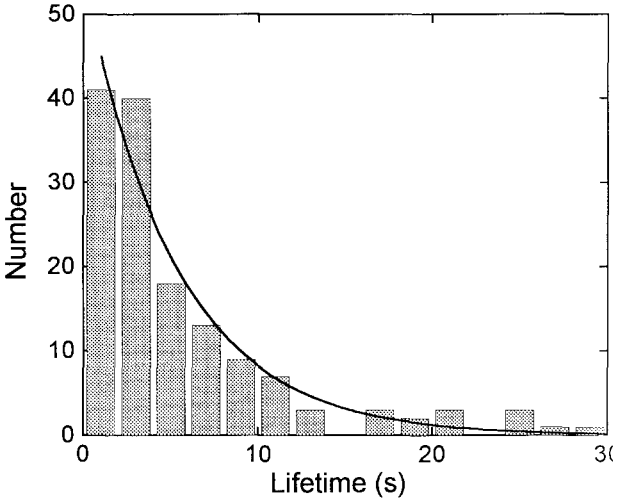

Fig. 4. Histogram of the lifetime of Cy3-nucleotides bound to S1dC-GFP molecules. The lifetime was determined by measuring the duration of fluorescence when Cy3-nucleotides bound to S1dC-GFPs and made clearly defined spots. The number of fluorescent spots of Cy3-ATP measured was 150. The lifetime was determined to be 5.3 s^{-1} from the exponential fitting.

sion. It is possible to detect the expression of chimeric GFP within 2.5 h by combining this technique with the in vitro expression system.

3.3. *Direct observation of individual ATP turnover by single S1dC molecules*

S1dC-GFP molecules, purified with Ni-chelating resins, were adsorbed onto a quartz slide and ATP turnover was observed. First, specimens were illuminated with a 488 nm line from an Ar laser and the positions of individual S1dC-GFP molecules were marked (Fig. 3B). Specimens were then illuminated with a 514.5 nm line from an Ar laser to visualize Cy3-ATP. The ATP turnover events were detected by directly observing association-(hydrolysis)-dissociation of Cy3-ATP [1,2]. When 30 nM Cy3-ATP was applied to S1dC-GFP on the surface, the background fluorescence due to free Cy3-ATP was low, because the illumination region was localized near the quartz slide surface (Fig. 3A). When Cy3-ATP or -ADP associated with the surface-bound S1dC-GFP, which had been previously recorded (Fig. 3B), it could be observed as a clearly defined in-focus fluorescent spot (Fig. 3C); free Cy3-ATP undergoing rapid Brownian motion, on the other hand, was not seen as a discrete spot. Hence, by observing the presence and lifetime of stationary, in-focus Cy3 molecules corresponding to the position of S1dC-GFP molecules on the surface, we could detect individual association-(hydrolysis)-dissociation of Cy3-ATP with single S1dC-GFP. As a control experiment, we confirmed that such ATP turnovers could not be observed when GFP without S1dC was used (data not shown).

S1dC-GFP molecules could be observed turning over Cy3-ATP for several minutes. This suggests that S1dC-GFP expressed in vitro had enzymatic activity and that the illumination of Cy3-nucleotide bound to S1dC-GFP did not diminish this enzymatic activity. Fig. 4 shows the histogram of the life time of bound Cy3-ATP or -ADP which reveals an exponential dissociation rate of 0.19 s^{-1}. This was higher than the ATP turnover rate of S1 suspended in solution (0.09 s^{-1}) [16]. The turnover rate of Cy3-ATP by S1 was similar to that of normal ATP under the same medium condition [2]. This difference may be attributed to the lack of the neck domain that binds to myosin light chains [4,17]. The photobleaching of Cy3-ATP hardly affected the results, as its lifetime (1/photobleaching rate) was ~60 s at the same laser power (5 mW).

It was difficult to produce an active S1dC in either the *E. coli* or baculovirus expression system because the expressed S1dC became insoluble (data not shown). However, S1dC-GFP could be synthesized in a soluble form using the in vitro expression system using rabbit reticulocyte lysate and ATP binding of S1dC-GFP was successfully demonstrated in this study. Moreover, whole myosin can be obtained in a soluble form using an in vitro expression system [18]. Thus, we predict that it will be relatively easy to obtain chimeric proteins of GFP and various other kinds of mutant myosins in vitro. The next step will be to measure the piconewton forces and nanometer displacements of mutant myosin at the level of single molecules [19–21]. The approach of expressing chimeric proteins of GFP in vitro and studying their function at the level of single molecule has potential applicability to many other kinds of proteins that have never been obtained in an active form in conventional expression systems.

Acknowledgements: We thank Drs. J. West (Monash University) and H. Higuchi (ERATO) for critically reading the manuscript, and the members of the ERATO project for valuable discussions.

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