Bioorganic & Medicinal Chemistry Letters 23 (2013) 5605–5608

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Affinity-based fluorogenic labeling of ATP-binding proteins with sequential photoactivatable cross-linkers

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ARTICLE INFO

Article history:
Received 7 May 2013
Revised 22 July 2013
Accepted 8 August 2013
Available online 16 August 2013

Keywords:
Diazirine
Photoaffinity labeling
ATP
Coumarin
Cinnamate

ABSTRACT

A specific illumination approach has been developed for identification of adenosine triphosphate (ATP)-binding proteins. This strategy utilizes a tandem photoactivatable unit that consists of a diazirine group as a carbene precursor and an *o*-hydroxycinnamate moiety as a coumarin precursor. The photolysis of diazirine induces a specific cross-link on target proteins and is followed by photoactivation of coumarin generation with a concomitant release of the pre-installed affinity ligand. The ATP, installed with this cross-linker at the γ-position, successfully transferred a coumarin onto ATP-binding proteins using only UV-irradiation.

Since the transporter responsible for the vesicular accumulation of adenosine triphosphate (ATP) was first identified,[1] ATP's essential roles as an extracellular signaling molecule, as well as an intracellular coenzyme in energy transfer reactions such as muscle contraction and active transport of biological material, have been revealed. Recently, a fluorescent protein has been developed that can show the location of ATP in a cell, and it is being applied to in situ, spatio-temporal functional analysis.[2] In addition to in situ imaging, the profiling of ATP-binding proteins has also been a very important issue in proteomic analysis to understand complex functions.[3] Since proteomic screening has become increasingly insightful with the arrival of novel analytical tools and technologies, affinity-based chemical tagging strategies, that is, activity-based protein profiling for enzymes, have also progressed greatly.[4]

Photoaffinity labeling (PAL) has been a powerful method for the specific cross-linking of interacting molecules, and PAL can profile binding proteins by using a ligand with a photoactivatable group. Because of the small quantity of labeled protein, the method usually requires the preparation of a photoprobe having a fluorescent, radioisotope, or biotin tag in advance for protein detection.[5] This sometimes causes high background noise, loss of affinity, and difficulties in probe synthesis. To avoid these problems, post-labeling methods for detection tags have been examined. This has been achieved by utilizing bio-orthogonal functions such as azido and ethynyl groups,[6] certain cleavable linkages between the cross-linker and ligand moieties,[7] etc. Recently, a unique PAL strategy for creating a fluorophore at the interacting interface of molecules using a tag-free chemical probe was reported.[8] It was applied to the detection of the anti-lysozyme expressed on a cell by a photoactivatable lysozyme bearing a diazirine-based photocross-linker including an *o*-hydroxycinnamate moiety in the structure. The *E* form of the cinnamate unit was isomerized to the *Z* form by irradiation with UV light. The *Z* form induces lactonization via intermolecular nucleophilic substitution of a hydroxy group at the *ortho* position, which results in the formation of a coumarin derivative. Therefore, this ligand probe with a photoactivatable unit can photochemically capture the target protein with a covalent bond, produce a coumarin fluorophore at the cross-linked position by releasing the ligand molecule, and enable specific and very sensitive detection of labeled products without necessitating a procedure for pre- or post-addition of a detection tag. In this Letter, an ATP probe **1** bearing a sequential photoactivatable unit at the γ-phosphate position was prepared and applied to the detection of ATP-binding proteins via fluorescent visualization. For probing of ATP, a photoactivatable group such as an azide, benzophenone, or diazirine has been commonly incorporated into the adenine ring,[9] ribose,[10] or phosphate linkage.[11] The phosphate-modified nucleotide analogs have been designed to exhibit resistance to hydrolysis, and they have been successfully applied to investigations of certain molecular interactions such as H-Ras (a small GTPase)[6b] and an actomyosin motor protein complex that includes a myosin exhibiting ATPase activity.[11c]

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http://dx.doi.org/10.1016/j.bmcl.2013.08.041

