



Published in final edited form as:

J Am Chem Soc. 2018 April 18; 140(15): 4995–4999. doi:10.1021/jacs.8b01087.

Genetically encoding fluorosulfate-L-tyrosine to react with lysine, histidine and tyrosine via SuFEx in proteins *in vivo*

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Abstract

Introducing new chemical reactivity into proteins in living cells would endow innovative covalent bonding ability to proteins for research and engineering *in vivo*. Latent bioreactive unnatural amino acids (Uaas) can be incorporated into proteins to react with target natural amino acid residues via proximity-enabled reactivity. To expand the diversity of proteins amenable to such reactivity *in vivo*, a chemical functionality that is biocompatible and able to react with multiple natural residues under physiological conditions is highly desirable. Here we report the genetic encoding of fluorosulfate-L-tyrosine (FSY), the first latent bioreactive Uaa that undergoes sulfur-fluoride exchange (SuFEx) on proteins *in vivo*. FSY was found nontoxic to *E. coli* and mammalian cells; after being incorporated into proteins, it selectively reacted with proximal lysine, histidine, and tyrosine via SuFEx, generating covalent intra-protein bridge and inter-protein crosslink of interacting proteins directly in living cells. The proximity-activatable reactivity, multi-targeting ability, and excellent biocompatibility of FSY will be invaluable for covalent manipulation of proteins *in vivo*. Moreover, genetically encoded FSY hereby empowers general proteins with the next generation of click chemistry, SuFEx, which will afford broad utilities in chemical biology, drug discovery, and biotherapeutics.

Graphical Abstract

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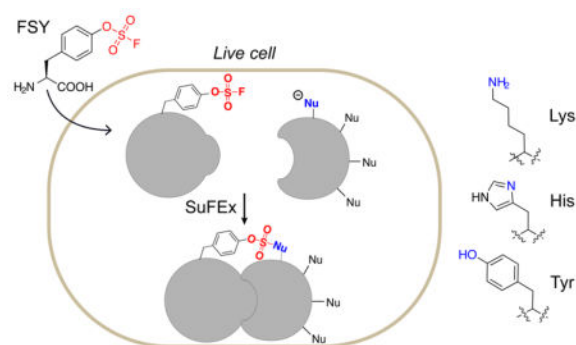
These authors contributed equally.

Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website. Experimental details, Figure S1–S3 (PDF)



The ability to endow proteins with new chemical reactivity will expand novel avenues for protein research and engineering, potentially impacting biological studies, biotherapeutics, and synthetic biology.¹ The genetic code has recently been engineered to encode a new class of unnatural amino acids (Uaas), the latent bioreactive Uaas.² These Uaas, after incorporation into proteins, react with a target natural amino acid residue via proximity-enabled reactivity, enabling selective formation of new covalent linkages within and between proteins. The new covalent bonding ability has been harnessed within proteins to enhance photostability,² increase thermostability,³ staple proteins recombinantly,⁴ and build optical nano-switches,⁵ and between proteins to pinpoint ligand-receptor interaction,⁶ target native receptors irreversibly,^{4,7} generate covalent macromolecular inhibitors,⁸ and capture weak protein-protein interactions.⁹ Bioreactive Uaas have initially been developed to target Cys² and subsequently to target Lys, His and Tyr.^{3-4,7,10} Reacting with Lys, His and Tyr often requires basic conditions that are infeasible *in vivo*.^{4,10b} At this burgeoning stage, most genetically encoded bioreactive Uaas react with 1 to 2 natural residues only, have modest to severe cytotoxicity, and are primarily used for *in vitro* applications.

An ideal chemical functionality for latent bioreactive Uaas should be stable, nontoxic, and nonreactive inside cells, yet upon placed in proximity to target residues becomes reactive under cellular conditions. Aryl fluorosulfates are weak electrophiles attracting great attention for the new generation of click chemistry:¹¹ they are relatively unreactive toward hydrolysis, thermolysis and reduction,¹¹⁻¹² and are unreactive toward free amino acids under physiological conditions,¹³ but form covalent bonds with Tyr, Lys and Ser through sulfur-fluoride exchange (SuFEx) reaction when used as war-heads in chemical probes specifically binding proteins,¹⁴ although a positively charged microenvironment is required for reactivity. Given the tremendous potential of aryl fluorosulfate for biocompatible reactivity emerging from recent small molecule studies, it is desirable to genetically install this functional group onto proteins so that its unique properties can be exploited in large biomolecules and *in vivo*.

