

Using Protein-Confined Proximity To Determine Chemical Reactivity

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S Supporting Information

ABSTRACT: Chemical reactivity is essential for functional modification of biomolecules with small molecules and the development of covalent drugs. The reactivity between a chemical functional group of a small molecule and that of a large biomolecule cannot be reliably predicted from the reactivity of the corresponding functional groups separately installed on two small molecules, because the proximity effect on reactivity resulting from the binding of the small molecule to the biomolecule is challenging to achieve by mixing two small molecules. Here we present a new strategy to determine the chemical reactivity of two functional groups in the context of close proximity afforded by proteins. The functional groups to be tested were separately installed at the interface of two interacting proteins in the format of amino acid side chains via the expansion of the genetic code. Reaction of the two functional groups resulted in covalent cross-linking of interacting proteins, readily detectable by gel electrophoresis. Using this strategy, we evolved new synthetases to genetically encode N^ϵ -fluoroacetyllysine (FAcK), an isosteric fluorine analogue of acetyllysine. We demonstrated that fluoroacetamide installed on FAcK, previously thought inert to biological functional groups, actually reacted with the thiol group of cysteine when in proximity. This strategy should be valuable for accurately evaluating chemical reactivity of small molecules toward large biomolecules, which will help avoid undesired side reactions of drugs and expand the repertoire of functional groups to covalently target biomolecules.

Chemical reactivities between small molecules and large biomolecules are invaluable for both basic biological studies and therapeutic development. Covalent reaction of small molecules toward biomolecules has been harnessed for functional modification of biomolecules such as labeling with biophysical probes,¹ activity-based protein profiling,² generation of antibody–drug conjugates,³ and development of covalent drugs.⁴ Compared with noncovalent drugs, resurgent covalent drugs show increased efficiency, lower risk of drug resistance, decreased sensitivity to pharmacokinetic parameters, and prolonged duration of inhibition.⁵ A major barrier to covalent drug development is the low specificity, which can lead to side effects such as hepatotoxicity, mutagenicity, and potential immunogenicity of the resulting nonspecific adduct. To achieve high specificity in covalent targeting, an attractive strategy is to down-tune the chemical reactivity of the small molecule as much

as possible to avoid undesired reactions, and then to regain its reactivity with the target through the proximity effect of binding. However, before a small molecule that is able to bind specifically to the target is designed and synthesized, it is challenging to predict the chemical reactivity of the functional warhead under proximity conditions. Proximity can dramatically increase the effective concentration of reactants, with effective molarity reaching as high as 10^5 M, which may not be replicated with small molecules in such high concentrations due to solubility issues.⁶ In addition, the beneficial entropy reduction and orientation effects afforded by proximity cannot be mimicked by simply mixing small molecules either.

Recent studies have shown that many electrophilic groups are more selective toward relevant targets than initially thought.⁷ Among them, α -haloacetamide is a well-known functional group used for covalent inhibition or labeling of thiol function in proteins. As iodo- and bromo-acetamide groups are highly reactive, they rapidly and nonspecifically form a covalent bond with all accessible free cysteines within proteome.⁸ A chloroacetamide group, a milder derivative, has been attracting much attention in the field of medicinal chemistry since it can site-specifically modify a cysteine residue in the desired site of enzymes or receptors to show the strong pharmacological activity.⁹ However, a fluoroacetamide group has been considered as a biologically inert functional group because of the poor leaving ability of the fluorine atom, as shown by the fact that many radioactive biomolecules with [^{18}F]-fluoroacetamide have been developed for diagnostic applications.¹⁰ To date, there is no report on fluoroacetamide reacting with the sulfhydryl group of cysteine.

Here we report a new strategy to evaluate the chemical reactivity of functional groups by placing them in proximity in the framework of two interacting proteins (Scheme 1). The warhead functional group to be tested was installed into one protein at the protein–interaction interface in the format of an unnatural amino acid (Uaa), whereas the other functional group is introduced at appropriate position into the partner protein. Incubation of these two interacting proteins will bring the functional groups into proximity, reaction of which will result in a covalent protein complex for ready detection by gel electrophoresis. Using this strategy we demonstrated that the “biologically inert” fluoroacetamide actually reacted with cysteine side chain selectively.

