Supporting Information

Using Protein-Confined Proximity to Determine Chemical Reactivity

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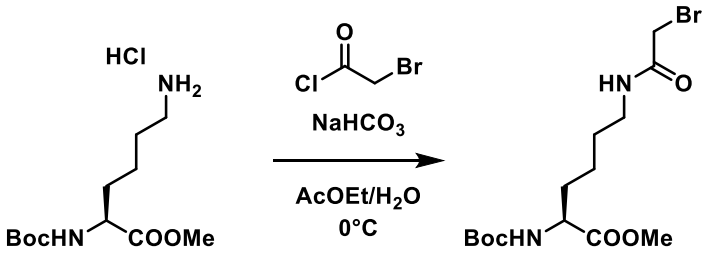
General Remarks

Chemicals were purchased from Sigma-Aldrich, Chem-Impex International, Fisher Scientific or ACROS and used without further purification. Solvents used were of reagent grade and without further purification. Reactions were monitored on Merck Silica 60 F254 TLC plates. Detection was done by staining with ninhydrin solution in butanol. NMR spectra were recorded on the Bruker AVANCE 300. The chemical shifts (δ) are given in ppm, the coupling constants (*J*) in Hz. The solutions are referred to residual undeuterated solvents as internal standard. LC-ESI MS analysis for FAcK was carried out using an Agilent 1290 LC system coupled to an Agilent 6530 QTOF with an Amide-80 4.6 x 150 mm column (TOSOH). Mobile phase consisted of H_2O with 7.5 mM ammonium acetate and 0.1% formic acid (A) and 5% aqueous acetonitrile with 0.1% formic acid (B). The chromatography was conducted with a linear gradient from 10% to 90% B for 12 min, then maintained at 90% B for 2 min and finally linear gradient from 90% to 10% for 2 min.

Primers were synthesized by Integrated DNA Technologies Inc. (U.S.A.). PCR was carried out with S1000™ Thermal Cycler (BioRad). Restriction enzymes, DNA polymerase, DNA ligase and reaction buffers were purchased from New England Biolabs. For DNA purification, TopVision Agarose Tablets (Thermo SCIENTIFIC), SYBR®Safe DNA gel stain (Invitrogen) and Nucleospin®Gel and PCR Clean-up (MACHEREY-NAGEL) were used. Electroporation was carried out by 2500V, 5 msec pulse in a 2 mm-gap cuvette with Eporator (Eppendorf). For plasmid extraction from bacteria, QIAprep®Spin Miniprep Kit (QIAGEN) was used. DNA sequencing analysis was carried out by Elim Biopharmaceuticals Inc. (Hayward, CA U.S.A.).

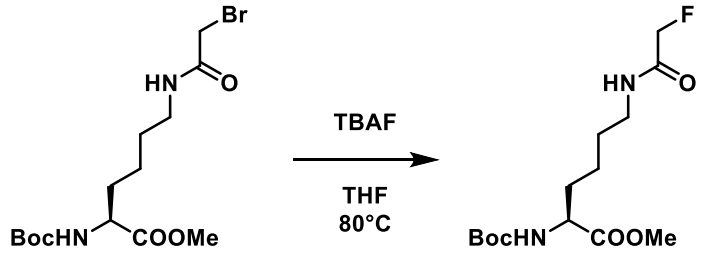
Plasmids (pTAK plasmid and pBK plasmid)[1] were prepared by standard overlap extension PCR procedures. 10% Tricine-SDS-PAGE was carried out according to the literature[2]. Gels were stained with standard procedure[3] and scanned with a scanner (HP Scanjet G4050) as a PDF file followed by processing with appropriate software. For western blot, SuperSignal West Pico Chemiluminescent Substrate® (Thermo SCIENTIFIC), 6x-His Epitope Tag Antibody HRP conjugate (Thermo SCIENTIFIC) and HyBlot ES™ (Denville Scientific, Inc.) were used. Protein concentration was measured by NanoDrop1000 (Thermo SCIENTIFIC). Intact protein MS analysis was carried out by Jadebio Inc. (La Jolla, CA U.S.A.). Protein cartoons in Figure 2 and 3 were drawn with PYMOL software (Schrödinger).

Chemical Synthesis of FAcK



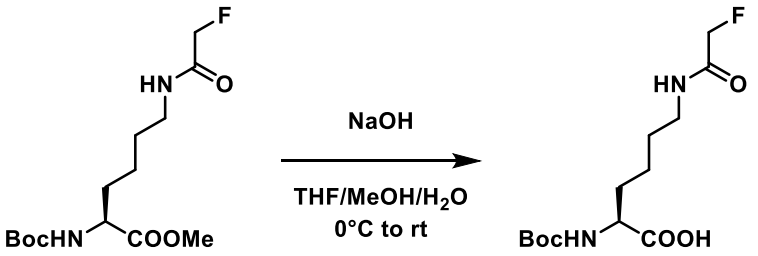
1 **2**

To a cold (0°C) biphasic solution of N^{α}-*tert*-butyloxycarbonyl (Boc)-L-lysine methyl ester hydrochloride (**1**, 5.00 g, 16.8 mmol), ethyl acetate (165 mL) and saturated aqueous sodium bicarbonate (50 mL) in a 500 mL-round bottomed flask was added bromoacetyl chloride (1.54 mL, 18.5 mmol) via a glass pipet with stirring. The mixture was stirred vigorously at 0°C for 1 h then layers were separated. The aqueous layer was added additional water (100 mL) and extracted with ethyl acetate (50 mL) twice then combined organics were washed with 0.5 M hydrochloric acid aqueous solution (100 mL) twice and dried with anhydrous sodium sulfate, filtered, and concentrated to dryness to afford the desired product (**2**) as a light brown oil (5.50 g) that was used in the next step.



