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Labeling study of avidin by modular method for affinity labeling (MoAL)

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ABSTRACT

We studied the specific labeling of avidin with biotinylated modular ligand catalysts via MoAL, which we recently established. The labeling yield was found to depend on the linker length connecting the catalytic site to biotin in the modular ligand catalyst **1**, and the maximum yield was obtained with **1d** possessing octamethylene linker. The labeling reaction reached a maximum rate with only 4 equiv of the ligand catalyst. Presumably, all the subunits of avidin with homotetrameric structure formed a stable complex with 4 equiv of the catalyst because of the extremely high affinity. The ligand catalyst bound to avidin first catalyzed N-triazinylation of the ε-amino group of Lys111, and the resulting regenerated catalyst then catalyzed the reaction of Asp108 and CDMT.

The elucidation of protein function is one of the most challenging and important issues in the post-genomic era. Affinity labeling (AL), which is based on the inherent interaction between proteins and their ligands, is a powerful tool for identifying target proteins and analyzing their binding sites.[1–5] In general, AL is performed using an affinity probe that contains three essential elements in a single molecule: a ligand with specific affinity for the target biomolecule; a labeling tag, such as an isotope or a fluorescent moiety, to facilitate the isolation and identification of the labeled biomolecule; and a reacting group, such as a photophore, to form a covalent bond at the binding site.[2] The complicated multifunctional structure of affinity probes sometimes makes their design and synthesis difficult, and this may deter the wide application of AL for a variety of protein/ligand systems.

To solve these problems, several useful methods for facilitating the design and synthesis of affinity probes have been reported.[4] Recently, our laboratory has established a convenient modular method for affinity labeling (MoAL),[5] which is based on a catalytic amide-forming reaction proceeding in an aqueous solution by using a combination of *N,N*-dimethylglycine (DMG) esters as a catalyst and 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) as a reactive molecule.[6] The distinctive advantages of MoAL are as follows. (1) Since the three essential elements of affinity probes are completely separated into individual module molecules (a modular ligand catalyst, a labeling module, and CDMT as a reactive module), design and synthesis of the simplified modules are easy. In fact, modular ligand catalysts can be readily prepared from ligands via the introduction of a DMG group as a catalytic site, and a variety of commercially available amines can be employed as labeling modules. (2) Proteins can be labeled specifically by only mixing them with these modules. The carboxyl group of Asp or Glu residues near the ligand-binding site can be specifically activated by the ligand catalysts into acyloxytriazines, which then undergo coupling with the amino group of labeling modules to give amide (Fig. 1). Thus, the site specificity of labeling should depend on the types of ligand catalysts but not on that of labeling modules.

In our previous report on MoAL using the avidin–biotin system, we revealed that the labeling reaction proceeded at only Asp108 among three acidic amino acid residues (Asp105, Asp108, and Asp109) included in the helical region between Ser104 and Arg114, which is located in the vicinity of the biotin binding site. In this Letter, we report further studies on avidin labeling with a biotinylated modular ligand catalyst by MoAL.

First, we examined the effect of the linker length connecting biotin and the catalytic site (DMG) on the labeling yield. Modular ligand catalysts (**1a** and **1c–1e**) possessing linkers of various lengths were synthesized according to the previously reported method for synthesizing **1b** (Fig. 2). Avidin was labeled by simply mixing it with the ligand catalyst, CBA (Cascade Blue® ethylenediamine, trisodium salt) as a labeling module, and CDMT as a reactive module. As shown in Table 1, the highest labeling yield (53%) per monomeric subunit was observed with ligand catalyst **1d**. Since avidin is a homotetrameric glycoprotein and each of its subunits can equally bind to biotin,[7] the labeling yield per protein should be 210%.[8]

Interestingly, LC–ESI–MS/MS analysis indicated that the labeling reaction proceeded at Asp108 with every ligand catalyst in

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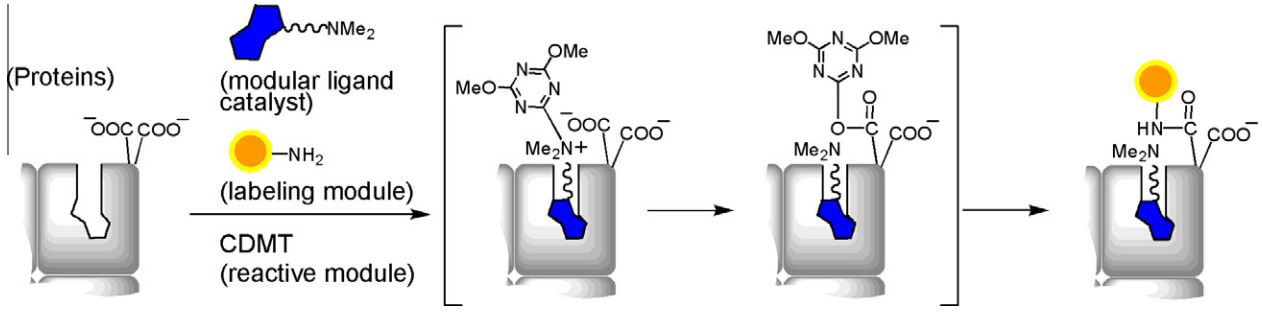


Figure 1. Specific labeling of proteins by MoAL.

