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Two-fold Bioorthogonal Derivatization by Different Formylglycine Generating Enzymes

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Abstract: *Formylglycine generating enzymes are of increasing interest in the field of bioconjugation chemistry. They catalyze the site-specific oxidation of a cysteine residue to the aldehyde containing amino acid C^{α}-formylglycine (FGly). This non-canonical residue can be generated within any desired target protein and subsequently be used for bioorthogonal conjugation reactions. The prototypic formylglycine generating enzyme (FGE) and the iron-sulfur protein AtsB display slight variations in their recognition sequences. We designed specific tags in peptides and proteins that were selectively converted by the different enzymes. The combination of the different tag motifs within a single peptide or recombinant protein enabled the independent and consecutive introduction of two formylglycine residues and the generation of heterobifunctionalized protein conjugates.*

Bioconjugation chemistry is a subject of growing interest, especially for the preparation of antibody drug conjugates (ADCs).[1–3] Selective introduction of bioorthogonal functionalities into the protein represents the main challenge in this research area.[4–11] Enzymatic modification avoids complex manipulations of the translational machinery and only requires a short amino acid sequence in the protein of interest, which is recognized by the enzyme to incorporate a bioorthogonal functionality or to connect the payload to the protein.[12,13]

The formylglycine generating enzyme (FGE) is a valuable tool for bioconjugation.[14–16] It catalyzes the oxygen-dependent oxidation of a cysteine residue within the recognition sequence CXPXR in type-I-sulfatases to C^{α}-formylglycine (FGly).[17] This recognition sequence can be introduced into recombinant proteins and converted by FGE in vivo or in vitro.[6,18] Eukaryotic and prokaryotic variants of FGE are known, and different catalytic mechanisms have been postulated.[19–22] In addition, the iron-sulfur protein AtsB of the radical-SAM (*S*-adenosyl methionine) protein superfamily is another FGly-generating system exclusively found in prokaryotes.[23-28] Unlike FGE, AtsB oxygen-independently catalyzes FGly formation with a broader substrate scope also accepting proline-free motifs. Some AtsB variants even accept serine for FGly-generation (Figure 1a).[29-32]

The aldehyde tag methodology has been proven highly efficient for the generation of site-specific ADCs with defined drug antibody ratios.[1,33–36] However, the introduction of different payloads remains a challenge. In order to overcome this problem, we present a new aldehyde tag methodology making use of two different recognition sequences selectively addressed by different formylglycine generating enzymes. Along this line, we also present the isolation of AtsB from *Methanosarcina mazei* (MM-AtsB), its characterization and application in bioconjugation.

The *M. mazei* sulfatase, endogenous substrate of MM-AtsB, contains the recognition sequence CTAGR with an alanine instead of a proline. We assumed that this proline-free motif might not be accepted by FGE and thus bioorthogonality between both enzymes could be achieved. Therefore, a combination of CTAGR for MM-AtsB with CTPSR for FGE and the consecutive application of both enzymes would allow for two-fold modification of target proteins. We first intended to test this predicted bioorthogonality by incorporating these two recognition sequences into a single peptide chain and analyze the specificity of MM-AtsB and FGE mediated FGly formation.

The *mm-atsb* gene was coexpressed with the *isc*-operon from *Azotobacter vinelandii* which encodes proteins supporting iron-sulfur cluster biosynthesis.[23,24] Cells were cultivated in minimal medium under micro-aerobic conditions followed by an anaerobic purification protocol using Ni^{2+}-sepharose chromatography. Typically, 10 mg/L of soluble MM-AtsB were obtained, which was purified to give concentrations up to 22 mg/mL (Figure 1b).

