Supporting Information

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SUPPORTING INFORMATION

Regioselective cysteine bioconjugation by appending a labeled cysteine tag to a protein using protein splicing *in trans*

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Modification of C-terminal DnaB fusion protein 2 with 5-Iodoacetamido-fluorescein

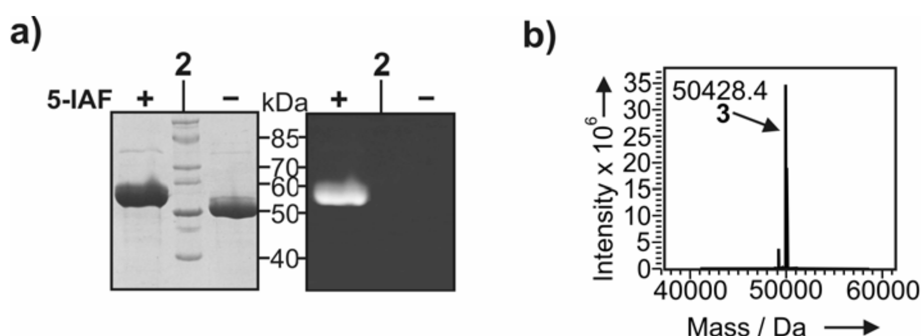

Figure S1: Labeling of the Cys-tag construct. Protein **2** (MBP-DnaB^C-Cys-His$_6$, 65µM) was reduced with TCEP and then reacted with 5-Iodoacetamido-fluorescein (5-IAF) to give modified protein **3**. (**A**) Coomassie brilliant blue stained (left) or UV-illuminated (right) SDS-PAGE gel of the modified and unmodified protein. (**B**) ESI-TOF-MS analysis of modified protein **3** (M^+[obs.] = 50428.4 Da; M^+[calc.] = 50428.58 Da).

Labeling with biotin

Maleimide-PEO$_2$-biotin (BM) was added from a 10 mM stock solution in a 25-fold molar excess to the TCEP-reduced protein **2**. Biotinylated protein **3a** (MBP-DnaB^C-Cys(BM)-His$_6$, 50.6 kDa) was separated from unlabeled protein using a Soft Link Soft Release Avidin resin (Promega) with subsequent elution with 5 mM biotin. Protein *trans*-splicing was performed by incubating constructs **1** and **3a** in equimolar concentrations of 6 µM at 25°C for 2h (see Figure S2a). Product formation was observed by Coomassie staining of a SDS-PAGE gel and immunoblotting of biotinylated splicing product **6a** (Figure S2b). Mass spectrometry further confirmed the identity of the expected biotinylated splicing product **6a** (Figure S2c).