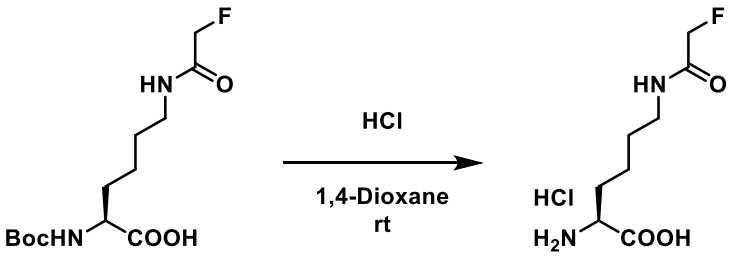
2 **3**

To N^{α}-Boc-L-bromoacetyl lysine methyl ester (**2**, 5.50 g, 14.4 mmol) in a 100 mL round-bottomed flask, 1M tetrabutylammonium fluoride in tetrahydrofuran solution (29 mL, 29.0 mmol) was added via syringe. The mixture was heated at 80°C for 4 h then cooled to room temperature. It was diluted with ethyl acetate (200 mL) then washed with water (100 mL) twice. The organic layer was washed with saturated sodium chloride brine (20 mL), dried with anhydrous sodium sulfate, filtered, and concentrated to dryness to afford the desired product (**3**) as a light brown oil (3.99 g) that was used in the next step.



3 **4**

To a cold solution (0°C) of N^{α}-Boc- L-fluoroacetyl lysine methyl ester (**3**, 3.99 g, 12.5 mmol), tetrahydrofuran (50 mL) and methanol (25 mL) in a 500 mL round-bottomed flask, 2M sodium hydroxide aqueous solution (25 mL, 50.0 mmol) was added via a glass pipet with stirring. The mixture was stirred at room temperature for 30 min and the resultant suspension was added water (200 mL). The aqueous layer was washed with diethyl ether (100 mL) then acidified with 2M hydrochloric acid aqueous solution (40 mL) with cooling on ice. It was extracted with ethyl acetate (100 mL) 3 times and the organic layer was washed with saturated sodium chloride brine (20 mL). The organic layer was dried with anhydrous sodium sulfate, filtered, and concentrated to dryness. The residue was added ethyl acetate (10 mL) then insoluble solid was filtered. The filtered solution was evaporated to dryness to afford the desired product (**4**) as an off-white solid (3.18 g) that was used in the next step.



4 **FAcK HCl**

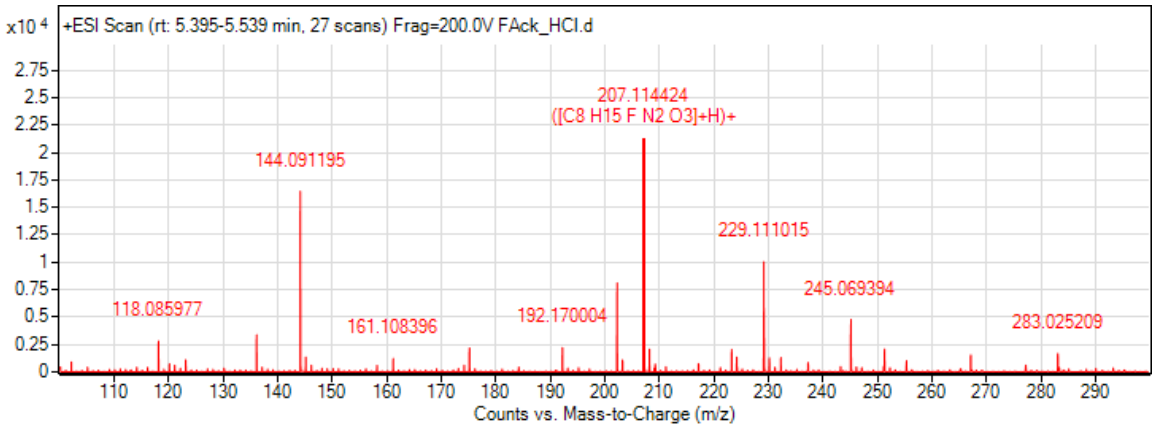
To N^{α}-Boc-L-fluoroacetyl lysine (**4**, 3.18 g, 10.4 mmol), 4M hydrogen chloride 1,4-dioxane solution (26 mL, 104 mmol) was added via syringe. The mixture was stirred at room temperature for 30 min then the supernatant was decanted. The residue was washed with tetrahydrofuran (20 mL) 3 times then sonicated in tetrahydrofuran (50 mL). The resultant was filtered and dried to afford the desired product as a white solid (**FAcK HCl**, 2.18 g, 53% in 4 steps).