Efficient in vitro conversion of cysteine within CTAGR by MM-AtsB was achieved for substrate peptide **P1** that contains additional auxiliary sequences flanking the core motif as present in the *M. mazei* sulfatase (Figure 1c). Product quantification was performed by MALDI-ToF-MS and integration of the mass signals generated by the cysteine (*m/z* 1580.7) and corresponding FGly variant (*m/z* 1562.8, Figures S1B, C). Comparable ionization efficiencies were verified by analyzing a 1:1 mixture of the short model peptide Ac-YLCTPSR-NH_2 and its FGly analog by MALDI-ToF-MS (Figure S1A). After 40 to 80 min 35-90% of the cysteine residue within **P1** had been converted, depending on the substrate/enzyme ratio (Figure S1D). In order to determine a maximum reaction rate and a K_M value, 1 µM MM-AtsB was incubated with 2.5-25 µM **P1**. Using a Lineweaver-Burk plot a rough V_{max} of 10.4 nmol/min·mg and a K_M of 4.1 µM could be extrapolated (Figures S1E, S1F). Thus, the k_{cat} value of cysteine modification in synthetic peptides by MM-AtsB (0.56 per min, at 30°C) is similar to that of AtsB from *Klebsiella pneumoniae* (1.14

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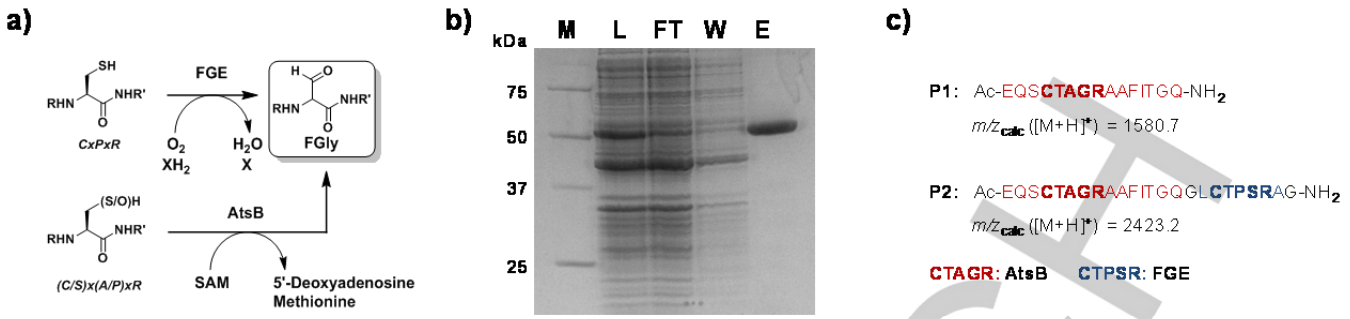

Figure 1: Purification and enzymatic characterization of AtsB from *Methanosarcina mazei* (MM-AtsB). a) The formylglycine generating systems FGE and AtsB have slightly different recognition sequences for the generation of C^{α}-formylglycine (FGly) (XH_2: reducing agent). b) SDS-PAGE (12.5%) analysis (Coomassie stain) of the purification of MM-AtsB (54 kDa) by Ni^{2+}-sepharose (**M**: Marker; **L**: Lysate; **FT**: Flow through; **W**: Wash; **E**: Elution). c) Model peptides for the enzymatic characterization of MM-AtsB with recognition sequences specific for AtsB (red) and FGE (blue) with the core motifs given in bold.

min^{-1}, at 37°C), but clearly higher than that of AtsB from *Clostridium perfringens* (0.048 min^{-1}, at 25°C).[23, 37]

In order to investigate the biocatalytic selectivity of MM-AtsB, peptide **P2** was prepared comprising the 15-mer recognition sequence of **P1** together with the FGE motif CTPSR (Figure 1c). We hypothesized that the specificity of MM-AtsB for the CTAGR sequence might be high enough for a selective introduction of one FGly residue into the peptide backbone (Figure 2a), while FGE should significantly prefer the CTPSR motif.