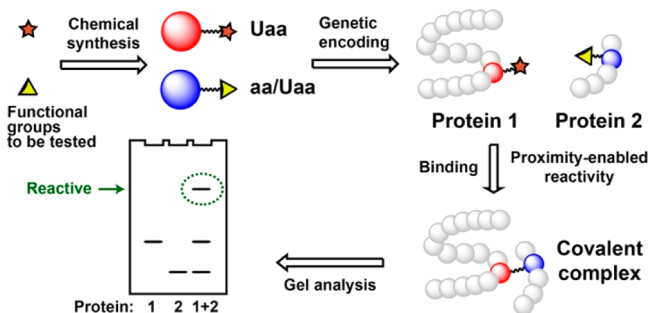
We designed N^ϵ -fluoroacetyllysine (FAcK, Scheme 2) as the Uaa bearing the fluoroacetamide group. Starting from a commercially available N^α -*tert*-butyloxycarbonyl (Boc)-L-lysine

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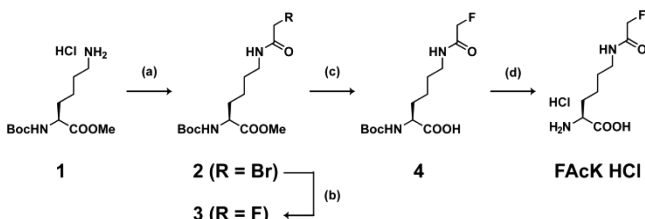
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Scheme 1. Determination of Chemical Reactivity of Two Functional Groups in the Context of Close Proximity Afforded by Proteins



Scheme 2. FAcK Synthesis



^aBromoacetyl chloride, sodium bicarbonate, ethyl acetate, water, 0 °C, 1 h. ^b1 M tetrabutylammonium fluoride, tetrahydrofuran, 80 °C, 4 h. ^c2 M sodium hydroxide aqueous solution, tetrahydrofuran, methanol, rt, 0.5 h. ^d4 M hydrogen chloride, 1,4-dioxane, rt, 0.5 h. 53% yield (4 steps).

methyl ester HCl salt (1), FAcK was synthesized by 4 steps, i.e., acylation with bromoacetyl chloride (2), conversion from bromine to fluorine (3), ester hydrolysis (4) and deprotection of the Boc group as HCl salt (Scheme 2). Pure FAcK HCl was obtained in 53% yield at multigram scale by simple synthetic procedures without silica gel column chromatography.

To incorporate genetically FAcK into proteins, we evolved the *Methanosarcina mazei* (Mm) tRNA^{Pyl}_{CUA}/PylRS pair to be specific for this Uaa. A mutant library of PylRS was generated with residues A302, L305, Y306, L309 saturatedly mutated,¹¹ L301M, C348F, Y384Y/W point-mutated, and then subjected to selections as described.¹² Two mutants, MmFAcKRS1 and MmFAcKRS2, were identified from the selection (Figure 1a, selection plates are shown in Figure S1). Together with the tRNA^{Pyl}_{CUA}, the mutant synthetases were able to incorporate FAcK into green fluorescent protein (GFP) at the TAG site with high specificity, as shown by Western blot analysis of GFP protein expressed in the presence and absence of FAcK in the growth media (Figure 1b). MmFAcKRS1 produced more proteins than MmFAcKRS2. The identity of FAcK at the TAG site of GFP was verified by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) of the GFP proteins (Figure 1c for MmFAcKRS1 and Figure S2 for MmFAcKRS2). A peak with average mass of 27957.18 was observed, corresponding to intact GFP with FAcK at position 182 (expected [M]⁺ = 27957.74); another peak at 27826.28 corresponds to the GFP182FAcK lacking the initiation Met (expected [M - Met]⁺ = 27826.54). No peaks corresponding to misincorporation of natural amino acids at 182TAG position was identified.