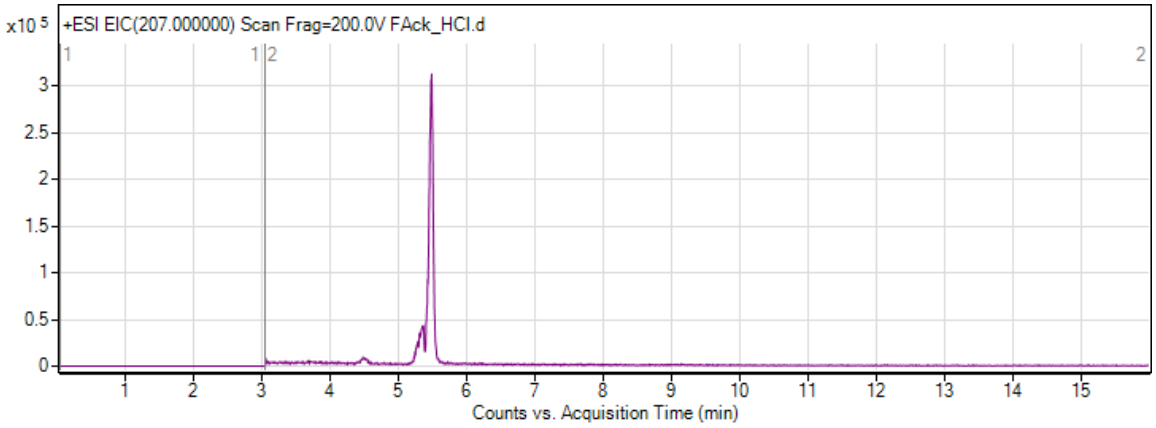
Chemical Properties of FAcK

^{1}H-NMR (DMSO-d_6) δ 13.69 (br, 1H, COOH^{Lys}), 8.67-8.37 (m, 3H, N$H_3^{+\,Lys}$), 8.32-8.20 (m, 1H, NHCO), 4.77 (d, J = 45 Hz, 2H, CH_2F), 3.88-3.70 (m, 1H, CH^{Lys}), 3.17-2.99 (m, 2H, CH_2^{Lys}), 1.88-1.68 (m, 2H, CH_2^{Lys}), 1.54-1.14 (m, 4H, 2 x CH_2^{Lys}) ppm.

^{13}C-NMR (DMSO-d_6) δ 171.0 (COOH^{Lys}), 167.0 (J = 18 Hz, NHCO), 80.1 (J = 179 Hz, CH$_2$F), 51.8 (CH^{Lys}), 37.7 (CH$_2^{Lys}$), 29.6 (CH$_2^{Lys}$), 28.5 (CH$_2^{Lys}$), 21.7 (CH$_2^{Lys}$) ppm.

HRMS (ESI$^+$): m/z calcd for [M+H]$^+$, 207.11450; measured 207.11442 (-0.08 mmu), Rt = 5.46 min





Synthetase Library Construction

For mutant library construction, mutations L301M, C348F, Y384Y/W were introduced into *M. mazei* PylRS through site-directed mutagenesis, and the resultant mutant PylRS were used as template to randomly mutate residues 302, 305, 306 and 309 by using the following oligonucleotide primers (randomized codons are underlined).

A_Fw: 5'-CGATGAGGACCTGAACAAATTCCTGAC-3'
B_Rev: 5'-CACGGTCCAGTTTACGMNNATAGTTMNNMNNGTTCGGMNNCATCATCGGACGCAGAC-3'
C_Fw: 5'-CGTAAACTGGACCGTG -3'
D_Rev: 5'-GAAACTGCAGCCACCCGCCTGGTTACAGGTTAGTAGAAATACCATTGTAATAGG-3'
D_Rev2: 5'-GAAACTGCAGCCACCCGCCTGGTTAC-3'

General PCR protocol

All materials below are mixed together in a PCR tube and used for a PCR reaction. PCR reactions were performed in 50 μL x 2 tubes under following condition: 98˚C for 30 sec, 35 x (98˚C for 10 sec, 50˚C for 30 sec, and 72˚C for 30 sec), 72˚C for 10 min.

1)	H_2O	32.5	μL	
2)	Template plasmid (1 ng/μL)	1	μL	
3)	Primer_Fw (10 μM)	2.5	μL	
4)	Primer_Rev (10 μM)	2.5	μL	
5)	dNTP stock solution (10 mM)	1	μL	
6)	5 x Phusion® HF buffer	10	μL	
7)	Phusion® DNA polymerase (2U/μL)	0.5	μL	Total 50 μL

General overlap extension PCR protocol

All materials below except for primers are mixed together in a PCR tube and used for a PCR reaction. PCR reactions were performed in 50 μL x 2 tubes under following condition: 98˚C for 30 sec, 35 x (98˚C for 10 sec, 50˚C for 30 sec, and 72˚C for 30 sec), 72˚C for 10 min. Primer_Fw and Primer_Rev were added at the end of 10th round of (98˚C for 10 sec, 50˚C for 30 sec, and 72˚C for

30 sec).

1)	H_2O	31.5	μL	
2)	Piece 1	1	μL	
3)	Piece 2	1	μL	
4)	dNTP stock solution (10 mM)	1	μL	
5)	5 x Phusion® HF buffer	0.5	μL	
6)	Phusion® DNA polymerase (2U/μL)	10	μL	
7)	Primer_Fw (10 μM)	2.5	μL	
8)	Primer_Rev (10 μM)	2.5	μL	Total 50 μL

The PCR amplified DNA fragment for mutant PylRS was double-digested with restriction enzymes (EcoO109I and BstXI), purified and ligated between EcoO109I and BstXI sites of pBK_MmNapRS[4] at molecular ratio of insert/vector (3/1) according to manufacturer's protocol. The ligated plasmid library (420 ng) was introduced into *E. coli* DH10β strain (100 μL) by electroporation to obtain the desired mutant library (pBK_TK2).

Synthetase Screening

The pREP-harboring DH10β cells [5] were transformed with the pBK_TK2 mutant library by electroporation, and the transformants were recovered in SOC media at 37°C for 1 hr. The recovered solution was diluted into LB-T12.5K25 (i.e. containing tetracycline hydrochloride 12.5 μg/mL and kanamycin sulfate 25 μg/mL) and plated onto a LB-T12.5K25Cm50 (i.e. containing tetracycline hydrochloride 12.5 μg/mL, kanamycin sulfate 25 μg/mL and chloramphenicol 50 μg/mL) agar plate (Φ150 mm) supplemented with FAcK 1 mM for positive selection. 80 green colonies were obtained and 72 out of 80 were re-streaked onto 3 different LB-agar plates containing 1) T12.5K25 2) T12.5K25Cm100 3) T12.5 K25Cm100 supplemented with 1 mM FAcK. After incubation for 48 hrs at 37°C, 2 clones showed FAcK-dependent survival and fluorescence (Figure S1). They were picked from the plate 3 and grown in LB- T12.5K25. The plasmids were extracted by miniprep and the pBK plasmid was separated from the pREP plasmid by 1% agarose gel electrophoresis. The purified pBK plasmid was analyzed for DNA sequencing and used for further protein expression experiments.