P2 was converted by MM-AtsB and after 80 min incubation time two MS peaks were observed and assigned to the substrate peptide (*m/z* 2422.9) and the 'mono-aldehyde' derivative (*m/z* 2404.9, Figures 2b, S2). In order to prove the substrate specificity of MM-AtsB, the FGly species were further investigated by MS/MS-experiments. We discovered that FGly-containing peptides show a specific fragmentation at the FGly peptide bond upon laser induced ionizations, thus forming distinct peptide fragments. The predicted aldehyde derivative **P2a** gives rise to a prominent fragment ion at *m/z* 1933.3 in MS/MS measurements (Figures 2c, S3). This can be assigned to a quasi-molecular ion $[M+H]^+$ of a y fragment which is generated from the (FGly)TAGR derivative by cleavage C-terminally to FGly.

In parallel, **P2** was converted by FGE from *Mycobacterium tuberculosis* (MtFGE) to give the aldehyde species **P2b** at *m/z* 2405.4 (Figure 2d).[38,39] MS/MS-analyses provided dominant y- and b-fragment ions at *m/z* 587.7 and 1818.8, respectively, originating from a (FGly)TPSR derivative (Figure 2e). Subsequently **P2b** was incubated with 0.5 equiv. of MM-AtsB to introduce the second FGly. After 80 min of incubation approximately 70% was converted to the corresponding 'di-aldehyde' **P2c** (*m/z* 2387.4, Figures 2f, S2). Thus, a step-by-step introduction of two FGly-residues into the peptide backbone was accomplished in a bioorthogonal manner.

The 15-mer MM-AtsB motif of **P1** and the dual tag of **P2** were attached to the C-terminus of the DARPin E01, which is directed against the epidermal growth factor receptor (EGFR), yielding the model proteins **D1**, **D2** and **D3**, respectively (Figures 3a, S4).[40–44]