Here we evolved a new tRNA-synthetase pair to genetically incorporate fluorosulfate-L-tyrosine (FSY, Fig. 1a) into proteins in *E. coli* and mammalian cells. We found that FSY was generally nontoxic to cells, and was able to react with Lys, His, and Tyr specifically via proximity-enabled SuFEx reaction within and between proteins under physiological conditions (Fig. 1b). We demonstrated the crosslinking of interacting proteins using FSY directly *in vivo*.

FSY was synthesized using the SO₂F₂/borax method (88% yield, Supplementary methods).^{11,15} To genetically encode FSY, we evolved a mutant pyrrolysyl-tRNA synthetase (PylRS) specific for FSY. A PylRS mutant library was generated by mutating residues Ala302, Leu305, Tyr306, Leu309, Ile322, Asn346, Cys348, Tyr384, Val401, and Trp417 of the *Methanosarcina mazei* PylRS using the small-intelligent mutagenesis approach,¹⁶ and subjected to selection as described.¹⁷ Six hits showing FSY-dependent phenotype were identified; they all converged on the same amino acid sequence (302I/346T/348I/384L/417K), which we named as FSYRS.

We evaluated the incorporation specificity of FSY into proteins in *E. coli*. The Z_{spa} affibody (Afb) gene containing a TAG codon at position 36 (Afb-36TAG) was co-expressed with the tRNA^{Pyl}_{CUA}/FSYRS pair in *E. coli*. In the absence of FSY, no full-length Afb was detected; when 1 mM FSY was added in growth media, full-length Afb36FSY was produced with a yield of 1.6 mg/L (Fig. 1c). The purified Afb36FSY was analyzed by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) (Fig. 1d). A peak observed at 7855.96 Da corresponds to intact Afb containing FSY at site 36 (Afb36FSY: expected 7856.69 Da). A peak measured at 7724.77 Da corresponds to Afb36FSY lacking the initiating Met (Afb36FSY-Met: expected 7725.50 Da). Two minor peaks observed at 7836.55 and 7705.16 Da correspond to Afb36FSY lacking F (expected 7836.69 Da) and Afb36FSY-Met lacking F (expected 7705.49 Da), respectively, suggesting slight F elimination during MS measurement. Notably, no peaks corresponding to Afb containing other amino acids at position 36 were observed. We also incorporated FSY at position 24 of the Z protein and analyzed with tandem MS. A series of b and y ions unambiguously indicate that FSY was incorporated at the TAG-specified position 24 (Fig. 1e). The presence of 1 mM FSY did not affect *E. coli* growth (Fig. S1), suggesting no obvious cytotoxicity. These results indicate that the evolved tRNA^{Pyl}_{CUA}/FSYRS pair was able to incorporate FSY with high efficiency and specificity in *E. coli*.

We next tested FSY incorporation into proteins in mammalian cells. We transfected HeLa-EGFP-182TAG reporter cells¹⁸ with plasmid pMP-FSYRS-3xtRNA, which expresses FSYRS and tRNA^{Pyl}_{CUA} genes. Suppression of the 182TAG codon of the genome-integrated EGFP gene would produce full-length EGFP rendering cells fluorescent. Strong EGFP fluorescence was measured from cells by flow cytometry only when FSY was added (Fig. 2a). The fluorescence intensity increased with FSY concentration and incubation time (Fig. 2b). As a positive control, we incorporated *p*-azido-L-phenylalanine (AzF) into reporter cells in parallel using plasmid pIre-Azi3 (Fig. S2), which is the most efficient Uaa incorporation system in mammalian cells in our hands.⁶ FSY incorporation reached 76% of the AzF level. Notably, while cellular toxicity is often an issue with bioreactive Uaas, we did not observe obvious toxicity of FSY to HeLa or HEK293T cells (Fig. S3), a valuable property of FSY possibly owing to the extremely low background reactivity of aryl fluorosulfate inside cells.^{14a} The above results were also confirmed by fluorescence confocal microscopy (Fig. 2c). In the presence of FSY, strong EGFP fluorescence was observed throughout the cells, and cell morphology remained normal. No fluorescence signal was detected when FSY was not added. We also incorporated FSY into proliferating cell nuclear antigen (PCNA) through

transient transfection of PCNA-165TAG and tRNA^{Pyl}_{CUA}/FSYRS genes into HEK293T cells. Full-length PCNA was produced in the presence of FSY, with incorporation efficiency reaching 41% of AzF (Fig. 2d). These results suggest that FSY was incorporated into proteins in mammalian cells with high efficiency and specificity without causing detrimental effects.