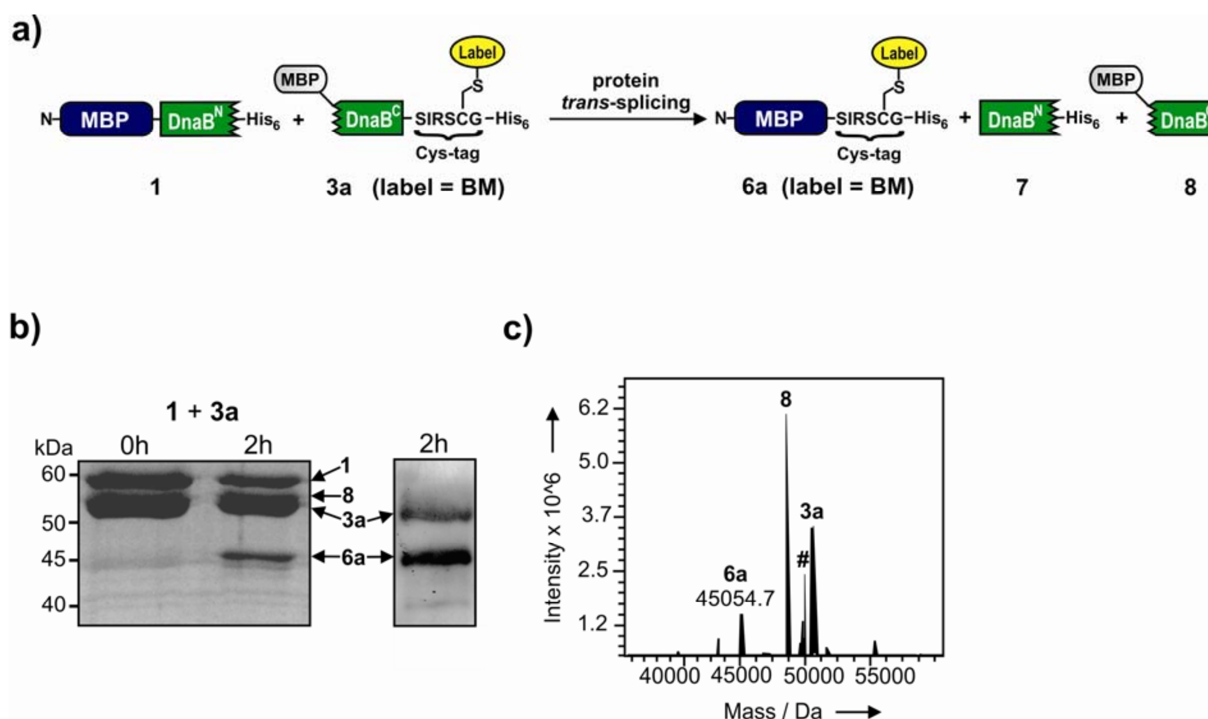

Figure S2: **Modification with biotin.** (A) Schematic illustration of the reaction with biotinylated protein. (B) Analysis of protein *trans*-splicing reaction mixtures at 0h and 2h on a Coomassie stained SDS-PAGE gel (left) and by Western blotting using Streptavidin-HRP (right). (C) ESI-TOF MS analysis of the reaction mixture shown in (A and B) after 2h. Calculated and observed molecular masses are: splice product **6a**, M^+[calc.] = 45055.0 Da; M^+[obs.] = 45054.7 Da; **8**, M^+[calc.] = 48613.7 Da, M^+[obs.] = 48613.1 Da; **3a**, M^+[calc.] = 50565.8 Da; M^+[obs.] = 50566.1 (# = impurity).

Labeling of thioredoxin (Trx)

The StreptagII-Trx encoding fragment from plasmid pCL14 (*1*) was cloned into vector pTK056 using *Nco* I and *Eco* RI restriction sites. From this plasmid the *Nco* I-*Hin*d III fragment was transferred into pET16b (Novagen) to give expression plasmid pTK077 for expression of protein StreptagII-Trx-DnaB^N-His$_6$ (**9a**, 26.9 kDa). This protein was expressed, purified, and used for protein *trans*-splicing as described for construct **9** (see Figure S3a and S3b). Enzymatic activity of fluorescein-modifed Trx (**13a**, 14.5 kDa) was monitored as

described previously (*1, 2*). Briefly, the reaction was started by adding 1 mM DTT to a mixture of insulin (0.1 % [w/v]), EDTA (2 mM) and Trx-protein (10 µM) in 20 mM Tris-HCl, 300 mM NaCl, pH 8.0 at room temperature. Precipitation of the insulin β-chain was monitored photometrically at 650 nm. In two control reactions, the activity of fusion protein **9a** was compared under the same conditions, and the reaction was performed without Trx, respectively. Figure S3c shows that fluorescein-modified protein **13a** was catalytically active. The slightly diminished activity compared to **9a** might result from small variations in protein concentration.