Gene sequence of MmFAcKRS1

ATGGATAAAAAGCCTTTGAACACTCTGATTTCTGCGACCGGTCTGTGGATGTCCCGCACCGGCACCATCC
ACAAAATCAAACACCATGAAGTTAGCCGTTCCAAAATCTACATTGAAATGGCTTGCGGCGATCACCTGGT
TGTCAACAACTCCCGTTCTTCTCGTACCGCTCGCGCACTGCGCCACCACAAATATCGCAAAACCTGCAAA
CGTTGCCGTGTTAGCGATGAGGACCTGAACAAATTCCTGACCAAAGCTAACGAGGATCAGACCTCCGTAA
AAGTGAAGGTAGTAAGCGCTCCGACCCGTACTAAAAAGGCTATGCCAAAAAGCGTGGCCCGTGCCCCGAA
ACCTCTGGAAAACACCGAGGCGGCTCAGGCTCAACCATCCGGTTCTAAATTTTCTCCGGCGATCCCAGTG
TCCACCCAAGAATCTGTATCCGTACCAGCAAGCGTGTCTACCAGCATTAGCAGCATTTCTACCGGTGCTA
CCGCTTCTGCGCTGGTAAAAGGTAACACTAACCCGATTACTAGCATGTCTGCACCGGTACAGGCAAGCGC
CCCAGCTCTGACTAAATCCCAGACGGACCGTCTGGAGGTGCTGCTGAACCCAAAGGATGAAATCTCTCTG
AACAGCGGCAAGCCTTTCCGTGAGCTGGAAAGCGAGCTGCTGTCTCGTCGTAAAAAGGATCTGCAACAGA
TCTACGCTGAGGAACGCGAGAACTATCTGGGTAAGCTGGAGCGCGAAATTACTCGCTTCTTCGTGGATCG
CGGTTTCCTGGAGATCAAATCTCCGATTCTGATTCCGCTGGAATACATTGAACGTATGGGCATCGATAAT
GATACCGAACTGTCTAAACAGATCTTCCGTGTGGATAAAAACTTCTGTCTGCGTCCGATGATGGCTCCGA
ACATTTTGAACTATGCGCGTAAACTGGACCGTGCCCTGCCGGACCCGATCAAAATTTTCGAGATCGGTCC
TTGCTACCGTAAAGAGTCCGACGGTAAAGAGCACCTGGAAGAATTCACCATGCTGAACTTCTTCCAGATG
GGTAGCGGTTGCACGCGTGAAAACCTGGAATCCATTATCACCGACTTCCTGAATCACCTGGGTATCGATT
TCAAAATTGTTGGTGACAGCTGTATGGTGTATGGCGATACGCTGGATGTTATGCACGGCGATCTGGAGCT
GTCTTCCGCAGTAGTGGGCCCAATCCCGCTGGATCGTGAGTGGGGTATCGACAAACCTTGGATCGGTGCG
GGTTTTGGTCTGGAGCGTCTGCTGAAAGTAAAACACGACTTCAAGAACATCAAACGTGCTGCACGTTCCG
AGTCCTATTACAATGGTATTTCTACTAACCTGTAA

Amino acid sequence of MmFAcKRS1

MDKKPLNTLISATGLWMSRTGTIHKIKHHEVSRSKIYIEMACGDHLVVNNSRSSRTARALRHHKYRKTCK
RCRVSDEDLNKFLTKANEDQTSVKVKVVSAPTRTKKAMPKSVARAPKPLENTEAAQAQPSGSKFSPAIPV
STQESVSVPASVSTSISSISTGATASALVKGNTNPITSMSAPVQASAPALTKSQTDRLEVLLNPKDEISL
NSGKPFRELESELLSRRKKDLQQIYAEERENYLGKLEREITRFFVDRGFLEIKSPILIPLEYIERMGIDN
DTELSKQIFRVDKNFCLRPMMAPNILNYARKLDRALPDPIKIFEIGPCYRKESDGKEHLEEFTMLNFFQM
GSGCTRENLESIITDFLNHLGIDFKIVGDSCMVYGDTLDVMHGDLELSSAVVGPIPLDREWGIDKPWIGA
GFGLERLLKVKHDFKNIKRAARSESYYNGISTNL

Gene sequence of MmFAcKRS2

ATGGATAAAAAGCCTTTGAACACTCTGATTTCTGCGACCGGTCTGTGGATGTCCCGCACCGGCACCATCC
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TGTCAACAACTCCCGTTCTTCTCGTACCGCTCGCGCACTGCGCCACCACAAATATCGCAAAACCTGCAAA
CGTTGCCGTGTTAGCGATGAGGACCTGAACAAATTCCTGACCAAAGCTAACGAGGATCAGACCTCCGTAA
AAGTGAAGGTAGTAAGCGCTCCGACCCGTACTAAAAAGGCTATGCCAAAAAGCGTGGCCCGTGCCCCGAA
ACCTCTGGAAAACACCGAGGCGGCTCAGGCTCAACCATCCGGTTCTAAATTTTCTCCGGCGATCCCAGTG
TCCACCCAAGAATCTGTTTCCGTACCAGCAAGCGTGTCTACCAGCATTAGCAGCATTTCTACCGGTGCTA
CCGCTTCTGCGCTGGTAAAAGGTAACACTAACCCGATTACTAGCATGTCTGCACCGGTACAGGCAAGCGC
CCCAGCTCTGACTAAATCCCAGACGGACCGTCTGGAGGTGCTGCTGAACCCAAAGGATGAAATCTCTCTG
AACAGCGGCAAGCCTTTCCGTGAGCTGGAAAGCGAGCTGCTGTCTCGTCGTAAAAAGGATCTGCAACAGA
TCTACGCTGAGGAACGCGAGAACTATCTGGGTAAGCTGGAGCGCGAAATTACTCGCTTCTTCGTGGATCG
CGGTTTCCTGGAGATCAAATCTCCGATTCTGATTCCGCTGGAATACATTGAACGTATGGGCATCGATAAT
GATACCGAACTGTCTAAACAGATCTTCCGTGTGGATAAAAACTTCTGTCTGCGTCCGATGATGGCTCCGA
ACGTTTTGAACTATGCTCGTAAACTGGACCGTGCCCTGCCGGACCCGATCAAAATTTTCGAGATCGGTCC
TTGCTACCGTAAAGAGTCCGACGGTAAAGAGCACCTGGAAGAATTCACCATGCTGAACTTCTTCCAGATG
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TCAAAATTGTTGGTGACAGCTGTATGGTGTATGGCGATACGCTGGATGTTATGCACGGCGATCTGGAGCT
GTCTTCCGCAGTAGTGGGCCCAATCCCGCTGGATCGTGAGTGGGGTATCGACAAACCTTGGATCGGTGCG
GGTTTTGGTCTGGAGCGTCTGCTGAAAGTAAAACACGACTTCAAGAACATCAAACGTGCTGCACGTTCCG
AGTCCTATTACAATGGTATTTCTACTAACCTGTAA