(*E*)-*N*-*tert*-Boc-*N*′-(2-methyl-3-(2-hydroxy-4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)cinnamoylethylenediamine[12] was synthesized by coupling 2-hydroxy-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]cinnamic acid[13] and *N*-Boc-ethylenediamine. The subsequent coupling with ATP after removal of Boc group, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, was carried out using a method modified from a previous work[6b] to give the corresponding phosphoramidate ATP probe **1** (Fig. 1). This product was purified by reverse-phase HPLC on ODS with a liner gradient of 0–40% acetonitrile-water containing 50 mM triethylammonium acetate (pH 7.0) over 40 min at a flow rate of 1 mL min^{-1}. The product fraction corresponding to the 30 min peak was collected and freeze-dried, and its structure was confirmed by NMR and high-resolution MS.[14]

Figure 2 shows the time course of coumarin formation from probe **1** under 360 nm light irradiation using black light lamps (15 W × 2). The fluorescence intensity remained constant at 0 °C for 60 min, and then, it significantly increased at 37 °C, suggesting that coumarin had formed. The reactions were monitored in detail by ^{1}H and ^{19}F NMR with ethyl 2-methyl-4-(3-trifluoromethyl)-3*H*-diazirin-3-yl)cinnamate (**4**, 20 mM in CD_3OD) under irradiation at 313 and 365 nm using a 250 W high-pressure mercury lamp through a bandpass filter (fwhm = 10 nm). Fluorine signals from the CF_3 group appeared at ca. −65, −57, and −77 ppm for the diazirine, diazo, and CD_3OD-adduct compounds, respectively (Fig. 3). When irradiated, the diazirine group decomposed to yield a carbene intermediate that reacts with the molecule in spatial proximity in picoseconds.[15] Rather than a decrease in peak intensity at −65 ppm due to the presence of diazirine compounds, the signal of the CD_3OD adducts at −77 ppm increased. On the other hand, proton signals from the ethyl group appeared at different chemical shifts for the *E* and *Z* isomers (ca. 4.25 and 4.08 ppm, respectively). This data suggested that the cinnamate unit could be similarly isomerized with both 313 and 365 nm radiation. Since the following nucleophilic substitution (lactonization) at the amide group was quite slow at 0 °C,[8] the data indicated that two photoreactions could be simply regulated using the reaction temperature.

Furthermore, photolysis of the diazirine derivatives (**5** and **6**) was examined in a way similar to that of **4** described above. Figure 4A shows decay curves of the diazirine groups of **4**–**6** during irradiation using black light lamps. The content percentage was calculated from integration values of the NMR signals, which were referenced to two standard signal values, that is, those of $Si(CH_3)_4$ in ^{1}H NMR and $CFCl_3$ in ^{19}F NMR. It became apparent that the photolysis of the diOH derivative (**6**) proceeded slowly compared to that of the OH-free derivative (**4**). This indicated that diazirine photolysis largely depends on the hydroxy substituent at the ortho position of the benzene ring, even though the π-conjugated system of benzene does not interact with that of diazirine. The *E*/*Z* ratio of compound **5** (mono-OH derivative) finally reached ca. 0.5, which was larger than the values for compounds **4** and **6** (Fig. 4B). Since compounds **5** and **6** in *Z* form undergo lactonization at 37 °C, the equilibrium should be shifted to produce the *Z* form. In this study, therefore, the following PAL experiments were carried out using the ATP probe **1** derived from the mono-OH cinnamate unit.

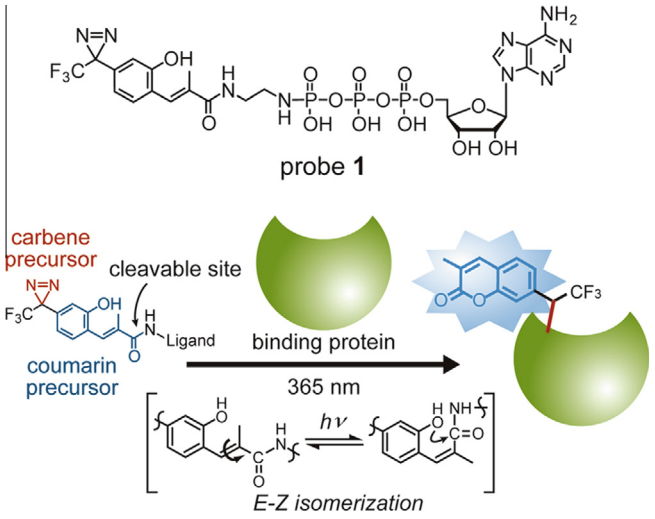

Figure 1. Structure of ATP probe **1** and the photochemical coumarin-tag installation into the binding protein.

