Bioorganic & Medicinal Chemistry Letters 25 (2015) 1675–1678

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

# Structure-assisted ligand-binding analysis using fluorogenic photoaffinity labeling

Souta Masuda, Takenori Tomohiro *, Shouta Yamaguchi, Shota Morimoto, Yasumaru Hatanaka

*Laboratory of Biorecognition Chemistry, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2603 Sugitani, Toyama 930-0194, Japan*

ARTICLE INFO

*Article history:*
Received 5 January 2015
Revised 2 March 2015
Accepted 4 March 2015
Available online 10 March 2015

*Keywords:*
Photoaffinity labeling
Diazirine
Fluorogenic
ATP
Glutamate dehydrogenase

ABSTRACT

Photoaffinity labeling (PAL) technique using a fluorogenic cross-linker is used to monitor the nucleotide-binding pocket within a protein. A coumarin fluorophore formed in the binding domain due to ultraviolet (UV) irradiation has been shown to accelerate the sequencing of the labeled peptide as well as identification of the labeled site by liquid chromatography (LC)–tandem mass spectrometry (MS), in addition to providing information on the ligand binding state. Selective monitoring of the predefined fluorescence peaks among the numerous digests obtained from high performance liquid chromatography (HPLC) clearly indicates the binding capability of the ligand to the entire protein as well as to the corresponding binding domain under various conditions. In the current study, ligand-binding analysis confirmed by the structural information of the binding state has been demonstrated using fluorogenic ATP/ADP photoactivatable probes under allosteric regulation of multiple substrates in the enzyme glutamate dehydrogenase (GDH).



The binding analysis of small molecules and proteins has been one of the important subjects for a fundamental understanding of the cellular functions.[1] Photoaffinity labeling (PAL) is an excellent method that can detect and identify most of the biomolecular interaction systems in the proteome by applying a combination of covalent cross-linking and attachment of a detectable tag to the labeled protein.[2] However, the general PAL method has a low labeling yield for binding analysis, which can be carried out by using an attachment of mainly radioisotopic, chemiluminescent or fluorescent reporter tags.[3] Alternatively, PAL has provided structural information of ligand binding at relatively high-resolution based on the mapping of the labeled amino acid residues. It has been particularly useful in the analysis of molecular interactions in the transition state or weak binding state, which otherwise would be difficult to access using the X-ray crystallography and NMR techniques.[4] In general, identification of the labeled sites has been achieved using tandem MS-based sequencing of purified and labeled peptides after digestion. To get better performance of PAL-based target identification, many efforts have been made aiming to purify negligible amounts of the labeled products from an enormous concomitant using several separation tags such as a biotin,[5] a perfluoroalkyl group,[6] a clickable tag.[7] Particularly biotinylated PAL probes have been used as a practical, non-radioactive way of detecting the corresponding interacting protein by the chemiluminescence method and to enrich the labeled protein using avidin-conjugated matrix. However, determination of the labeled sites at picomolar concentrations has been still difficult due to the interference of trace amounts of certain unavoidable adsorption materials.

To minimize the interferences arising from the further attachments, such as high background noise, loss of affinity, and difficulties in probe synthesis, a unique tag-free cross-linker that photochemically gets transformed into a coumarin fluorophore has been developed for fluorescence spectroscopic analysis of ligand binding with the corresponding protein (Fig. 1).[8] The fluorogenic property showed great potential for simplifying the discriminating process of the labeled peptides in HPLC without the necessity of comparing with repeated control experiments, where the fluorescent peaks could be directly identified using LC–MS apparatus.[9] Furthermore, the cleavability in this PAL system was an advantage for MS/MS-based sequencing, because a small and stable coumarin tag was attached in the protein accompanied with removal of the large and unstable ligand molecule. Since the labeled amino acid residues form a part of the binding domain, the fluorescence intensities indicate the binding capability of the probe to the binding domain, while the retention time reflects the differences between the cross-linked sites that suggest change in the binding positions. Therefore, once the fluorescence peak is assigned to a particular peptide of the target protein, the ligand interactions can be spectroscopically determined in that particular binding domain; whereas, the SDS-PAGE based analysis could be

* Corresponding author. Tel.: +81 764347518; fax: +81 764345063.
*E-mail address:* ttomo@pha.u-toyama.ac.jp (T. Tomohiro).