Amino acid sequence of MmFAcKRS2

MDKKPLNTLISATGLWMSRTGTIHKIKHHEVSRSKIYIEMACGDHLVVNNSRSSRTARALRHHKYRKTCK
RCRVSDEDLNKFLTKANEDQTSVKVKVVSAPTRTKKAMPKSVARAPKPLENTEAAQAQPSGSKFSPAIPV
STQESVSVPASVSTSISSISTGATASALVKGNTNPITSMSAPVQASAPALTKSQTDRLEVLLNPKDEISL
NSGKPFRELESELLSRRKKDLQQIYAEERENYLGKLEREITRFFVDRGFLEIKSPILIPLEYIERMGIDN
DTELSKQIFRVDKNFCLRPMMAPNVLNYARKLDRALPDPIKIFEIGPCYRKESDGKEHLEEFTMLNFFQM
GSGCTRENLESIITDFLNHLGIDFKIVGDSCMVYGDTLDVMHGDLELSSAVVGPIPLDREWGIDKPWIGA
GFGLERLLKVKHDFKNIKRAARSESYYNGISTNL

Protein Expression Protocol

EGFP182FAcK expression

BL21 (DE3) electrocompetent *E. coli* cells transformed with both pTAK_EGFP182TAG_His and pBK_MmFAcKRS1 or 2 were plated on an LB-K50Cm40 agar plate and cultured overnight at 37°C. Single colony was picked up from the plate and grown in 2xYT-K50Cm40 (5 mL) overnight at 37°C.

Aliquot (1 mL) of this startup culture was diluted into 50 mL 2xYT-K50Cm40 and it was incubated at 37°C until OD_{600nm} reached 0.4~0.5. The flask containing bacteria was taken out, added 2M nicotinamide solution (0.5 mL), 100 mM FAcK aqueous solution or water (0.5 mL) and allowed to stand for 5 min at room temperature. After adding 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG) solution (50 μL), the baterial solution was incubated at 37°C for 6 hrs. After measuring OD_{600nm} of 10 x diluted solution (i.e. bacterial solution 100 μL/2xYT 900 μL), the appropriate volume of the bacterial solution was transferred into a 50 mL tube as each tube had the same number of cells. Cell pellet was collected by centrifugation (4200 rpm, 30 min, 4°C) and stored at −80°C overnight.

The cell pellet was added lysis buffer (5 mL) containing 50 mM TrisHCl pH8.0, 500 mM NaCl, 20 mM imidazole pH8.0, 1%v/v Tween20, 10%v/v glycerol, Lysozyme 1 mg/mL, DNase 0.1 mg/mL, Tris (2-carboxyethyl)phosphine hydrochloride 0.6 mg/mL, cOmplete™ Mini EDTA-free 1/2 tablet, and 20 mM Nicotinamide. The *E. coli* suspension was transferred to a 15 mL tube and incubated at 4°C for 1 hr. The lysed solution was sonicated with a Sonic Dismembrator (Fisher Scientific, 30%output, 2 min, 1 sec off, 1 sec on) with cooling on an ice-water bath. The sonicated solution was split into 1.5 mL tubes and spinned down (15,000 rpm, 30 min, 4°C).

The supernatant was collected into a 15 mL tube and added Protino®Ni-NTA Agarose 30 μL. The mixture was incubated at 4°C for 1 hr, and the solution was filtered by a Poly-Prep® Chromatography Column. The filtered bead was washed with wash buffer 1000 μL (containing 50 mM TrisHCl pH8.0, 500 mM NaCl, 20 mM imidazole pH8.0, 10%v/v glycerol) 4 times and eluted with elution buffer 200 μL (containing 50 mM TrisHCl, pH8.0, 500 mM NaCl, 250 mM imidazole pH8.0, 10%v/v glycerol) 4 times to obtain the desired protein solution. The whole amount was loaded onto a 10K Amicon Ultra column and concentrated into about 100 μL. 1x phosphate buffered saline (PBS, pH7.4) 400 μL was added onto the concentrated solution, mixed well and concentrated into about 100 μL. Above steps were repeated 4 times to completely exchange the buffer into 1x PBS. The protein solution was messed up to 30 μL with 1x PBS and used for 10% Tricine-SDS-PAGE, western blot and intact protein MS analysis (Figure 1).

Affibody (Afb) 36FAcK expression

Afb36FAcK was expressed in 400 mL bacterial culture according to the same procedure as the EGFP182FAcK except for expressed at 30°C. The eluted protein solution was charged onto a 30K Amicon Ultra column and the flow through was collected to remove >30K impurities. The obtained flow through was loaded onto a 3K column, concentrated and buffer-exchanged as the EGFP182FAcK. The obtained protein was used for crosslinking experiments (Figure 2).