We then determined whether the incorporated FSY could react with natural amino acid residues via proximity-enabled reactivity directly in *E. coli*. Afb binds to its substrate Z protein with a moderate affinity, providing a suitable protein framework to study FSY crosslinking *in vivo*. In light of the crystal structure of Afb-Z complex,¹⁹ we introduced FSY at position 24 of Z protein and the target natural residue at position 7 of Afb, placing the two residues in close proximity upon Afb-Z binding (Fig. 3a). As aryl fluorosulfate is a weak electrophile, we decided to test FSY's reactivity toward Lys, His, Tyr, Cys, Ser, and Thr using Ala as a negative control. To better separate the Afb and Z proteins of similar molecular weights, we fused maltose binding protein (MBP) to the N-terminus of Z (MBP-Z). MBP-Z and Afb were both appended with a 6xHis-tag at C-terminus. To determine whether chemical crosslinking could occur in living cells, we co-expressed MBP-Z24FSY and Afb-7X (X = target residue) in *E. coli*. After culturing at 37 °C for 6 h, the same number of cells were analyzed using Western blot under denatured conditions. From cells expressing Afb-7Lys, Afb-7His, or Afb-7Tyr, crosslinking bands were observed with molecular weight corresponding to MBP-Z24FSY and Afb adducts (Fig. 3b). We then purified 6xHis-tagged proteins from cells and analyzed with SDS-PAGE. Consistently, a protein band corresponding to the crosslinked MBP-Z with Afb was clearly observed for Afb-7Lys, Afb-7His, and Afb-7Tyr (Fig. 3b), with crosslinking efficiency (determined by band intensities) of 59%, 53% and 35%, respectively. In contrast, no cross-linking bands were observed when MBP-Z24FSY was co-expressed with Afb-7Cys, Afb-7Ser, Afb-7Thr, or Afb-7Ala. While aryl carbamate requires basic pH to crosslink Lys or Tyr at Afb/Z interface *in vitro*,^{10b} FSY was able to crosslink Lys, His or Tyr directly in live *E. coli* cells.

To further validate the *in vivo* chemical crosslinking ability of FSY, we analyzed the purified proteins using tandem MS. As expected, strong signals corresponding to the covalently-linked peptides of MBP-Z24FSY and Afb-7Lys were identified (Fig. 3c). A series of b and y fragmented ions clearly indicate that the incorporated FSY crosslinked exclusively with Lys 7 of Afb. Similar MS results were also obtained for MBP-Z24FSY co-expressed with Afb-7His or Afb-7Tyr, confirming FSY crosslinked with the target His7 (Fig. 3d) or Tyr7 (Fig. 3e), respectively. Meanwhile, consistent with Western and SDS-PAGE results, no crosslinked peptides of MBP-Z24FSY with Afb-7Ser, Afb-7Thr, Afb-7Cys, or Afb-7Ala were detected by tandem MS.

In addition, we incorporated FSY and Tyr into a single protein to test intramolecular crosslinking *in vivo*. We co-expressed in *E. coli* the tRNA^{Pyl}_{CUA}/FSYRS pair with a mutant calmodulin gene (CaM-76TAG), which encoded 76TAG for FSY incorporation and Tyr at a nearby site 80 (Fig. 4a). This CaM protein was expressed in the presence of 1 mM FSY, purified (Fig. 4b), and analyzed with tandem MS (Fig. 4c). A series of b and y fragment ions unambiguously show that FSY formed a covalent linkage with Tyr80 via SuFEx.

