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Kinetic controlled affinity labeling of target enzyme with thioester chemistry

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ABSTRACT

High specificity has been an important feature in affinity labeling for target profiling. Especially, to label targets via rapidly progressing reactions with consumption of ligand (probe), high specificity of reaction with common functional groups of target protein should be achieved without reactions with similar groups of non-target proteins. Herein, we demonstrate the kinetic controlled affinity labeling of acyl CoA synthetase using a fatty acid analogue containing a phenylthioester linkage. High specificity was attained by accelerating the labeling rate in the binding pocket. This approach could be useful for profiling a series of target enzymes and transporters in signal transduction pathways.

1. Introduction

Identifying interacting proteins based on specific labeling has been a crucial objective in the drug discovery process and in fundamental biochemical research for elucidating a protein's complex biological roles.[1] Photochemical affinity labeling (PAL), the light-regulated cross-linking of molecules via a covalent bond, has been recognized as a suitable method for identification/profiling of known or unknown interacting partners, especially in cases of using diazirines.[2] Especially membrane proteins and weak-interacting proteins, that are hardly addressed by conventional affinity purification protocols, are effectively studied with this approach. Various multifunctional photocross-linkers have been developed to increase efficiency by attaching functions such as biotin,[3] clickable,[4] and fluorogenic[5] properties. Yet, it has been difficult to apply PAL to enzymes or transporters, the major targets of drug discovery, since the PAL probe must remain bound to (or within) the protein during light irradiation. Alternatively, chemical affinity labeling theoretically can trap even such rapid processes. However, the uncontrollable high reactivity of cross-linkers (such as active esters) often causes increased levels of hydrolyzed and non-specific labeled products, which interfere with target identification.

The recent developments in chemical affinity labeling methods have significantly reduced non-specific labeling using activity-based probes. Such activity-based probes contain a mechanism-based reactive group such as fluorophosphates for hydrolase profiling,[6] or a leaving group such as ester,[7] tosylate[8] and thioester,[9] and a catalytic group of acylation.[10] This development has provided useful tools for profiling proteins (such as kinases[11]) and spatiotemporal cellular imaging of receptors (for example, fluorescence-based sensing of sugar binding[8a]). The former is suitable for enzyme profiling by trapping reaction intermediates since the probe acts as an irreversible inhibitor by reacting with the active residue. The latter method would be suitable for receptor or transporter profiling. These methods kinetically regulate the reactivity of their functional groups (cross-linkers) to increase both the specificity and the yield due to the near-field effect between the reactive group for cross-link and the residue mediating the enzyme reaction and the concentration effect that accelerates the substitution reaction with the amino groups of lysine residues on the protein surface that is protonated in a neutral media. In this report, we employed an activity-based affinity method with thioesters, since thioester biomolecules were synthesized as active intermediates by coupling with CoA such as lipid metabolism. It was evaluated in a molecular system with relatively low specificity and applied to label a dynamically progressing enzyme process, namely, labeling acyl CoA synthetase (ACS) with a palmitic acid analogue bearing a biotin, via a phenylthioester linkage.

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2. Results and discussion

2.1. Probe design and synthesis

Myristoyl probe **1** was synthesized by coupling with a biotin moiety through a phenylthioester linkage (Fig. 1). Arylthioester derivatives have generally shown higher reactivity to nucleophiles than alkylthioesters, as demonstrated in native chemical ligation. In addition, a previous study on coumarin-labeling of a maltose-binding protein suggested a relatively unreactive alkylthioester.[9] The biotin moiety was employed as a tag for detection and bovine serum albumin (BSA) was employed as a receptor with low affinity to evaluate labeling in the steady state. It has the ability to enclose several fatty acids within its structure. The X-ray crystallography of complexes with human serum albumin and six myristic acids (1O9X[12] or 2XVV[13]) indicated that the carboxylate groups of some bound myristic acids are located close to lysine residues (that are likely to be labeled). Biotin tags were used to form thioester linkages as an acyl compound, to introduce them into the target protein by a nucleophilic substitution reaction.

Probe **2** consists of palmitate and biotin molecules connected via a thioester bond on a Lys scaffold that has a carboxyl group as the enzyme reaction site. This probe was designed according to our previous PAL study on the in vitro peroxisomal β-oxidation of fatty acids by rat liver.[14] A photoreactive phenyldiazirine group was added at the α-position of the fatty acid moiety, to prepare the 'photoactivatable' palmitoyl analogue. The probe rapidly labeled multifunctional enzyme type 2 (MFE2), an enzyme that catalyzes the peroxisomal β-oxidation of fatty acids. Specifically, this probe should be effectively adenylated, displaced with CoA, and transported through the peroxisome membrane before reaching MFE2. Thus, these proteins could recognize and modify the probes at their active centers. The side chain was utilized as a spacer (ca. 1.45 nm) between the carboxylate and the thioester group, which could react with some Lys residues in the biding site. Crystallography data from long-chain fatty acyl-CoA synthetase and fatty acids derived from *Thermus thermophilus* showed amino groups of two Lys residues in the pocket were located 0.93 and 1.60 nm from the carboxyl group of the substrate, although one amino group interacted directly with the carboxyl group (PDB ID: 1V26).[15] The protected Lys was coupled with palmitic acid to yield compound **5**, and a thiophenol moiety was then introduced on the side chain. After hydrolysis, a biotin moiety was added at the thiol group to produce the corresponding probe **2**.