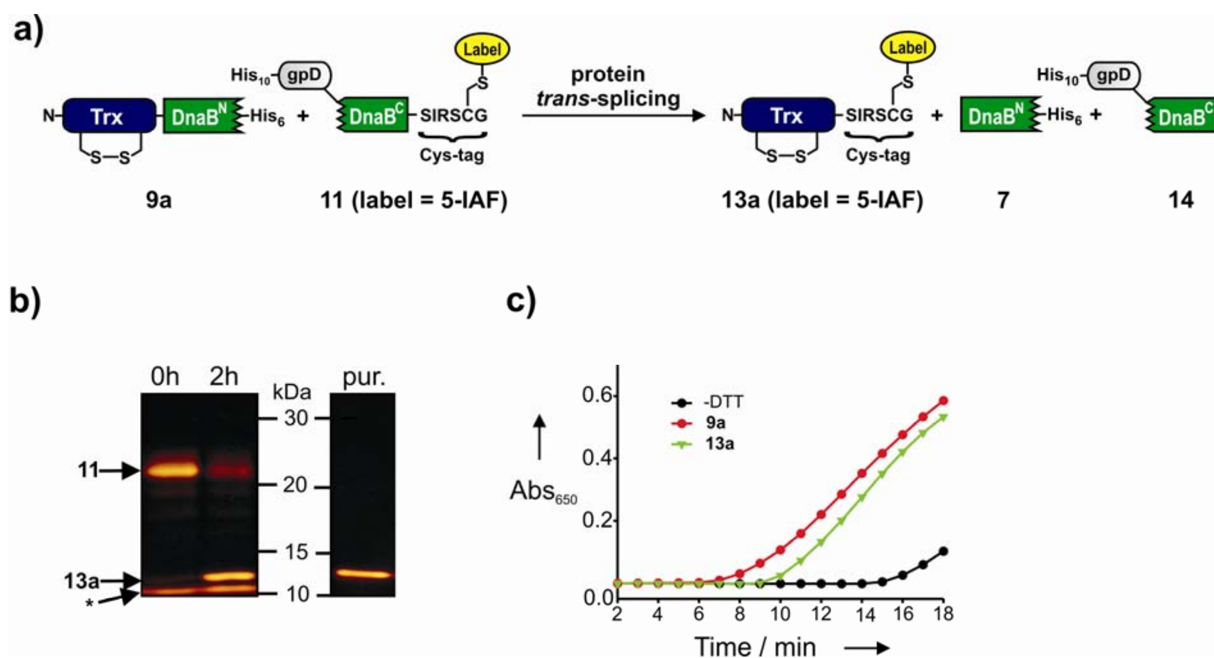

Figure S3: Preparation and enzymatic characterization of fluorescein-labeled thioredoxin. (A) Reaction scheme. (B) Shown are the SDS-PAGE gels under UV illumination of samples taken from the protein *trans*-splicing reaction after 0 and 2h (left), as well as the purified splice product (right). The asterisk denotes excess labeling reagent in the running front of the gel. (C) Photometric assay of thioredoxin activity by monitoring precipitation of the insulin β-chain.

Materials and Methods

General techniques and materials

5-Iodoacetamido-fluorescein (5-IAF), Alexa Fluor C_5 maleimide (AF), Texas Red C_2 maleimide (TR) were purchased from Molecular Probes, Invitrogen, Fluorescein-5-maleimide (FM) and Maleimide-PEO_2-Biotin (BM) were from Pierce. Nitrocefin was ordered from Calbiochem and insulin was purchased from Sigma-Aldrich. For DNA handling standard protocols were applied. Ampicillin was used in a concentration of 100 µg/mL. Oligonucleotides were purchased from Operon (Köln, Germany). All plasmids were verified by DNA sequencing. Standard chemicals were from Sigma Aldrich (Munich, Germany), Roth (Karlsruhe, Germany) or AppliChem (Darmstadt, Germany). Figure S4 shows the chemical structures of the reagents for cysteine modification.