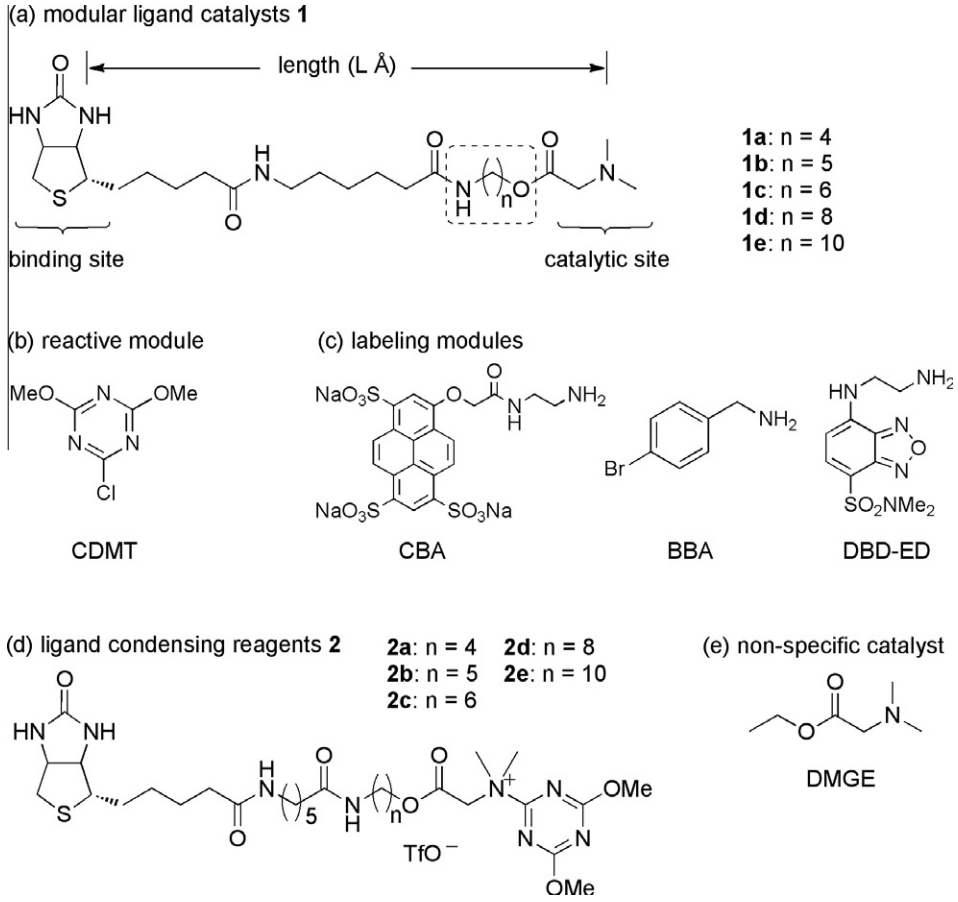


Figure 2. Chemical structure of module molecules.

Table 1
Effect of linker length on labeling yield of avidin

Entry	Ligand catalyst	L (Å)	Yield (%)
1	**1a**	25.8	25
2	**1b**	27.0	29
3	**1c**	28.2	36
4	**1d**	36.6	53
5	**1e**	33.0	52

The labeling reaction was performed using avidin (20 μM), **1** (80 μM), CBA (1.6 mM), and CDMT (1.6 mM) in phosphate buffer (pH 8.0) for 8 h at rt. The labeling yield was determined by UV–vis spectral analysis of labeled avidin,[5] and represents the yield per monomeric subunit.

