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Rapidly photoactivatable ATP probes for specific labeling of tropomyosin within the actomyosin protein complex

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ABSTRACT

Tropomyosin-specific photoaffinity adenosine triphosphate (ATP) probes have been first developed, in which a diazirine moiety is incorporated into the γ-phosphate group as a rapidly carbene-generating photophore. These probes clearly labeled tropomyosin in the presence of other actomyosin components, that is, myosin, actin, and troponins. The specific labeling of tropomyosin was easily identified by selective trapping of the photo-incorporated ATP probe on Fe^{3+}-immobilized metal ion affinity chromatography (IMAC) beads. The characteristic nature of tropomyosin-specific photocross-linking was further confirmed with a biotin-carrying derivative of the ATP probe. These data suggest that the tropomyosin on the actin filament assembly is located in close proximity to the ATP binding cavity of myosin.

Adenosine triphosphate (ATP) is one of the most important molecules in cell biology; it plays roles in various cell functions, including extra- and intracellular signaling, biosynthesis, active membrane transport of macromolecules, and protein-based molecular motors. Myosins, a family of molecular motors, are ATPases that transduce the energy from ATP hydrolysis to mechanical movement on actin thin filaments in muscle contraction. Despite the recent developments in crystallography, it is difficult to analyze the moving conformation of the actin–myosin system,[1] and chemical cross-linking is required for crystallization of the ATP binding state.[2] Thus, various site-specific probes have been developed to investigate the dynamic nature of the actin system;[3] of these, ATP photoprobes are especially useful for cross-linking protein–protein interactions as well as mapping the ATP binding sites.[4]

The ATP probes include a photoreactive moiety within the ligand structure, in which azido- or benzophenone groups are commonly used. These photophores can be incorporated into the adenine ring,[5] within the ribose structure,[6] or on the phosphate linkage.[7] Among of these three types of ATP probes, γ-phosphate-modified probes can be a powerful tool for analyzing the force generation process of the actin–myosin motor derived from ATP hydrolysis.[2,8] Because a phosphoramide[9] or thiophosphate[10] group resists to hydrolysis under physiological conditions, several photoactivatable ATP analogs modified at the γ-phosphate have been used as cross-linking reagents for binding analysis of kinases.[7] Although benzophenone and arylazide have been used in the ATP probe synthesis as photoactivatable species, the use of diazirine-based ATP analogs has not yet been reported, primarily because of the inconvenience experienced in probe synthesis. Trifluoromethyl phenyldiazirine, however, has become increasingly important because it rapidly produces very reactive carbene upon photolysis at 360 nm.[11] The generated carbene introduces specific and stable cross-links on the target protein, and the availability of the diazirine and its probes is greatly improved.[12] We previously used new diazirine-carrying guanosine triphosphate analogs for the rapid proteomic analysis of G-coupled proteins.[13] In this Letter, we describe an efficient method for detection of tropomyosin within the actomyosin complex using novel diazirinyl ATP probes.

4-[3-(Trifluoromethyl)-3*H*-diazirin-3-yl]benzylamine **2a** was derived from the corresponding bromide[14] in 2 steps via synthesis of phthalimide derivative **1a** based on the Gabriel method and was then coupled with ATP using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) by a method of modifying the previous work[15] to give the corresponding phosphoramidate probe **3a** (Fig. 1). This product was then purified by reverse-phase HPLC on ODS with a liner gradient of 0–50% acetonitrile-water containing 50 mM triethylammonium acetate (pH 7.0) over 20 min at a flow rate of 1 mL/min. The 16.0 min peak was collected and freeze-dried. The probe **3b** bearing a biotin moiety was also prepared in a similar way. The structure of probes **3a** and **3b** was confirmed by NMR and high-resolution MS.[16]

Photolabeling of actomyosin obtained from chicken skeletal muscle with the probe **3a** was carried out under UVA (~360 nm) irradiation for 10 min at 0 °C in the absence of Ca^{2+} by addition