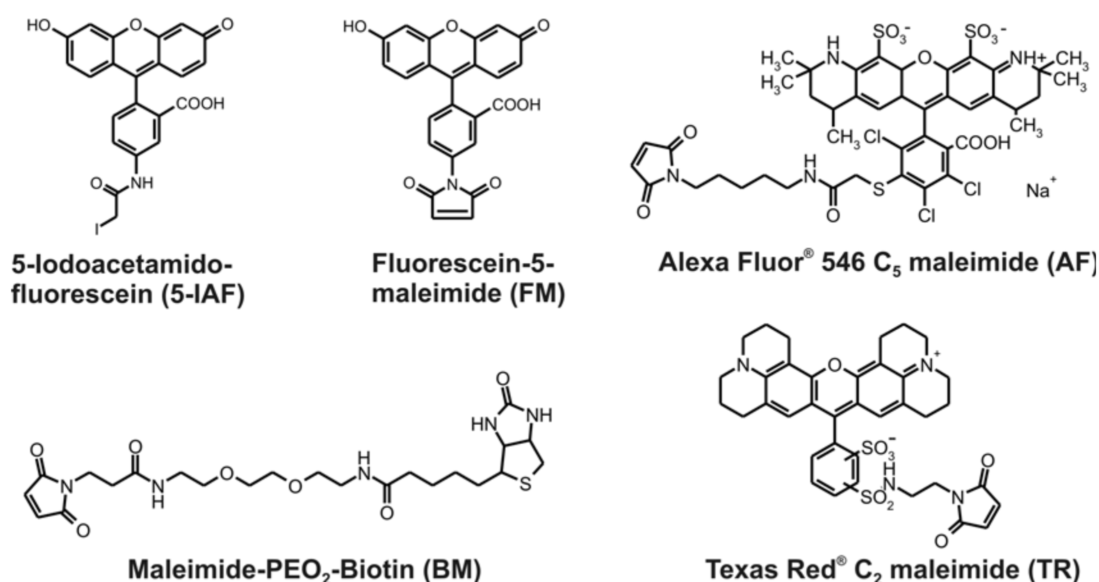

Figure S4: Chemical structures of cysteine modification reagents.

Construction of expression plasmids

Plasmid pTK054 for expression of **2** was obtained by site-directed mutagenesis of pTK055 (*23*) using oligonucleotide 5'-CATAACAGTATTAGATCCTGCGGTCATCACCATCACCATC-3' and the reverse complement by the QuikChange protocol (Stratagene). To construct pSB34 for the expression of **10**, a DNA fragment was amplified from pTK054 using oligonucleotides 5'-AATGGATCCTCTAGTTCACCAGAAATAG-3' and 5'-ATAAAGCTTAACCGCAGGATCTAATACTGTTATGG -3' and cloned into pAT37 (*27*) (kindly provided by Frank Bernhard) via *Bam* HI and *Hin*d III restriction sites. The Bla encoding *Bam* HI-*Hin*d III fragment of the previously reported plasmid pHM137 (*22*) was moved into pQE60 (Qiagen) to give pCL39 encoding Bla-His_6. Subsequently, a fragment encoding $DnaB^N$ was inserted into the *Bgl* II site of pCL39. Finally, the *Nco* I- *Hin*d III fragment of this plasmid was cloned into pET16b (Novagen) to obtain pTK083 for the expression of **9**.

Expression and purification of proteins

For the expression of constructs **1**, **2**, **4**, and **9** *E. coli* BL21 cells were transformed with the plasmids pTK056, pTK054, pTK055, pTK083, respectively. Construct **10** was expressed in *E. coli* XL-Gold cells using plasmid pSB34. Expression was carried out in LB-medium containing ampicillin (100 µg/mL) and glucose (0.2 %) for the MBP carrying constructs. Cells were grown at 37°C to an OD (600 nm) of 0.7, when the temperature was lowered to 30°C, and expression was induced by adding isopropyl-β-thiogalactopyranoside (IPTG, 0.4 mM) for 3-5h. Cells were harvested by centrifugation, resuspended in buffer containing 50 mM Tris, 300 mM NaCl, pH 8.0 and lysed by two passages through an emulsifier. Insoluble material was removed by centrifugation at 30,000 g for 30 min. Proteins **1**, **2**, and **4** were purified from the soluble fraction by loading it consecutively on a Ni^{2+}-NTA (Qiagen) and