To evaluate the potential reactivity between fluoroacetamide and thiol group, we began by incorporating FAcK into the Z_{spa} Affibody (Afb) protein.¹³ The Afb gene encoding an amber TAG

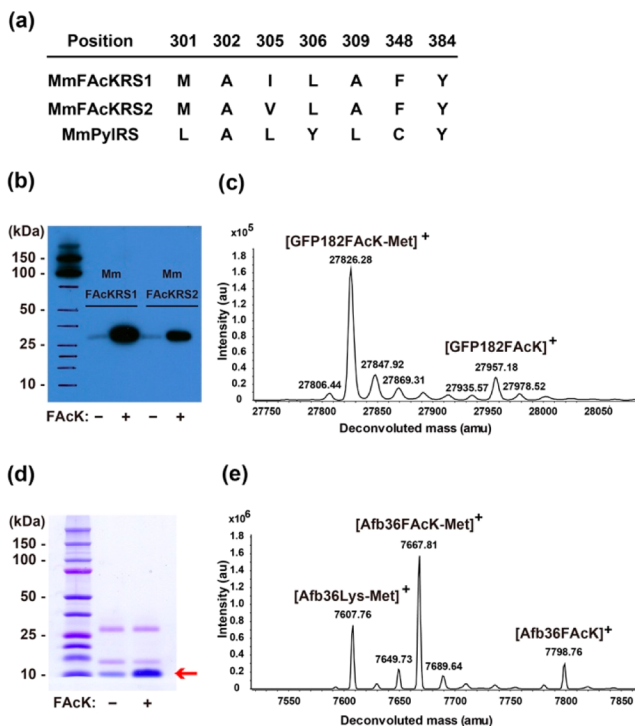


Figure 1. Genetically encode FAcK into proteins. (a) Amino acid sequence of MmFAcKRS1 and MmFAcKRS2 along with that of MmPylRS. (b) Western blot analysis of GFP182FAcK expressed in the presence of tRNA^{Pyl}_{CUA}/MmFAcKRS1 or MmFAcKRS2 supplemented with or without 1 mM FAcK. Samples were normalized for constant cell numbers for each lane. (c) ESI-TOF MS analysis of intact GFP182FAcK expressed in the presence of tRNA^{Pyl}_{CUA}/MmFAcKRS1 supplemented with 1 mM FAcK. (d) 10% Tricine-SDS PAGE analysis of Afb36FAcK expressed in the presence of tRNA^{Pyl}_{CUA}/MmFAcKRS1 supplemented with or without 1 mM FAcK. Samples were normalized for constant cell numbers for each lane. (e) ESI-TOF MS analysis of intact Afb36FAcK expressed in the presence of tRNA^{Pyl}_{CUA}/MmFAcKRS1 supplemented with 1 mM FAcK.

codon at position 36 and a C-terminal Hisx6 tag together with the tRNA^{Pyl}_{CUA}/MmFAcKRS1 were expressed in *Escherichia coli* (*E. coli*) BL21 cells. From Tricine-SDS-PAGE analysis of the expressed products, the production of full-length Afb was markedly increased (3.3-fold) when 1 mM FAcK was included in the growth media (Figure 1d). The purified Afb was obtained in the yield of 0.19 mg/L of culture, and was analyzed by ESI-TOF MS. An observed peak with average mass of 7798.76 Da (Figure 1e) corresponds to the intact Afb with the FAcK residue at position 36 (Afb36FAcK, expected 7798.82 Da). A second observed peak corresponds to the FAcK-containing Afb lacking the initiating Met (Afb36FAcK-Met: expected, 7667.62 Da; measured, 7667.81 Da). Interestingly, there was another peak that corresponds to the lysine-containing Afb lacking the initiating Met (Afb36Lys-Met: expected, 7607.60 Da; measured, 7607.76 Da). Because we did not observe Lys misincorporation into GFP by the same tRNA^{Pyl}_{CUA}/MmFAcKRS1 pair, we postulate that Lys at the 36 site of Afb could result from the hydrolysis of FAcK by deacetylases inside *E. coli* cells.