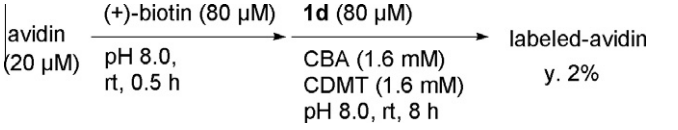
spite of the different linker lengths, and we did not observe labeling at Asp105 or Asp109. The labeling reaction for MS analyses was conducted using 4-bromobenzylamine (BBA) instead of CBA as a labeling module because BBA facilitated the MS analysis of the labeled amino acid.[5,9] We should note that although the X-ray structure of avidin (PBD ID: 1LEL) suggests that the length of linker in **1a** is shorter than the distance between the biotin binding site and Asp108, labeling at Asp108 is also observed with **1a**. As the ligand catalyst is tightly affixed to the biotin binding site of avidin because of the high affinity between biotin and avidin (vide infra), the activated triazino group bound to the catalytic site cannot reach Asp108. Thus, alternatively, the amino acid undergoing labeling would need to approach the triazino group at the binding site, indicating that the conformation of avidin would move during the labeling reaction. This may indicate that the linker length of a ligand catalyst used in MoAL need not be designed strictly.

Furthermore, we carried out MS analysis in detail to confirm the site specificity of the amino acid labeling of avidin by MoAL. The

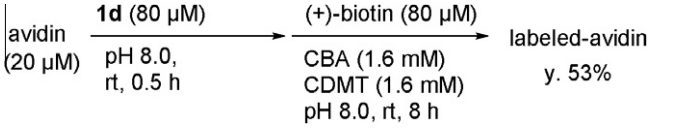
labeled avidin obtained from **1b** and 4-(*N*,*N*-dimethylaminosulfonyl)-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DBD-ED) as a labeling module as well as the above avidin proteins labeled using **1** and BBA were analyzed. We detected 10 acidic amino acids (both Asp and Glu, except for Asp13 and Glu128) among 12 acidic amino acids contained in the avidin monomeric subunit in the peptide fragments obtained by tryptic digestion. As a result, no signal indicating the introduction of DBD-ED or BBA to the 9 acidic amino acids (other than Asp108) was detected. Therefore, we can conclude that the specific labeling of avidin occurred selectively at Asp108 in MoAL.

The relationship between the labeling yield and the amount of ligand catalyst **1d** was linear and reached a plateau at 4 equiv of **1d**. We did not observe any significant increase in the yield up to 16 equiv of **1d** (Fig. 3). In contrast, the labeling yield increased in a linear fashion with the amount of *N*,*N*-dimethylglycine ethyl ester (DMGE), albeit in a low yield even though it exceeded 4 equiv. In general, in enzyme-like reactions involving the formation of an enzyme–substrate complex (ES complex), the reaction rate increases with increasing concentration of the complex. However, when all enzymes are converted into ES complexes by saturation at high concentrations of the substrate, the reaction rate reaches a maximum. From the observed results, we may conclude that all four subunits constituting the avidin molecule are converted into the complexes with only 4 equiv of ligand catalyst **1d** because of the large binding affinity between avidin and biotin ($K_a = 10^{15}\ M^{-1}$).[10] In other words, the biotinylated catalysts would bind to avidin in an irreversible fashion. The ligand catalyst added exceeding 4 equiv did not bind to avidin, and therefore, it could not contribute to labeling.[11]

In order to examine whether the binding of the ligand catalysts with avidin was irreversible during the labeling reaction, we employed two different procedures for the labeling reaction. First, avidin was treated with 4 equiv of biotin for 30 min at rt followed by the addition of the same amount of ligand catalyst **1d**, and the resulting mixture was subjected to the labeling reaction under the standard conditions (Eq. (1)). As a result, the labeling yield dropped to only 2%. On the other hand, when avidin was pre-treated with 4 equiv of **1d** for 30 min followed by addition of the same amount of biotin and then labeling reaction was carried out (Eq. (2)), the observed labeling yield was 53%, the same as that in Table 1, entry 4. These results clearly indicate that avidin interacts with **1d** as well as biotin in an irreversible fashion under the reaction conditions, and that the resulting avidin modified at Asp108 still retains the large binding affinity for **1d**. Because of the low concentration of employed avidin, the reaction of the ligand catalyst binding to avidin with CDMT becomes slow.

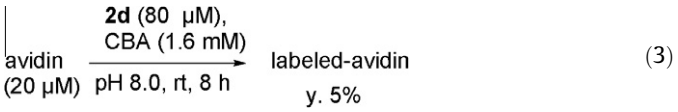


(1)

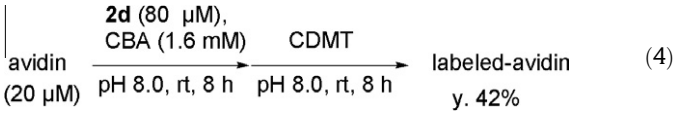


(2)