To demonstrate that FSY could crosslink interacting proteins through targeting a native Tyr residue, we tested whether FSY-armed thioredoxin (Trx) could covalently capture 3'-phosphoadenosine-5'-phosphosulfate (PAPS) reductase. Trx interacts with PAPS reductase to facilitate the reduction of adenylated sulfate to sulfite for *de novo* cysteine biosynthesis.²⁰ On the basis of the complex structure of PAPS reductase with Trx1,²⁰ we incorporated FSY into *E. coli* Trx1 at site 62 to target the proximal Tyr191 of PAPS reductase (Fig. 5a). We expressed and purified Trx1(62FSY) and WT PAPS reductase, and incubated them in Tris buffer at pH 7.4 or 8.0 for 12 h. SDS-PAGE showed clear bands corresponding to the covalent complex of Trx1 with PAPS reductase (Fig. 5b, Fig. S4). Tandem MS analysis of the sample unambiguously indicate that FSY of Trx1 covalently crosslinked with the target Tyr191 of PAPS reductase via SuFEx reaction (Fig. 5c). Taken together, the intramolecular CaM crosslinking and intermolecular Trx1-PAPS reductase crosslinking further corroborate that FSY reacted with Tyr in proximity via SuFEx reaction.

In summary, we genetically encoded the live-cell friendly FSY into proteins in *E. coli* and mammalian cells, which selectively reacted with proximal Lys, His and Tyr residues via SuFEx directly in live *E. coli* cells. Intermolecular crosslinking using bioreactive Uaas has been mainly limited to *in vitro* usage with few exceptions targeting Cys;^{6,9a} FSY enables intermolecular crosslinking of interacting proteins *in vivo* and targeting three different residues. Since FSY's target residues are often found at protein surface and interface, FSY will dramatically expand the diversity of proteins amenable to covalent bonding and enable creative *in vivo* applications to exploit protein covalent bonding ability. Moreover, genetically encoding FSY now empowers proteins with the new generation of click chemistry, SuFEx, which will find broad applications in chemical biology, drug discovery, and biotherapeutics.

Supplementary Material

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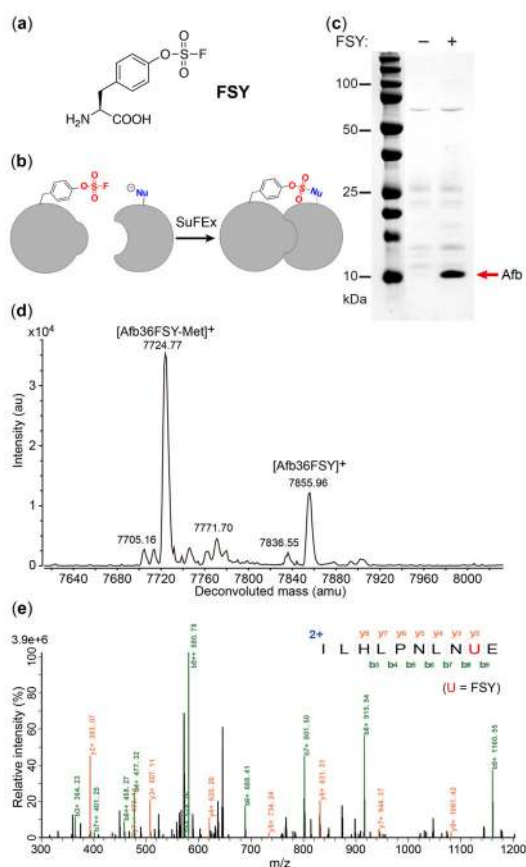
Acknowledgments

L.W. acknowledges the support of the NIH (R01GM118384, RF1MH114079).

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**Figure 1.**

Genetically encode FSY into proteins in *E. coli*. (a) Structure of FSY. (b) Scheme showing proximity-enabled SuFEx reaction between FSY and a natural nucleophilic residue. (c) SDS-PAGE showing FSY incorporation into Afb(36TAG) in *E. coli*. (d) ESI-TOF MS spectrum of intact Afb-36FSY. (e) Tandem MS spectrum of Z-24FSY.