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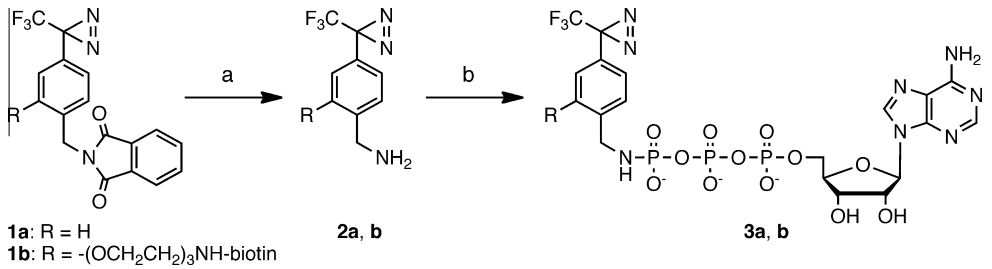

Figure 1. Synthesis of photoactivatable probes **3**: (a) 40% $MeNH_2$/MeOH, rt, (b) ATP, EDCI, pH 6.8, 25 °C.

of ethylene glycol tetraacetic acid (EGTA). The sample was treated with biotin *N*-hydroxysuccinimide for chemiluminescent detection of protein bands using avidin-horseradish peroxidase (HRP) conjugate. The products were subjected to Fe^{3+}-IMAC, and the photolabeled proteins were isolated by chelating a photo-incorporated ATP tag with Fe^{3+} on the resin.[13] Coomassie brilliant blue (CBB) staining of actomyosin after SDS–PAGE showed that actomyosin is mainly made up of a mix of proteins such as myosin, actin, and tropomyosin (lane 8). A band around 37 kDa was clearly detected as a major photolabeled product (Fig. 2, lane 2), which corresponds to tropomyosin among the actomyosin components. It was detected by Western blot analysis using anti-tropomyosin (lane 6) as the same position of tropomyosin in actomyosin (lane 7). The binding specificity of the probe to each of these proteins was evaluated on the basis of the inhibitory effects of γ-thio-ATP on the photoaffinity labeling because γ-thio-ATP is an ATP analog that is stable against hydrolysis.[17] As shown in Figure 2, the labeled amount of 37 kDa protein was reduced by increasing the concentration of γ-thio-ATP (lanes 3–5).

The labeled 37-kDa protein was identified by peptide mass fingerprinting analysis of the trypsin-digested sample by using MALDI-TOF MS. Some peptides were detected at $m/z = 1157$, 1285, 1400, and 1612, and MS/MS analysis of the peak at m/z 1285 indicated that this peptide consisted of the amino acids KLVIIEGDLER. This sequence exists in part of tropomyosin obtained from chicken skeletal muscle (NCBI gi|833617).[18] Consequently, we concluded that **3a** specifically labeled tropomyosin in the actomyosin complex. In binding analysis of the myosin motor protein complex, tropomyosin was labeled by photoactivatable ATP analogs for the first time.

However, the procedures for detection and purification of products labeled with **3a** retain the possibility of including phosphorylated proteins. These proteins are also able to bind to Fe-resin and may still adhere to the resin after washing. To avoid both nonspecific biotin labeling and the following IMAC purification steps, the biotinylated probe **3b** was used for the detection of labeled products in the actomyosin motor system. The biotinylated products in this case should be only those molecules cross-linked with the probe. In addition, this procedure would simplify the analytical processes for detecting or purifying labeled products. Photoaffinity labeling with probe **3b** and inhibition assays showed similar results to those obtained by the procedure using probe **3a** (Fig. 3), regardless of the presence of the biotin moiety. Tropomyosin was primarily detected in UV-irradiated samples (Fig. 3, lane 2), and the labeled amount decreased depending on the concentration of γ-thio-ATP. This result indicates that the biotin tag on probe **3b** does not have a large effect on the myosin–actin filament interaction, and the binding of these photoprobes within the actomyosin complex are similarly allocated to that of natural ATP, directing the phosphate terminal to the actin filament, around which the tropomyosin is twisted.[19]

Tropomyosin is composed of 2 α-helixes that form an elongated coiled-coil structure and lie along the helical strand of the actin thin filament. Tropomyosin induces molecular switching in response to Ca^{2+} concentration changes to regulate actomyosin motility. At low Ca^{2+} concentrations, tropomyosin blocks the myosin-binding site of the actin filament in cooperation with troponin, which is a tropomyosin-binding protein consisting of 3 subunits (TnT, TnI, and TnC). Upon Ca^{2+} influx, calcium-bound TnC causes TnI to release the hold on actin. This allows tropomyosin to slide and thus open the myosin-binding site,[20] and myosin can bind strongly to the actin filament in the ATP hydrolysis cycle. Since we have confirmed specific labeling of tropomyosin in the actomyosin complex by the ATP analogs bearing a diazirine at the γ-phosphate **3a** or **3b** in the absence of Ca^{2+}, the probe **3a** was used for photolabeling actomyosin under conditions that included 1 mM $CaCl_2$. Unexpectedly, tropomyosin was mainly labeled by the ATP probe, which is independent of Ca^{2+} concentration (Fig. 4). Little labeled actin or myosin was observed.