http://dx.doi.org/10.1016/j.bmcl.2015.03.008
0960-894X/

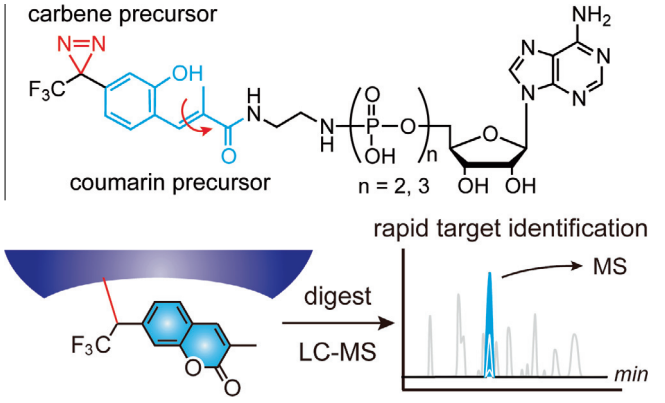


**Figure 1.** Structure of the nucleotide photoprobes and photochemical tagging of coumarin within the binding domain. Fluorescence-labeled peptides can be directly identified by performing LC–MS of the proteolytic digest.

used to monitor the binding of the ligand with the entire protein. In this study, the fluorogenic labeling method is applied for the identification of the labeled site to monitor and evaluate the nucleotide binding to the interacting site within a protein.

L-Glutamate dehydrogenase (GDH) is a homohexameric enzyme that catalyzes the reversible oxidative deamination of L-glutamate to 2-oxoglutarate using $NAD^+$ or $NADP^+$ as a coenzyme. The allosteric effects of the interaction of L-glutamate with GDH have been studied using coexisting substrates such as ADP, GTP, NADH, and ATP.[10] Both NADH and GTP inhibit the oxidative reaction by keeping the catalytic cleft of GDH in a close conformation, whereas ADP activates the oxidative reaction.[11] Further, ATP has also been suggested to inhibit the reaction; however, the binding site for ATP has not been clearly identified. Both ADP[12] and NADH[13] bind and interact with GDH through the adenine moiety oriented at the same site of the allosteric domain placed behind the active center. Thus, in this study, we have synthesized fluorogenic ATP and ADP probes, γDAcinATP **1**[8b] and βDAcinADP **2**, by incorporating diazirine (DA) as a photoactivatable unit into the terminal phosphate group of the nucleotides via an amide linkage to determine the nucleotide-binding pocket within the GDH enzyme.

A buffer solution (15 μL, 50 mM HEPES, pH 8.0) containing γDAcinATP **1** (80 μM) and bovine GDH (125 μM, EC 1.4.1.2-3, 56 kDa) was irradiated with 365 nm light using a 250 W high-pressure mercury lamp through a bandpass filter (FWHM = 10 nm) for 15 s at 0 °C followed by 15 min at 40 °C. The photoproduct was proteolyzed with lysyl endopeptidase (Lys-C) overnight at 37 °C. The monomer unit of glutamate dehydrogenase has 32 possible digestion sites. Reverse-phase HPLC of the digests was carried out on an ODS (ZORBAX 300SB-C18, 5 μm, 2.1 × 250 mm) with a linear gradient of 10–80% acetonitrile–water containing 0.1% formic acid for over 60 min at a flow rate of 0.4 mL $min^{-1}$ by monitoring the absorption at 215 nm and emission at 420 nm ($\lambda_{ex}$ = 320 nm for the detection of coumarin fluorescence). The peptide coverage was ca. 73%. A major fluorescence peak was observed at 21.1 min (indicated by the black arrow in Fig. 2), and the peak area decreased depending on the concentration of ATP without changing the retention time. On the other hand, the HPLC profiles that were concurrently detected using UV absorption for all the peptide fragments, did not show significant change with respect to the ATP concentrations. These results suggest that the probe specifically interacts with GDH at a particular binding site in where ATP interacts. The coumarin-labeled peptide at 21.1 min was identified using MS and gave a signal with $m/z$ 871.9 ($z$ = 2, Fig. 3), which corresponds to a peptide fragment comprising of the residues 489–501 of GDH (1742.8 for $MH^+$ + coumarin). The MS/MS spectrum acquired for the singly