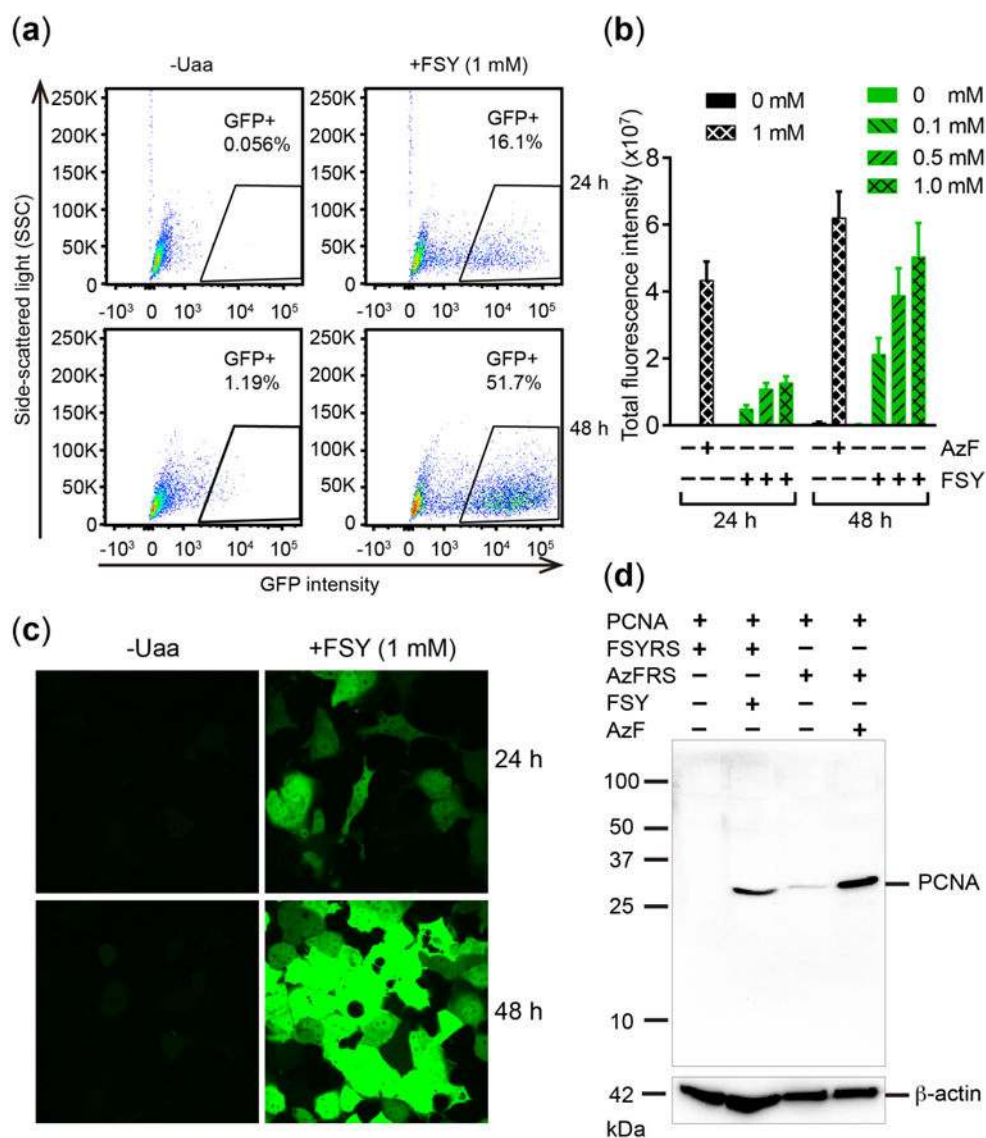


Figure 2. Genetically encode FSU into proteins in mammalian cells. (a) FACS analysis of FSU incorporation into EGFP-182TAG in HeLa cells. (b) Total EGFP fluorescence intensity measured from the same number of HeLa-EGFP-182TAG reporter cells. Error bar: s.e.m., $n = 6$. (c) Fluorescence images of HeLa-EGFP-182TAG reporter cells. (d) Western blot analysis of FSY and AzF incorporation into PCNA-165TAG in HEK293T cells. An anti-His antibody was used to detect the 6xHis-tag appended at the C-terminus of PCNA.