Thus, we expected that the labeling reaction would proceed rapidly in a high yield when the ligand catalyst prospectively incorporating a 1,3,5-triazino group (ligand condensing reagent **2d**), which can be prepared from **1d** and 2,4-dimethoxy-6-trifluoromethanesulfonyloxy-1,3,5-triazine,[12] is directly treated with avidin. However, the reaction of avidin with **2d** and CBA at rt for 8 h actually resulted in only a 5% labeling yield (Eq. (3)). The labeling yield improved to 42% by further treatment of the resulting mixture with CDMT for another 8 h (Eq. (4)). On the basis of above MS analysis, we concluded that the triazino group of **2d** bound to the biotin binding site preferably reacted with Lys111 over Asp108, and therefore, the labeling yield decreased. After transferring the triazino group to Lys111, the *N*,*N*-dimethylamino group of the regenerated **1d**, tightly bound to the avidin molecule, could then catalyze the conversion of the carboxylate of Asp108 into a triazino ester, and therefore, the labeling of avidin proceeded only in the presence of CDMT. The observed preference for the amine is in marked contrast to that using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM) and other ammoniotriazines, in which the triazino group reacts exclusively with carboxylic acids over amines.[13] Since Asp108 and Lys111 are known to form a salt bridge in the X-ray structure of avidin,[14] the observed unusual amine selectivity with **2d** in the present system may be attributed to the specific interaction between the two residues. In fact, it was reported that Lys111 readily underwent intermolecular arylation with 2,4-dinitro-1-fluorobenzene.[14b]



(3)



(4)

In the cyclotransferase model involving a tertiary amine catalyst coupled to 18-crown-6, which is based on a similar mechanism as that of MoAL, 96% of the substrate selectivity with 78% yield was realized on the basis of a binding constant of $\sim 10^4\ M^{-1}$ between 18-crown-6 and the primary ammonium in MeOH.[15] In marked contrast, in spite of the extremely high affinity between avidin and biotin, the observed labeling yield was not as high as we had expected. In the case of 18-crown-6 system, all the catalyst molecules can work repetitively for the reaction and contribute to increasing the yield because of the reversible formation of the complex between the catalyst and substrates. In the present study, on the other hand, the ligand catalysts **1** do not work very efficiently presumably due to both the irreversible binding of **1** to avidin and the preferential reactivity of Lys111 over Asp108. Since the ligand catalyst **1** (80 μM) should immediately bind to avi-

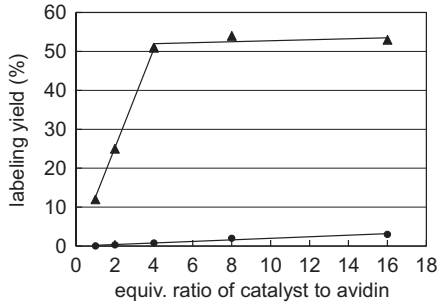


Figure 3. Correlation between the amount of *tert*-amine catalyst and labeling yield. The labeling reaction was performed using **1**, avidin (20 μM), CBA (1.6 mM), and CDMT (1.6 mM) in phosphate buffer (pH 8.0) for 8 h at rt, and the concentration of **1** was changed from 20 μM to 320 μM. The labeling yield was determined by UV–vis spectral analysis of labeled avidin,[5] and represents the yield per monomeric subunit. The labeling yield was plotted against the amount of the catalyst in equiv (▲: modular ligand catalyst **1d**; ●: DMGE).

din prior to reaction with CDMT to give **2**, the actual concentration of **1** could be as low as that of avidin itself (20 μM). Thus, the rate of generation of **2** from **1** at the biotin binding site of avidin should much decrease. In addition, this slow step is necessary to repeat at least twice because the intended labeling of Asp108 takes place only after the reaction of **2** with Lys111. Decomposition of the ligand catalyst by the hydrolysis or N-demethylation of **2**,[13] or the hydrolysis of the ester group of **1**[16] during the slow labeling reaction might be responsible for decreasing the labeling yields. Thus, the too strong binding ability of biotin with avidin does not allow replacement of **1** bound to avidin with free **2**, and therefore, would be paradoxically responsible for the suppression rather than the promotion of the labeling reaction. After all, the labeling yield increased with both the increasing concentration of CDMT (3.2 mM, y. 63%) or a prolonged reaction time (24 h, y. 77%).

In summary, we revealed the details of the specific labeling of avidin by MoAL. The labeling reaction occurred specifically at Asp108 at a significant level even if the linker lengths of the ligand catalysts differed. This may indicate that the linker length of the ligand catalyst does not need to be strictly designed. MoAL would be useful not only in searching for target proteins but also in the conformational analysis of the proteins.

Acknowledgment

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Supplementary data

Supplementary data (general methods, experimental and analytical data for the synthesized compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.109.

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