2.2. Affinity labeling of BSA

To evaluate the strategy for protein labeling in steady binding state, reaction of BSA with probe **1** was examined under various conditions. All reactants including probe **1** (1 μM) and BSA (1 μM) were mixed at 0 °C in phosphate buffer (pH 6.9, 20 mM), and the solution was then incubated at 37 °C. The reaction was quenched by adding SDS sample buffer. After denaturing at 95 °C for 5 min, the product was subjected to SDS–PAGE, followed by blotting onto a PVDF membrane. The biotinylated BSA was detected via a chemiluminescence method, using an avidin–horseradish peroxidase (HRP) conjugate. The protein band indicated a size of approximately 66 kDa, and the emission intensity increased with incubation time and reached its maximum at 15 h. The yield was calculated to be ca. 84% using the emission intensity of the protein band and comparing it with that of biotin-labeled BSA. Optimization of the reaction condition was determined by the yields at 30 min as the initial stage, because the labeling yield showed a proportionate relationship with reaction time.

Figure 2A demonstrates the temperature-dependent labeling signatures, revealing that the labeling did not occur at 0 °C, proceeded slowly at room temperature and the reaction efficiency increased at temperatures above 37 °C. In addition, BSA could be labeled with biotin with up to two mole equivalents when excess amount of probe **1** (Fig. 2B) was given, although there were multiple fatty acid binding sites and 59 Lys residues within the protein (that were potential labeling sites). These results indicated that there was little non-specific labeling with probe **1** on functional groups such as amino groups of Lys residues on the protein surface, which should be protonated in neutral aqueous solutions. Furthermore, the emission intensity clearly decreased in the presence of the competitor myristic acid, in a concentration-dependent manner (Fig. 2C). Hence, longer incubations in the presence of an excess amount of myristic acid gradually increased the labeling yield, as shown in Figure 2D. These results also suggested site-specific labeling, as probe **1** competed with myristic acid in the binding pocket within BSA. Thus, thioester does not show enough activity to react with protonated amino groups, as is common when using active esters. However, labeling in/near the pocket can be accelerated due to the concentration effect of the reactant, namely, the near-field effect induced by their specific binding and deprotonation induced by the equilibrium shift in the hydrophobic conditions within the pocket, during ligand binding. The labeled BSA was then digested with trypsin and subjected to LC–MS analysis to identify the labeled site. The sequence coverage was ca. 35%. However, the labeled peptide could not be detected.

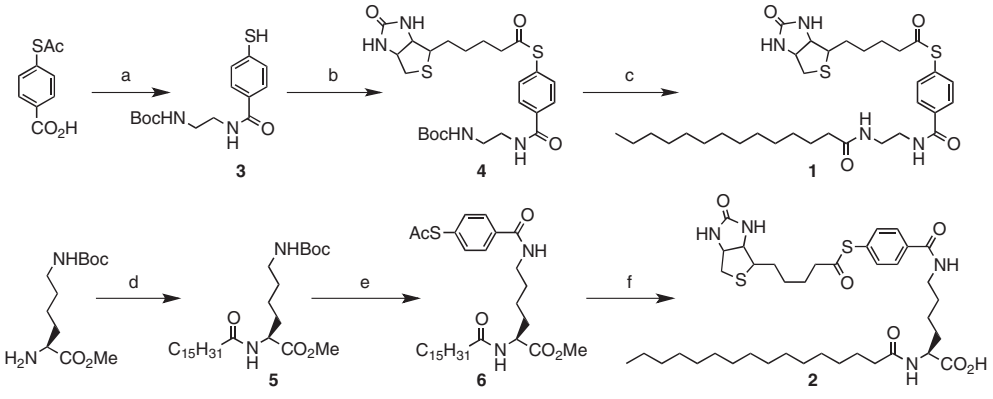

Figure 1. Synthesis of probes **1** and **2**. Reagents and conditions: (a) $BocNHC_2H_5NH_2$, EDCI, Et_3N, rt, then 1 M NaOH–MeOH (50 w/w%); (b) biotin–Cl, DMAP, rt; (c) 20% TFA–$CHCl_3$, then myristic acid, DIC, DIEA, rt; (d) palmitic acid, EDCI, Et_3N, rt; (e) 50% TFA–$CHCl_3$, then 4-(acetylthio)benzoic acid, PyBOP, rt; (f) 1 M NaOH–MeOH (50 w/w%), then NHS–biotin, rt.

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