Afb36AcK expression

Afb36AcK was prepared according to the same procedure as the Afb36FAcK in 400 mL bacterial culture expressing pTAK_Afb36TAG_His and pBK_MmAcKRS1. The obtained protein was used for crosslinking experiments (Figure 2).

MBP-Z6FAcK expression

MBP-Z6FAcK was prepared according to the same procedure as the Afb36FAcK in 400 mL bacterial culture expressing pTAK_MBP-Z6TAG_His and pBK_MmFAcKRS1, and concentrated with 30K Amicon Ultra column. The obtained protein was used for crosslinking experiments (Figure S4).

MBP-Z24FAcK expression

MBP-Z24FAcK was prepared according to the same procedure as the MBP-Z6FAcK in 400 mL bacterial culture expressing pTAK_MBP-Z24TAG_His and pBK_MmFAcKRS1, and concentrated with 30K Amicon Ultra column. The obtained protein was used for crosslinking experiments (Figure S5).

Calmodulin (CaM) 76FAcK expression

CaM76FAcK was prepared according to the same procedure as the expression of Afb36FAcK except for expressed in Nico21 (DE3) E. coli cells, additionally purified with a chitin resin column and concentrated with a 10K Amicon Ultra column. The obtained protein was used for 10% Tricine-SDS-PAGE and intact protein MS analysis (Figure 3).

MBP-Z6Cys and MBP-Z11Cys expression

MBP-Z6Cys and MBP-Z11Cys were prepared according to the previous report[6].

Afb36Cys expression

Afb36Cys was prepared according to the same procedure as the Afb36FAcK without unnatural amino acid and nicotinamide in 400 mL bacterial culture expressing pTAK_Afb36Cys_His. The obtained protein was used for crosslinking experiments (Figure S3).

Afb7Cys expression

Afb7Cys was prepared according to the same procedure as the Afb36Cys in 400 mL bacterial culture expressing pTAK_Afb7Cys_His. The obtained protein was used for crosslinking experiments (Figure S4).

Crosslinking Experiments Protocol

Intermolecular crosslinking with Afb36FAcK and MBP-Z6Cys

Both Afb36FAcK 1.47 μg and MBP-Z6Cys 1.0 μg (molar ratio Afb36FAcK/MBP-Z6Cys = 10/1) were dissolved in 7.5 μL 1x PBS solution and incubated at 37°C for 24 hrs. *N*-ethylmaleimide (NEM) was treated (5 mM) for 1hr at 37°C before Afb36FAcK addition. The reaction mixture was added 4x Laemmli sample buffer 2.5 μL containing 10%v/v 2-mercaptoethanol and heated at 95°C for 5 min. The whole amount was applied to 10% Tricine-SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. Other crosslinking experiments were also carried out according to the same procedure shown here unless otherwise noted.

Amino Acid Sequence of Expressed Proteins

EGFP182FAcK (**U** = FAcK)

MTSVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYG
VQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNIL
GHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADH**U**QQNTPIGDGPVLLPDNHYLSTQSAL
SKDPNEKRDHMVLLEFVTAAGITLGMDELYKHHHHHH

Afb36FAcK (**U** = FAcK)
Afb36AcK (**U** = AcK)
Afb36Cys (**U** = C)

MTSVDNKFNKELSVAGREIVTLPNLNDPQKKAFIFSLW**U**DPSQSANLLAEAKKLNDAQAPKGSHHHHHH

Afb7Cys

MTSVDNKFN**C**ELSVAGREIVTLPNLNDPQKKAFIFSLWDDPSQSANLLAEAKKLNDAQAPKGSHHHHHH

CaM76FAcK80Cys (**U** = FAcK)

MTSHDQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIYFPEF
LTMMARK**U**KDTCSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNY
EEFVQMMTAKHHHHHH

MBP-Z6Cys (**R¹** = C, **R²** = N)

MBP-Z11Cys (**R¹** = N, **R²** = C)

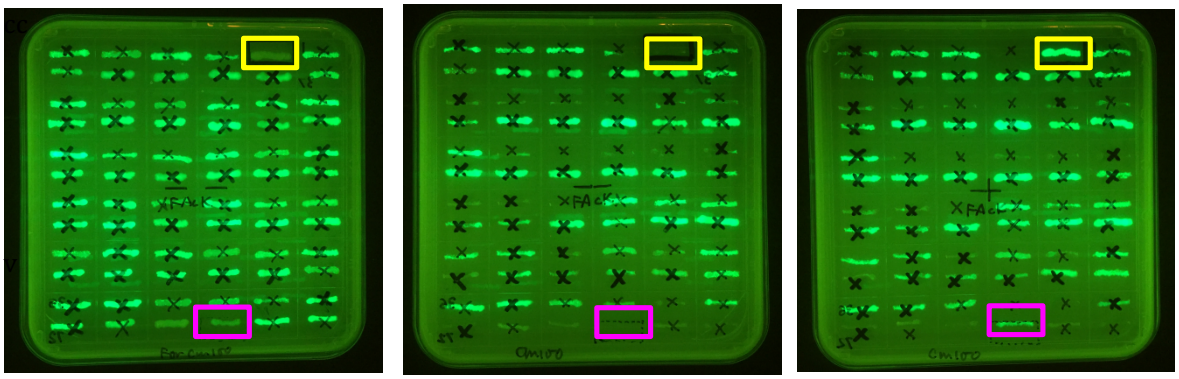
MKHHHHHHHHHGGPCMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATG
DGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPK
TWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDL
IKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAA
SPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFW
YAVRTAVINAASGRQTVDEALKDAQTNSSSNNNNNNNNNNLGSSGLVPRGSHGTSVDNKF**R¹**KEQQ**R²**AFY
EILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAQAPKLEHHHHHH