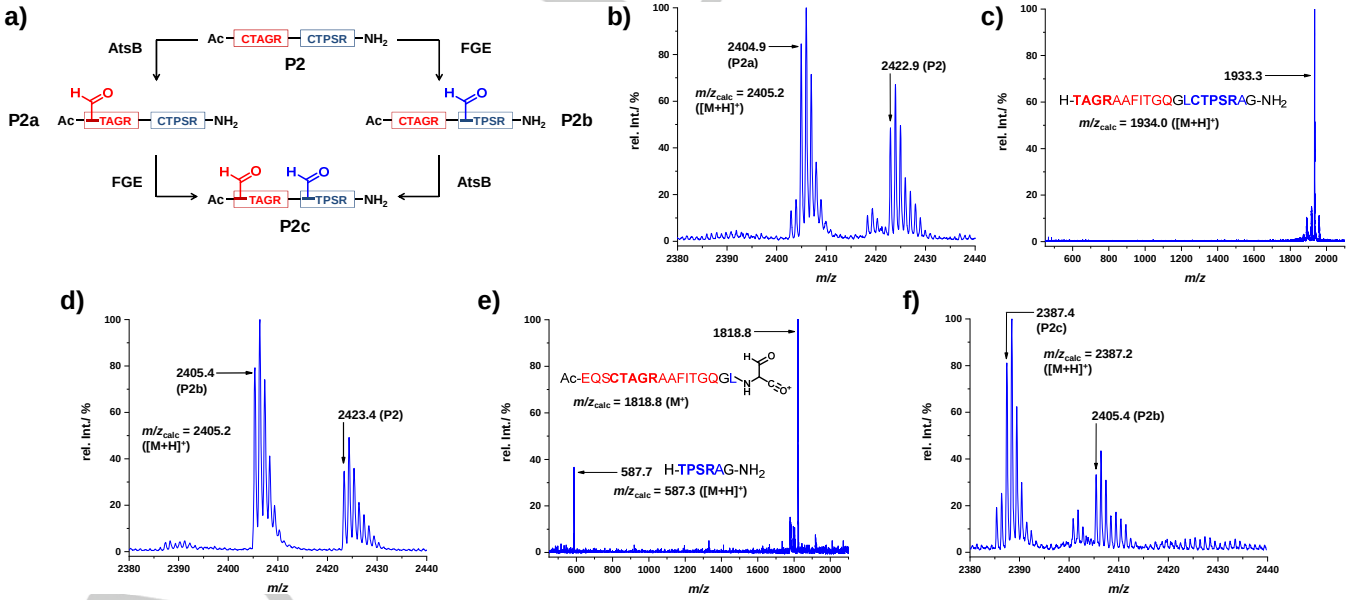

Figure 2: a) Two FGly residues can be successively introduced into a model peptide exploiting the enzymatic preferences of different formylglycine generating systems. b) Enzymatic modification of **P2** by MM-AtsB. The FGly species **P2a** can be observed at *m/z* 2404.9. c) MS/MS (LIFT) analysis of **P2a** yielding a prominent fragment ion at *m/z* 1933.3. d) Enzymatic modification of **P2** by MtFGE yielding the aldehyde **P2b** at *m/z* 2405.4. e) MS/MS (LIFT) analysis of **P2b** gives rise to a fragment ion at *m/z* 1818.8. f) The FGly-containing peptide that was previously prepared with MtFGE (see SI) was modified by MM-AtsB affording the corresponding di-aldehyde species **P2c** (*m/z* 2387.4).

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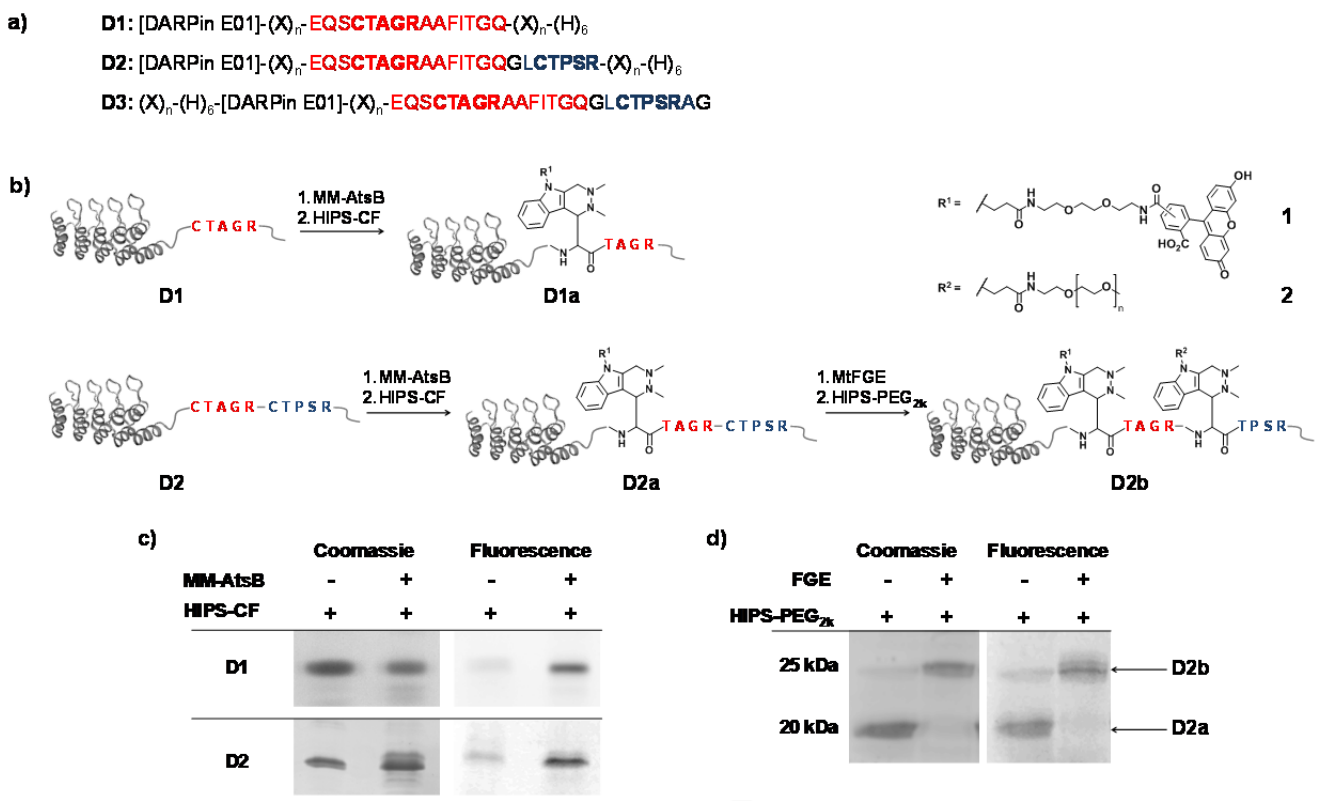