We next tested whether FAcK incorporated into Afb could react with a proximal Cys incorporated into the Z protein. We decided to use this Z_{spa} Afb and its substrate protein Z as the protein framework to create proximity, which bind with a K_d of 6 μ M. On the basis of the structure of the Afb-Z protein complex,¹³ we introduced Cys at residue 6 of the Z protein, placing it in

proximity to the FAcK36 of Afb (Figure 2a). As Afb has similar molecular weight with the Z protein, we fused the Z protein to the maltose binding protein (MBP-Z) for clear separation by molecular weight. The purified Afb36FAcK was incubated with the purified MBP-Z6Cys in a 10:1 ratio in phosphate buffered saline (pH 7.4) at 37 °C for 24 h, and the reaction mixture was subjected to Tricine-SDS-PAGE under denaturing conditions. We observed a band with a molecular weight corresponding to the MBP-Z in complex with the Afb, thus indicating that the two proteins were covalently cross-linked (Figure 2b, Lane 3). When the MBP-Z protein was pretreated with *N*-ethylmaleimide (NEM), a thiol blocking reagent, the complex band was completely gone (Lane 4). In addition, this complex band did not form when 36FAcK was replaced by the nonfluorinated isosteric acetyllysine (AcK) in the Afb (Figure 2c). Moreover, when we placed the Cys at position 11 of the Z protein instead of position 6, no covalent Afb/MBP-Z complex was formed (Figure 2d). Taken together, these results indicate that FAcK was able to react with Cys but only when these two residues were in close proximity.

We characterized the reactivity of FAcK with Cys by varying concentration and time. As shown in Figure 2e, increasing the concentration of FAcK-containing Afb resulted in more covalent complex formation. Figure 2f shows that the reaction occurred under mild conditions (mimicking physiological conditions) in 1 h, and the reaction extent increased with time. The reaction did not reach 100% possibly because some FAcK was hydrolyzed into the nonreactive Lys, as revealed by mass spectrometric analysis of Afb36FAcK. These results on concentration and time dependence further support that FAcK reacted with Cys in proximity under mild conditions.

We then measured the cross-linking efficiency of FAcK with Cys at various sites and pH. At pH 7.4, Afb36FAcK cross-linked with MBP-Z6Cys in the yield of 33% (Figure 2b). As Cys reactivity is pH dependent, we incubated Afb36FAcK with MBP-Z6Cys in weakly basic condition (tris-buffered saline, pH 8.8), and observed that the cross-linking efficiency was indeed increased to 88% (Figure 2g). To show the general utility of FAcK cross-linking with Cys, we tried other sites for intermolecular cross-linking in the Afb/MBP-Z system. We first used the same sites but swapped FAcK with Cys generating Afb36Cys and MBP-Z6FAcK. In this case, we measured 24% cross-linking yield at pH 7.4 and 64% at pH 8.8 (Figure S3), showing that FAcK also reacted with Cys after switching positions. We next placed FAcK and Cys at the other side of the Afb-Z complex, generating Afb7Cys and MBP-Z24FAcK (Figure S4). Incubation of these two mutant proteins also resulted in 12% cross-linking at pH 7.4 and 34% at pH 8.8. The WT Afb binds with the Z protein with a K_d of 6 μ M; most drugs and ligands would interact with their targets in a comparable or higher affinity. The considerable cross-linking yields we measured for the Afb-Z system at various proximal sites suggest that FAcK would be useful to cross-link Cys in other systems.

To test further whether FAcK could react with a proximal Cys within the same protein, we incorporated FAcK into calmodulin (CaM) at site 76 and placed a Cys at site 80 on the central linker region of CaM (Figure 3a). After expressing in *E. coli* the tRNA^{Pyl}_{CUA}/MmAcKRS1 pair with the CaM mutant gene (76TAG/80Cys and a C terminal Hisx6 tag),¹⁴ full-length CaM was produced in the presence of FAcK (Figure 3b). Analysis of the Ni-NTA purified CaM using ESI-TOF MS confirmed the incorporation of FAcK (CaM76FAcK: expected, 18 008.00 Da; measured, 18 008.02 Da) and, more importantly,