MBP-Z6FAcK (**U¹** = FAcK, **U²** = E)

MBP-Z24FAcK (**U¹** = N, **U²** = FAcK)

MTSKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFG
GYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELK
AKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDY
SIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLE
NYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAAS
GRQTVDEALKDAQTNSSSNNNNNNNNNNLGSSGLVPRGSHGVDNKF**U¹**KEQQNAFYEILHLPNLN**U²**EQR
NAFIQSLKDDPSQSANLLAEAKKLNDAQAPKHHHHHH

Supplementary Figures



1) Tet/Kan **2) Tet/Kan/Cm** **3) Tet/Kan/Cm + FAcK**

Figure S1. Fluorescence images of 72 green colonies from the 1st positive selection plate on 3 different LB-agar plates containing 1) Tet 12.5 μg/mL, Kan 25 μg/mL, 2) Tet 12.5 μg/mL, Kan 25 μg/mL, Cm 100 μg/mL and 3) Tet 12.5 μg/mL, Kan 25 μg/mL, Cm 100 μg/mL, FAcK 1 mM. Two hits highlighted in yellow and pink squares were further analyzed by DNA sequencing for pBK and the two mutants were identified as MmFAcKRS1 and MmFAcKRS2, respectively.

Tet: Tetracycline hydrochloride

Kan: Kanamycin sulfate

Cm: Chloramphenicol

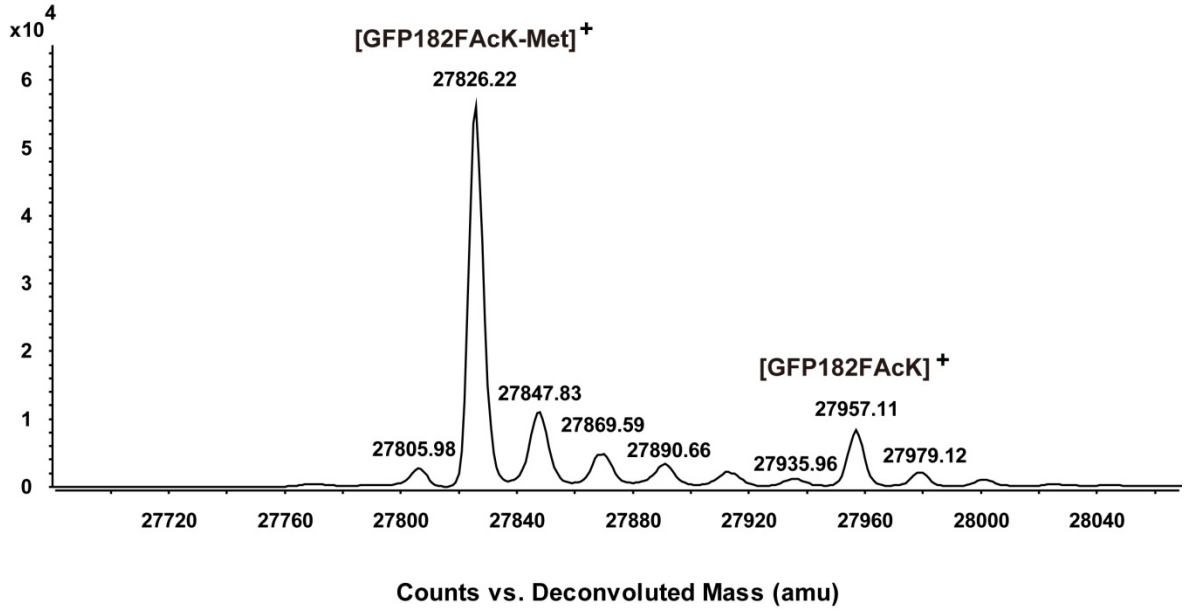


Figure S2. ESI-TOF MS analysis of intact GFP182FAcK expressed in the presence of MmFAcKRS2/$tRNA^{Pyl}_{CUA}$ supplemented with 1 mM of FAcK.