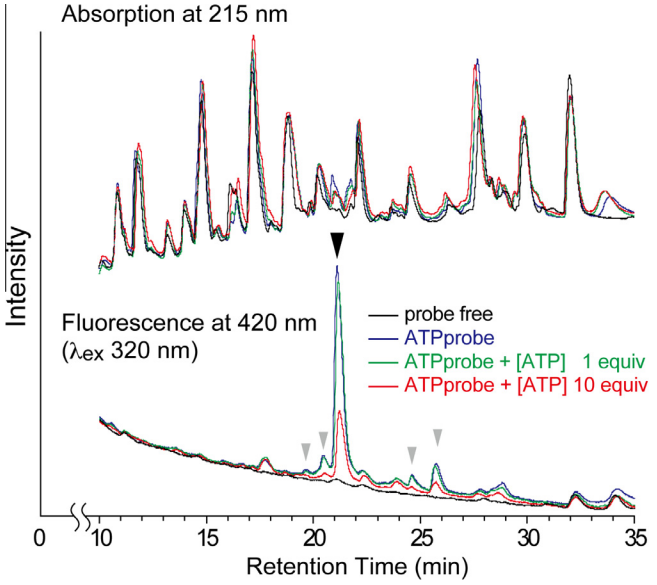


**Figure 2.** Reverse-phase HPLC profiles of the Lys-C digest of GDH after photolysis with γDAcinATP **1** in the presence of ATP at 0, 1, and 10 mol ratio to the probe as indicated by the blue, green, and red lines, respectively, and without the probe indicated by the black lines. These profiles were detected by monitoring the UV absorption at 215 nm (upper) and fluorescence emission at 420 nm with an excitation at 320 nm (lower).

charged $[M+H]^+$ ions displayed a series of $\mathbf{b_n}$ + 240 ions and $\mathbf{y_n}$ + 240 ions with a coumarin modification (detailed mass numbers are listed in Fig. S1). The labeled site was clearly identified as the residue Arg491 (indicated by an asterisk) at the third position from the N-terminus of the 13-amino acid peptide fragment (VFR*VYNEAGVTFT). Arg491 is located along the inlet port of the binding cavity for the adenine moiety of ADP or NADH within the allosteric domain, which is in agreement with the X-ray crystallography data of ADP–GDH complex (PDB ID: 1NQT)[12] and NADH-Glu-GTP-GDH complex (PDB ID: 3MW9).[13]

The PAL with βDAcinADP **2** also resulted in similar HPLC profiles (Fig. S2) as those obtained for γDAcinATP **1** although the labeling efficiency is about 7 times lower. A major fluorescence peak was observed at the same retention time, and the tandem MS analysis also assigned the labeled residue as Arg491 (Fig. S3).[14] These data suggested that both the probes as well as ATP bind to the same domain that acts as the allosteric ADP/NADH site. In addition, the HPLC profiles of PAL showed a similar inhibition pattern as indicated by the other fluorescence peaks (indicated by gray arrows in Fig. 2) although the labeling efficiency of these peaks was different; thus, indicating that the phenyldiazirine moiety of both the probes pivots in the direction of the Arg491 residue along with preserving the orientation of the adenine moiety in the same binding cavity (Fig. S4). Therefore, these HPLC profiles should reflect these binding states. Initial PAL studies have been well carried out to clarify the role of these small molecules in GDH catalysis using a variety of photoactivatable nucleotides with a radioisotope such as [α-$^{32}$P]2-$N_3$$NAD^+$,[15] [γ-$^{32}$P]8-$N_3$GTP,[16] [α-$^{32}$P]8-$N_3$ADP,[17] and [β-$^{32}$P]2-$N_3$ADP[17] (where an azide group was modified in the nucleobase); and GTPγBP[18] and ATPγBP[17] (where a benzophenone (BP) group was modified at the terminal phosphate). However, identification of the labeled amino acid residue has often proved to a failure although selective isolation of the nucleotide probe-labeled protein by Fe(III)-IMAC[19] was performed prior to the proteolytic digestion. The fluorogenic method used in this study results in the rapid and proper identification of the infinitesimal target peptides without the enrichment and also remarkably