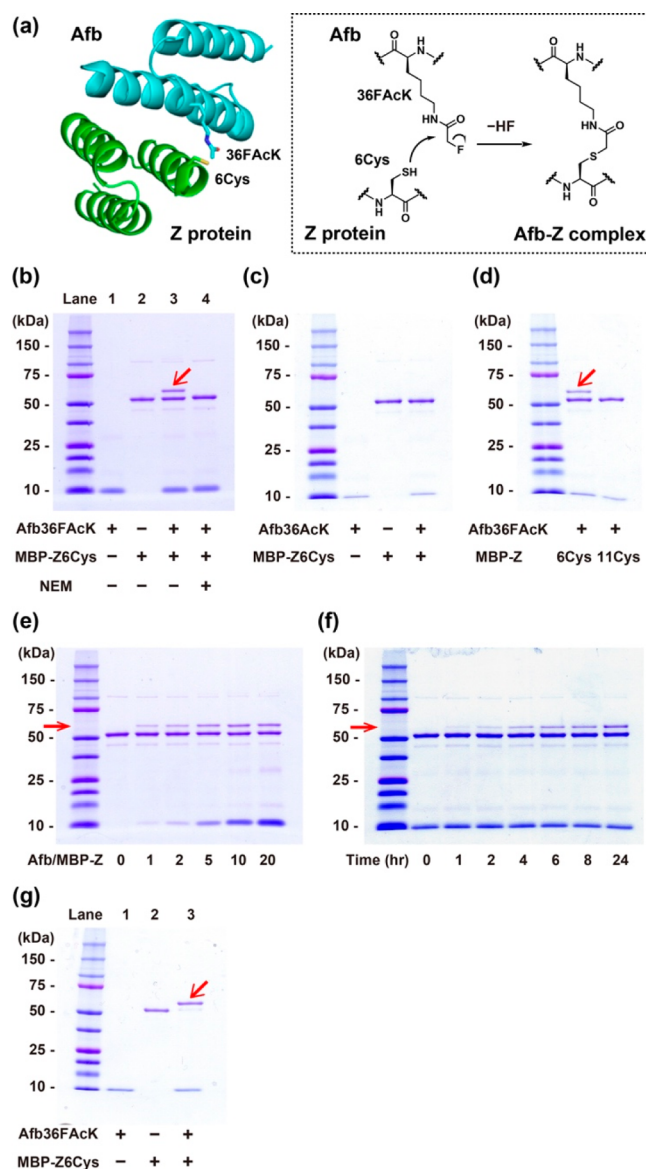


Figure 2. Fluoroacetamide of FAcK reacted with thiol of Cys in proximity created by the Afb-Z protein complex. (a) Structure of the Afb-Z protein complex (PDB ID 1LP1), with 36FAcK in the Afb and 6Cys in the Z protein highlighted. (b) Formation of the Afb/MBP-Z covalent complex after incubation of the Afb36FAcK with the MBP-Z6Cys, indicated by the red arrow in a 10% Tricine-SDS-PAGE gel with Coomassie Brilliant Blue staining (Lane 3). NEM: *N*-ethylmaleimide. + indicates the protein or the reagent added, - not added. (c) Cross-linking reaction of the Afb36FAcK with the MBP-Z6Cys (molar ratio 10/1) in PBS solution was incubated at 37 °C for 24 h. (d) Cross-linking reaction of the Afb36FAcK with the MBP-Z6Cys or the MBP-Z11Cys. Afb36FAcK/MBP-Z (molar ratio 10/1) in PBS solution was incubated at 37 °C for 24 h. (e) Concentration dependency for cross-linking reaction of the Afb36FAcK with the MBP-Z6Cys. Molar ratio of Afb36FAcK/MBP-Z6Cys was changed from 0/1 to 20/1 in PBS solution and incubated at 37 °C for 21 h. (f) Incubation time dependency for cross-linking reaction of the Afb36FAcK with the MBP-Z6Cys. Afb36FAcK/MBP-Z6Cys (molar ratio 10/1) in PBS solution was incubated at 37 °C for the specified amount of time. (g) Cross-linking of the Afb36FAcK with the MBP-Z6Cys (molar ratio 10/1) in 50 mM tris(hydroxymethyl)aminomethane buffered saline (pH 8.8) in the presence of 10 mM TCEP (to prevent thiol oxidation) for 24 h at 37 °C.