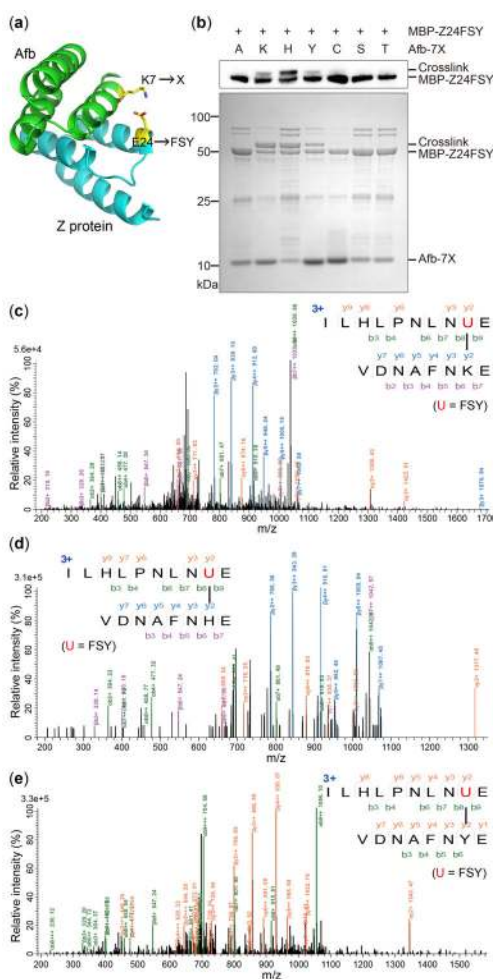


Figure 3. FSY crosslinks proximal Lys, His and Tyr via SuFEx directly in *E. coli* cells. (a) Structure of Afb-Z complex showing two proximal sites for FSY and target residue X incorporation. (b) Top: Western blot of *E. coli* cell lysates; Bottom: SDS-PAGE of proteins His-tag purified from *E. coli*. (c–e) Tandem MS spectrum of MBP-Z-24FSY/Afb-7Lys (c), MBP-Z-24FSY/Afb-7His (d) and MBP-Z-24FSY/Afb-7Tyr (e).