Figure 3: FGly dual tag system in DARPin E01 constructs. a) Three different DARPin constructs **D1**, **D2** and **D3** contain the MM-AtsB and FGE recognition sequences. b) Bioconjugation experiments: **D1** and **D2** were enzymatically modified first by MM-AtsB and fluorescently labeled by HIPS ligation with the carboxyfluorescein derivative **1** (HIPS-CF). Fluorescently labeled **D2** was then further modified by MtFGE followed by PEGylation with **2** (HIPS-PEG_{2k}). c) SDS-PAGE analysis of fluorescently modified **D1** and **D2** (Coomassie stain and in-gel fluorescence detection). Residual labeling in the negative controls is due to endogenous *E. coli* FGly generating system.[45] d) SDS-PAGE analysis of fluorescently modified and pegylated **D2** (Coomassie stain and in-gel fluorescence detection).

The constructs were analyzed by CD spectroscopy using the tag-less DARPin E01 as a reference in order to address potential changes in stability and folding.[46] The CD spectra suggest negligible impact of the recognition sequences on the DARPin secondary structure (Figure S5).

The enzymatic conversions of **D1**, **D2**, and **D3** by MM-AtsB were performed at a 1:1 to 2:1 DARPin/enzyme ratio for 2 h at 30 °C. In order to confirm FGly formation, the reaction mixtures were treated with trypsin either directly or after separation by SDS-PAGE and were analyzed by MALDI-ToF-MS. The data confirm that MM-AtsB accepts the CTAGR motif within **D1** and, furthermore, nearly exclusively modifies CTAGR, but not CTPSR in **D2** and **D3** (Figures 3b, S6-9). Some minor conversion of the CTPSR motif was observed only in the presence of higher concentrations of MM-AtsB.

The enzymatic conversions of **D2** with FGE were performed at a 10:1 of DARPin/enzyme ratio for 1.5 h at 25 °C. Besides *M. tuberculosis* FGE, the homolog from *Streptomyces coelicolor* was used which is known to display a more stringent substrate specificity for proline-containing motifs.[38] However, under the aforementioned reaction conditions, both enzymes almost exclusively converted cysteine within CTPSR (Figure S7).

Proof-of-concept bioconjugation reactions were initiated with the enzymatic modification of **D1** and **D2** by MM-AtsB followed by the FGly-specific Hydrazino-*iso*-Pictet-Spengler ligation (HIPS ligation, Figure 3b).[35] The FGly-containing DARPins were incubated with the carboxyfluorescein derivative **1** (HIPS-CF) for 2 days at 22 °C and pH 6.0, and the success of the conjugation reaction was confirmed by SDS-PAGE and in-gel fluorescence imaging (Figures 3b, c). Afterwards, the conjugates were purified by anion exchange chromatography to remove remaining HIPS reagent and MM-AtsB. The fluorescently labeled DARPin **D2** (**D2a**) was subsequently incubated with MtFGE followed by a second HIPS ligation with HIPS-PEG_{2k} (**2**, Figure 3b). The reaction products were separated by SDS-PAGE to evaluate the success of the two-fold conjugation. **D2a** which had been incubated with MtFGE prior to the addition of **2** showed a significant mass shift from 20 to 25 kDa (Figure 3d), indicating that a second payload was attached in a bioorthogonal way. The overproportional increase of the molecular mass can be explained with the larger hydrodynamic radius of the attached PEG_{2k} moiety.[47]