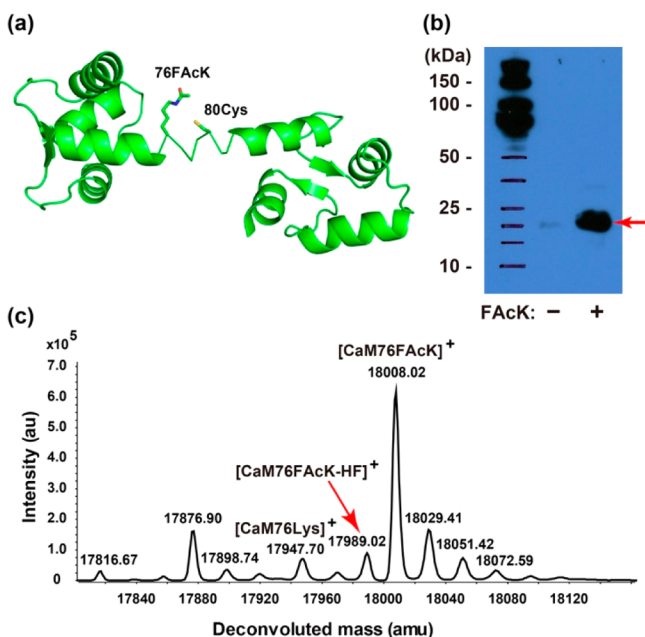


Figure 3. Fluoroacetamide of FAcK reacted with thiol of Cys in proximity within protein CaM. (a) Structure of the CaM (PDB ID 3cln) with 76 FAcK and 80Cys highlighted. (b) Western blot analysis of CaM76FAcK expressed in the presence of tRNA^{Pyl}_{CUA}/MmFAcKRS1 supplemented with or without 1 mM FAcK. Samples were normalized for constant cell numbers for each lane. An antibody specific for the His6 tag was used for detection. (c) ESI-TOF MS analysis of intact CaM76FAcK80Cys expressed in the presence of tRNA^{Pyl}_{CUA}/MmAcKRS1 supplemented with 1 mM FAcK. The red arrow indicates the peak of the CaM in which 76FAcK reacted with 80 Cys.

the formation of a protein bridge through FAcK reacting with 80Cys (Figure 3c). The mass peak detected at 17 989.02 Da corresponds to CaM bearing the covalent bridge (expected 17 987.99 Da). Peaks were also observed corresponding to lysine at the 76TAG site (expected, 17 947.96 Da; measured, 17 947.70 Da) as seen in the expression of the Afb protein. The yield of intramolecular cross-linking was estimated as about 14% based on the mass peak intensity. The reaction did not go to completion due to two possible reasons: (1) Hydrolysis of FAcK to the nonreactive Lys by cellular deacetylases, and (2) The central region of CaM where FAcK and Cys reside is a flexible loop, which may not provide optimal orientation for the side chains to react. Nonetheless, these results clearly indicate that FAcK reacted with a proximal Cys within the same protein as well.

In summary, we genetically encoded FAcK, an isosteric fluorine analog of acetyllysine. Although there are many reports about reactive iodo-, bromo-, and chloroacetamide-containing small molecules or peptides designed for covalent labeling or inhibition of thiols, the fluoroacetamide group has been considered as a biologically inert group. Our results showed that when in close proximity, a condition often encountered when small molecules bind with large biomolecules, fluoroacetamide reacted with the Cys thiol group readily under mild physiological conditions. This mild reactive functional group can be useful as a warhead in small molecules, peptides, and proteins for specific labeling or inhibiting thiol function in proteins, promoting the development of novel covalent drugs that are more specific to relevant targets. Our protein-confined proximity strategy will enable researchers to evaluate chemical reactivity of

functional groups under proximity conditions, which can be different from that observed in the conventional mixture of small molecules. As proximity effect can unexpectedly occur to small molecules *in vivo*, knowledge of proximity reactivity will be invaluable for assessing potential side effects of drugs previously deemed impossible and for building a repertoire of warhead functional groups that are highly specific with minimal undesired side reactions.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b08656.

Experimental details (PDF)

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Notes

The authors declare no competing financial interest.

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