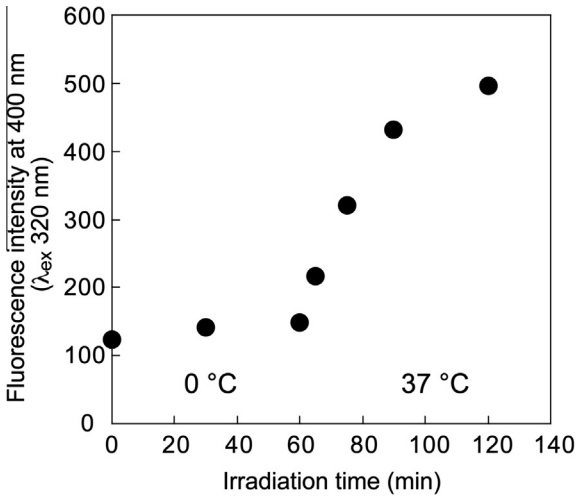

Figure 2. Time course of coumarin formation from probe **1** (1 μM in 50 mM Tris–HCl, pH 7.4, containing 30% ethanol) under irradiation with 360 nm light. The sample solution was irradiated at 0 °C and then at 37 °C. Fluorescence at 400 nm (λ_{ex} = 320 nm) was measured at 20 °C.

PAL of L-glutamate dehydrogenase (GDH, EC 1.4.1.2–3, 0.2 mg mL^{-1}, 1 μg for each lane) with probe **1** (100 μM) was carried out in 0.1 M phosphate buffer (pH 7.3) under 360 nm light irradiation at 0 °C for 60 min, after incubation in the absence or presence of inhibitor at 37 °C for 1 h in the dark. The enzymatic activity of GDH is allosterically regulated by a wide array of metabolites such as ADP and leucine as activators, and GTP, palmitoyl CoA, and ATP as inhibitors.[16] The photoproduct was then subjected to 10% SDS–PAGE (Bio-Rad TGX gel plate) after treatment with the SDS sample buffer at room temperature. Emission was detected from the gel through a bandpass filter (420 nm, fwhm = 10 nm) under UV irradiation (black light lamps) through a second bandpath filter (320 nm, fwhm = 10 nm). The labeled GDH was clearly detected at approximately 50 kDa (Fig. 5, lane 4), and the emission decrease corresponded to the amount of ATP in the solutions added as an inhibitor (lanes 5–7). As a reference, a diOH-type ATP analog (probe **2**, $R^1 = R^2 = OH$, HRMS (ESI-) 832.0875 calculated for $[M-H]^-$, found 832.0881) also labeled GDH, but the yield decreased significantly (lanes 1 and 2).

Since probe **1** was resistant to hydrolysis, it was used to label another protein, that is, heat shock protein 90 (Hsp90) having ATPase activity.[17] Hsp90 is a molecular chaperone that manages conformational maturation of multiple client proteins upon ATP binding and hydrolysis. PAL of Hsp90 (2 μg per lane) was carried out with probe **1** (200 μM) in a 35 mM Tris–HCl solution (MP Biomedicals ultra-pure grade, pH 7.4, containing 4 mM $MgCl_2$), which resulted in a coumarin-labeled Hsp90 (lanes 3 and 4 in Fig. 6). The emission intensity of the Hsp90 band decreased in the presence of γ-thio-ATP as a competitive inhibitor (lanes 2–4). γ-Thio-ATP is an ATP analog that is stable against hydrolysis.[18] The Hsp90 intensity data indicated that Hsp90 was specifically labeled by probe **1**.