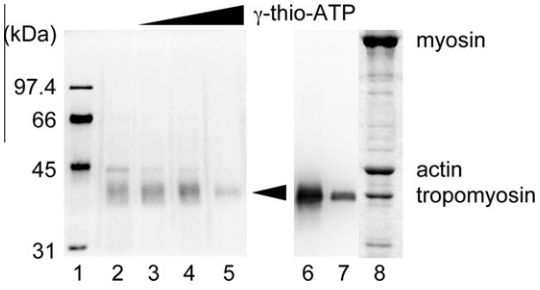

Figure 2. Photoaffinity labeling of actomyosin with probe **3a**. Actomyosin (3.9 μg) was incubated with **3a** (125 μM) in a solution containing 30 mM Tris–HCl (pH 7.2), 5 mM $MgCl_2$, and 1 mM EGTA for 1 h followed by irradiation with 360 nm light. The biotinylated samples isolated by Fe^{3+}-IMAC were subjected to 10% SDS–PAGE and then blotted onto a polyvinylidene difluoride (PVDF) membrane. Lane 1: size markers; lanes 2–5: chemiluminescent detection of photolabeled samples using avidin-HRP conjugate in the presence of 0, 0.125, 1.25, and 12.5 mM γ-thio-ATP, respectively; lanes 6 and 7: Western blot analysis of photolabeled sample isolated by Fe^{3+}-IMAC and actomyosin (78 ng) using anti-tropomyosin, respectively; lane 8: CBB-stained PAGE separation of actomyosin components.

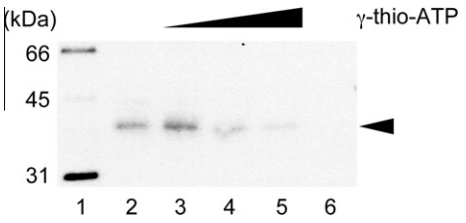

Figure 3. Photoaffinity labeling of actomyosin with biotinylated probe **3b**. Photolabeling was carried out under the same condition as that of **3a** in the absence (lane 2) or presence (lanes 3–6) of γ-thio-ATP. Photoproducts were separated by 10% SDS–PAGE and then blotted on a PVDF membrane. The labeled products were detected by a chemiluminescent method using avidin-HRP conjugate. Lane 1: size markers; lanes 2–5: photolabeled samples in the presence of 0, 0.05, 5, and 12.5 mM γ-thio-ATP, respectively; lane 6: sample without irradiation.

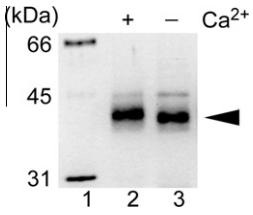

Figure 4. Effect of calcium on labeling of tropomyosin. Actomyosin (3.9 μg) was incubated with probe **3a** (125 μM) in a solution containing 30 mM Tris–HCl (pH 7.2), 1 mM $CaCl_2$ or 1 mM EGTA, and 5 mM $MgCl_2$. Photolabeling and detection were performed by using the same method described in Figure 2. Lane 1: size markers; lanes 2 and 3: chemiluminescent detection of photolabeled samples in the presence of 1 mM $CaCl_2$ (lane 2) and in the absence of Ca^{2+} (lane 3).

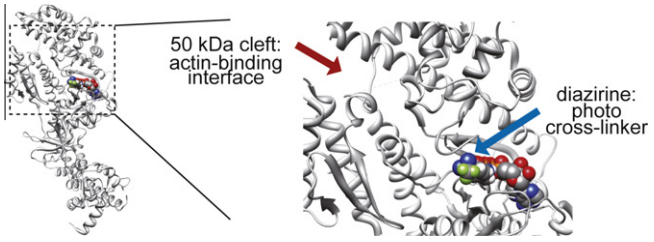

Figure 5. Docking model of the photoprobe **3a** binding to chicken myosin. The probe **3a** is shown as a CPK model, in which the nitrogen (blue) of the diazirine is indicated by a blue arrow. The probe binds to the ATP binding site and directs its photoreactive diazirine to the 50-kDa cleft (actin-binding interface) indicated by the red arrow. Original data refers to PDB: 1W7I.