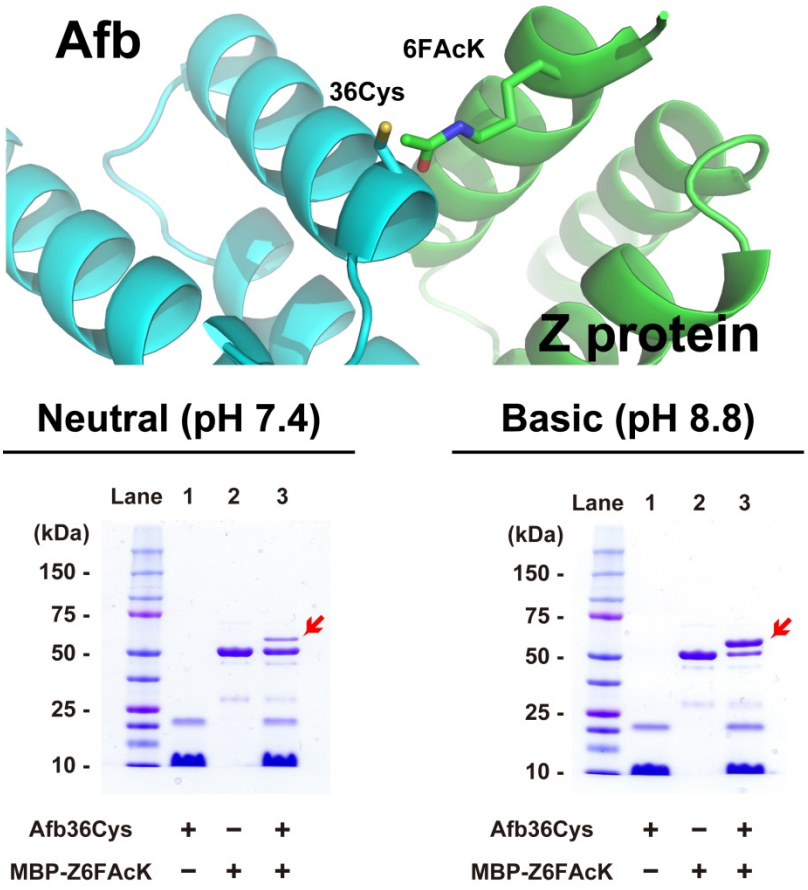


Figure S3. Formation of the Afb/MBP-Z covalent complex after incubation of the Afb36Cys with the MBP-Z6FAcK (molar ratio Afb36Cys/MBP-Z6FAcK = 30/1), indicated by the red arrow in a 10% Tricine-SDS PAGE gel with Coomassie Brilliant Blue staining (Lane 3). The crosslinking reaction was carried out in 50 mM Tris/HCl buffered saline (pH 7.4 or pH 8.8) in the presence of 10 mM TCEP for 24 hrs at 37°C. The crosslink yield was determined as 24 % (pH 7.4) and 64 % (pH 8.8) respectively, calculated from the intensity of the bands on the gel.

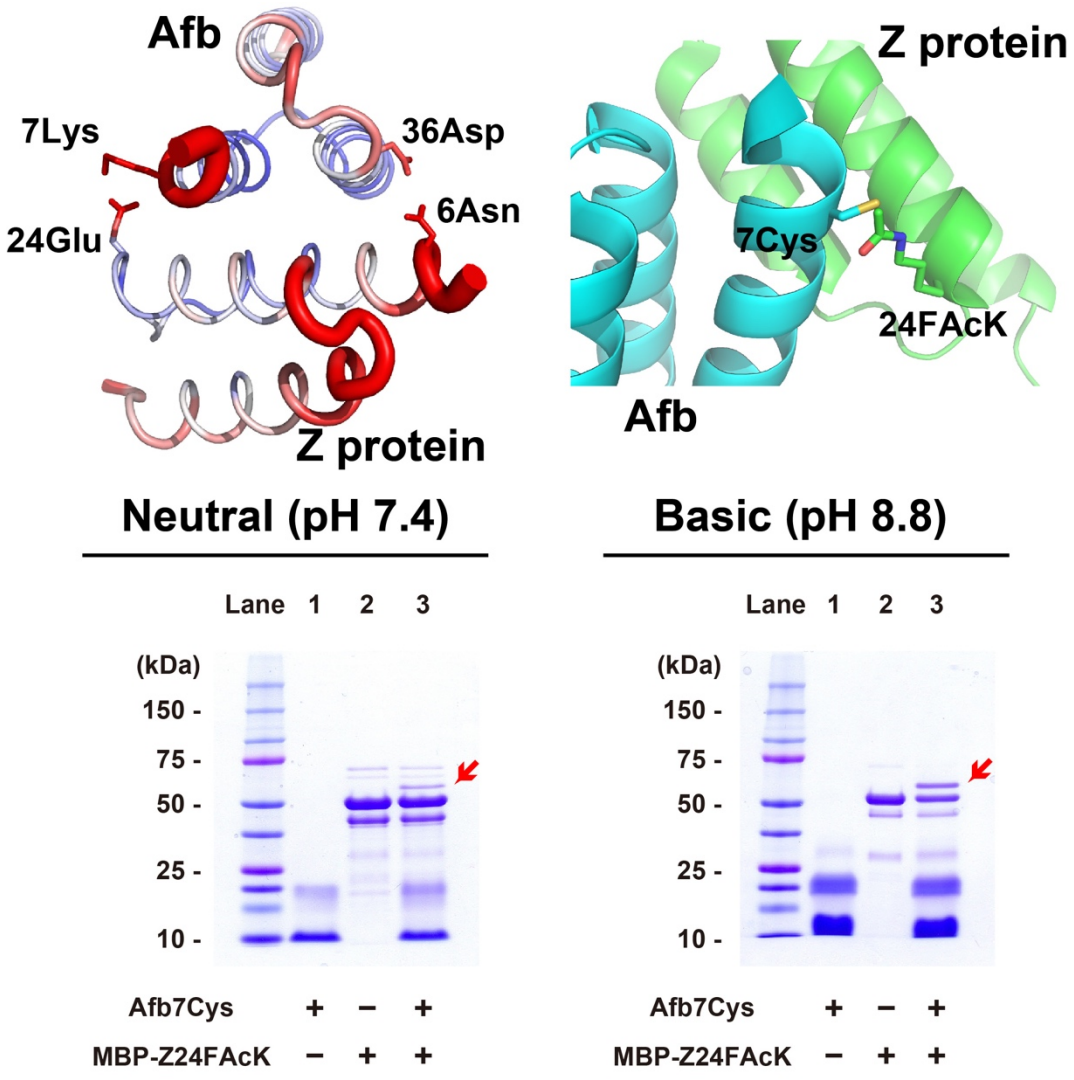


Figure S4. Formation of the Afb/MBP-Z covalent complex after incubation of the Afb7Cys with the MBP-Z24FAcK (molar ratio Afb7Cys/MBP-Z24FAcK = 50/1), indicated by the red arrow in a 10% Tricine-SDS PAGE gel with Coomassie Brilliant Blue staining (Lane 3). The crosslinking reaction was carried out in 50 mM Tris /HCl buffered saline (pH 7.4 or pH 8.8) in the presence of 10 mM TCEP for 24 hrs at 37°C. The crosslink yield was determined as 12 % (pH 7.4) and 34 % (pH 8.8) respectively, calculated from the intensity of the bands on the gel. The picture on the top left shows the two sets of sites chosen for incorporating FAcK and Cys. The *B* factors of the complex structure are colored as blue-white-red, with red being highest.

Supplementary References

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