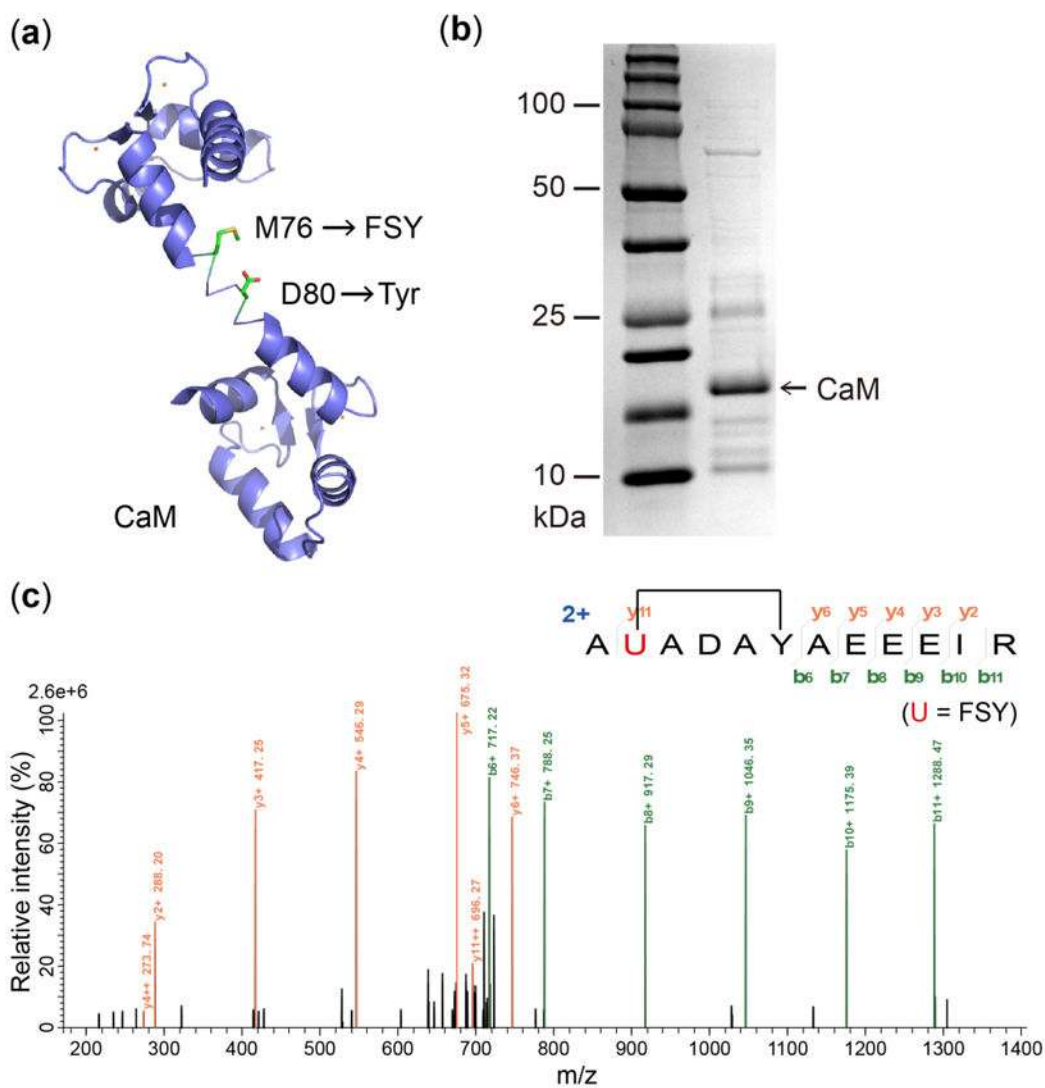


Figure 4. FSY crosslinks Tyr via SuFEx intramolecularly in *E. coli* cells. (a) Structure of CaM showing sites for FSY and target Tyr. (b) SDS-PAGE and (c) tandem MS spectrum of purified CaM-76FSY-80Tyr.

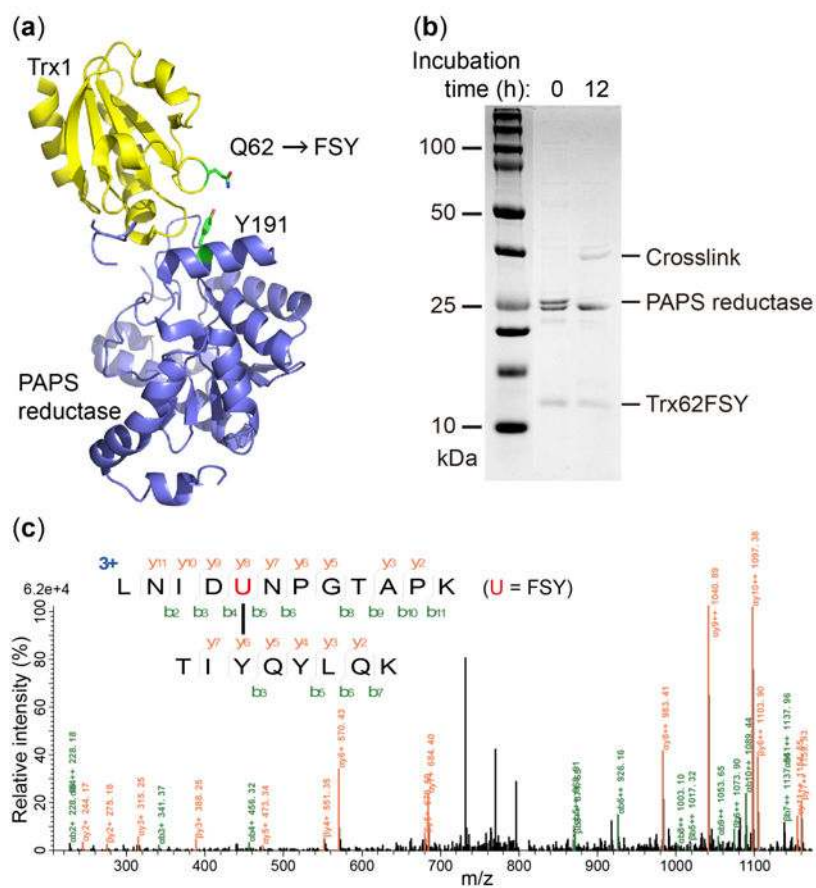


Figure 5. FSU crosslinks Tyr via SuFEx intermolecularly. (a) Structure of Trx1 in complex with PAPS reductase showing FSU site and the native Tyr191. (b) SDS-PAGE and (c) tandem MS spectrum of Trx1 crosslinked with PAPS reductase.