Our ATP probes clearly showed the tropomyosin-specific nature of labeling in actomyosin. Previous reports showed that photoactivatable ATP analogs labeled myosin or myosin S1, in which the photophore was attached on the adenine ring[5c] or in the ribose ring,[21] respectively. We examined the molecular docking of photoprobe **3a** with the reported X-ray crystallographic structure of myosin. Since the ATP-bound crystal structure has only been revealed for scallop myosin,[2] we used the chicken myosin–ADP complex from the Protein Data Bank (PDB) 1W7I dataset which reflects the ATP binding state.[22] The probe **3a** manually docked into 1W7I by replacing bound ADP (Fig. 5). The probe binds to the ATP binding site of myosin by directing the photoactivatable diazirine to actin within the actomyosin complex, and the distance from the diazirine to the actin filament could be estimated at a few nm.[23] Since the short-lived carbene intermediate immediately cross-links to a spatially closed molecule,[11] our result of probing the tropomyosin on the actin filament suggests that the diazirine moiety of **3a** and **3b** should exist near to the tropomyosin coil on the actin filament through binding with myosin. This suggests the hypothesis that the tropomyosin coil remains in the 50-kDa cleft throughout the ATP binding stage regardless of the presence of Ca^{2+}.

In conclusion, we have synthesized diazirine-based photoactivatable ATP probes modified at the γ-phosphate, and these probes provided the first case showing that tropomyosin is specifically labeled by ATP analogs in the actomyosin complex. Our probe could therefore be a useful tool to analyze the role of tropomyosin during the conformational change of the actomyosin complex.

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16. *Compound* ***3a***: ^{1}H NMR (500 MHz, D_2O, $Me_3SiCD_2CD_2CO_2Na$ (TSP-d_4)): δ 8.41 (1H, s), 8.18 (1H, s), 7.13 (2H, d, *J* = 8.3 Hz), 6.84 (2H, d, *J* = 8.3 Hz), 5.97 (1H, d, *J* = 5.6 Hz), 4.59 (1H, t, *J* = 5.3 Hz), 4.48 (1H, t, *J* = 4.3 Hz), 4.36–4.42 (2H, m), 4.24–4.30 (1H, m), 3.92 ppm (2H, d, *J* = 8.1 Hz); ^{19}F NMR (376 MHz, D_2O-CD_3OD, $CFCl_3$): δ −64.2 ppm (3F, s); HRMS (FAB^+): Calcd for $C_{19}H_{22}O_{12}N_8F_3P_3$ $[M+H]^+$ 705.0601. Found 705.0624; **3b**: ^{1}H NMR (500 MHz, CD_2OD): δ 8.66 (1H, br s), 8.16 (1H, s), 7.61 (1H, br s), 6.79 (1H, d, *J* = 7.0 Hz), 6.63 (1H, S), 6.09 (1H, d, J = 5.6 Hz), 4.57 (1H, br s), 4.44 (1H, dd, J = 4.7, 8.1 Hz), 4.18–4.30 (5H, m), 4.11 (2H, t, J = 4.7 Hz), 3.87 (2H, t, J = 4.7 Hz), 3.71–3.73 (2H, m), 3.63–3.66 (2H, m), 3.55 (2H, t, J = 5.1 Hz), 3.34 (2H, t, J = 5.1 Hz), 3.10–3.15 (1H, m), 2.88 (1H, dd, J = 5.1, 12.8 Hz), 2.67 (1H, d, J = 12.8 Hz), 2.16–2.20 (2H, m), 1.38–1.62 (2H, m), 1.35–1.40 (2H, m), 1.28–1.31 ppm (2H, m); ^{19}F NMR (367 MHz, CD_3OD, $CFCl_3$): δ −65.2 ppm (s, 3F); HRMS (FAB^+): Calcd for $C_{35}H_{52}O_{17}N_{11}F_3P_3S$ $[M+H]^+$ 1080.2428. Found 1080.2465.
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