In order to assess whether the CTAGR-tag or the enzymatic conversion by MM-AtsB would impair the ability of the DARPin to bind EGFR, cell binding assays with fluorescently labelled **D1** (**D1a**) by confocal microscopy and flow cytometry were carried out. The microscopy experiments showed distinct accumulation of fluorescent dye on the EGFR-rich surface of A431 cells (Figure S10A). Binding of **D1a** was not observed for MCF7 cells due to their much lower expression level of the receptor. These results were validated by flow cytometry analyses in which A431 cells showed a higher fluorescence signal shift after incubation with **D1a** compared to MCF7 cells (Figure S10B).

In summary, we present the first dual aldehyde tag motif which incorporates two different recognition sequences that can

be selectively modified by different formylglycine generating enzymes. AtsB from *Methanosarcina mazei* (MM-AtsB) nearly exclusively converts the CTAGR motif within a dual tag that also contains a classical CTPSR site. In contrast, FGE almost exclusively converts cysteine within CTPSR. We incorporated the newly designed tag sequences into the DARPin E01 for proof-of-concept bioconjugation experiments. The results indicate that the consecutive enzymatic conversion by different formylglycine generating enzymes allows for a successive introduction of two distinct payloads into a target protein. Moreover, the model protein retains its function despite the addition of the tag and the enzymatic transformation.

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Conflict of Interest

The authors declare no competing financial interest.

Keywords: bioconjugation • enzyme catalysis • formylglycine • peptides • radical-SAM proteins

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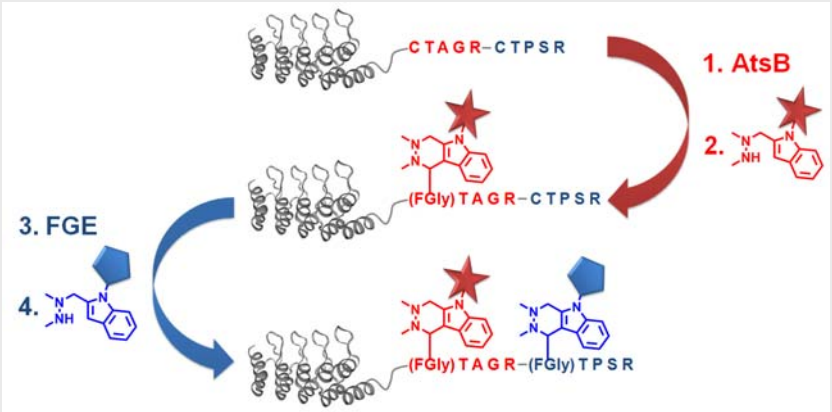

Tobias Krüger, Stefanie Weiland, Georg Falck, Marcus Gerlach, Mareile Boschanski, Sarfaraz Alam, Kristian Müller, Thomas Dierks*, and Norbert Sewald**

Page No. – Page No.

Two-fold Bioorthogonal Derivatization by Different Formylglycine Generating Enzymes

The formylglycine (FGly)-based bioconjugation strategy was expanded by utilizing the iron-sulfur protein AtsB. Combined with the prototypic formylglycine generating enzyme (FGE) the independent introduction of two aldehyde moieties into peptides and proteins was accomplished. This improved enzymatic system represents a new strategy for dual protein labeling at two specific sites.