amylose column (New England Biolabs) according the manufacturer's recommendations. Fractions containing pure protein were pooled and dialysed against splicing buffer (50 mM Tris, 300 mM NaCl, 1 mM EDTA, 10% glycerol, pH 7.0). For constructs **1** and **4**, 2 mM DTT was present in all dialysis steps. Construct **2** was dialysed once against DTT-containing splicing buffer and three times against splicing buffer without DTT. Protein **10** was purified only by Ni^{2+}-NTA-chromatography and dialysed against splicing buffer. Protein **9** was purified from the insoluble fraction of the *E. coli* lysate under denaturing conditions by solubilization in buffer containing 8 M urea and subsequent Ni^{2+}-NTA-chromatography. The protein was refolded by dialysis at 4°C against splicing buffer containing 2 mM DTT. All proteins were stored at -80°C. Protein concentrations were determined using the calculated molecular extinction coefficient at 280 nm.

Cysteine Bioconjugation with fluorescein reagents

Constructs **2** (65 µM) and **10** (71 µM) were reduced with a 10-fold molar excess of reducing reagent (TCEP or DTT). Subsequently, the labeling reagent was added in a 25-fold molar excess from stock solutions (10 mM for 5-IAF and biotin maleimide, 1 mM for fluorescein maleimide [FM]; Stock solutions were always prepared freshly in 50 mM phosphate buffer [pH 7.2] containing 150 mM NaCl. 5-IAF and FM were dissolved in DMF before diluted in phosphate buffer to give a final DMF concentration of 5%). This mixture was incubated for 2h at 25°C. Then the reaction was quenched with 2-10 mM DTT and excess of labeling and reducing reagent were removed by gel-filtration using a Hi-Trap sephadex G-25 superfine column.

Protein *trans*-splicing and purification of splice products

Reactions were performed in splicing buffer at 25°C. For analysis by SDS-PAGE, aliquots were removed at different time points, mixed with 4x SDS-PAGE loading buffer and boiled before applying on a gel. For proteins modified with a fluorescein reagent, gels were illuminated on a UV-screen (λ = 312 nm) prior to staining with Coomassie brilliant blue. Gels were scanned or photographed and the relative intensities of the protein bands densitometrically analyzed using the program "Scion Image" (http://www.scioncorp.com). Reactions were performed at least in duplicate and error bars represent standard deviations. For the purification of splice products **12** and **13**, the reaction mixtures were mixed with Ni^{2+}-NTA-matrix for 1h and then loaded onto a gravity flow column. The flow-through was collected, concentrated, and stored on ice for further analysis and enzymatic assays.

Cell lysate experiments

E. coli BL21 cells expressing or not expressing **1** were grown, harvested, and lysed as described above. The soluble fraction of the lysate was mixed with **3** and 2 mM DTT and incubated for 1h at 25°C. For product purification, the mixture was subjected to Ni-NTA-chromatography.

Mass spectrometry analysis

Protein solutions (100 µL) were desalted using an 1100 HPLC-system (Agilent) on a 2.1 x 50 mm Zorbax 300SB-C8 column (Agilent). Protein masses were measured by ESI-MS with a QStar Pulsar I TOF mass-spectrometer (Applied Biosystems) or by ESI-FTICR-MS using a Finnigan-LTQ-FT mass spectrometer (Thermo Electron Group.).

Nitrocefin hydrolysis assay

β-lactamase activity was measured by photometrically monitoring hydrolysis of nitrocefin at 486 nm and by using the molar extinction coefficient of hydrolyzed nitrocefin of 20,500 cm^{-1} M^{-1} (*25*). Kinetic investigations were performed by mixing 150 pM of enzyme with increasing concentrations of nitrocefin (10-500 μM) in 50 mM Tris-HCl (pH 7.0). Assays were performed at least in duplicate. Error bars represent standard deviations.

References

1. Ludwig, C., Pfeiff, M., Linne, U., and Mootz, H. D. (2006) Ligation of a synthetic peptide to the N terminus of a recombinant protein using semisynthetic protein trans-splicing, *Angew Chem Int Ed Engl 45*, 5218-5221.
2. Holmgren, A. (1979) Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide, *J Biol Chem 254*, 9627-9632.