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Expanding the Scope of Protein *Trans*-Splicing to Fragment Ligation of an Integral Membrane Protein: Towards Modulation of Porin-Based Ion Channels by Chemical Modification

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The selective chemical modification of proteins is crucial for many aspects of basic protein research as well as biotechnological and biomedical applications. We have recently introduced a new strategy for the chemo- and regioselective chemical modification of proteins that relies on the covalent labeling of a cysteine residue and a subsequent protein *trans*-splicing step.[1,2] The latter reaction serves to link the chemically modified polypeptide segment with the protein of interest, which therefore can contain additional cysteines that will remain unmodified. This approach extends the application range of regioselective cysteine bioconjugation to proteins with more than one or essential cysteines. We demonstrated the utility of this strategy for the attachment of a short, labeled peptide sequence, termed the Cys-tag, to the C terminus of various globular and soluble proteins, including β-lactamase and a multidomain nonribosomal peptide synthetase.[1,2] In order to manipulate the conductance pathway of ion channel proteins by chemical modification, we were interested to further expand the scope of this approach to a porin protein and to the incorporation of one or more chemical labels at internal positions of a protein.

Our synthetic target was the porin OmpF from the outer membrane of *Escherichia coli*. OmpF forms a homotrimer with each monomer constituting a large water-filled pore built up by a 16-stranded β-barrel structure (see below and Figure 1).[3,4] In general, an internal cysteine in a protein with multiple cysteines could be selectively targeted if the protein was split into two fragments—one of which would contain the single cysteine to be modified. However, protein folding might be disrupted by the internal split position due to the high probability of aggregation and the likely insolubility of the N- and C-terminal fragments. This creates a major challenge for the reassembly of the two fragments. Moreover, as the splice product OmpF itself would be insoluble in aqueous buffer without additives, we expected that a simple renaturation protocol, as previously reported for several artificially split inteins, would not be successful.[2,5] Indeed, to the best of our knowledge, protein *trans*-splicing has not yet been applied to assemble an integral membrane protein. If membrane proteins are prepared from smaller fragments by native chemical ligation (NCL) or expressed protein ligation (EPL), this problem can be circumvented because these chemical reactions can be performed in the presence of high concentrations of denaturants (for example, guanidinium hydrochloride or urea).[6] In keeping with this line of thought, we were aware of earlier work by Perler and coworkers on the artificially split *Psp*-GBD Pol intein. This intein from the thermophilic strain *Pyrococcus sp.* GB-D was even reported to be active in buffer containing 6 M urea.[7] However, it was only used to ligate two highly soluble and unrelated proteins as well as intact SH3 and SH2 domains from the same protein by protein *trans*-splicing. We therefore set out to test if a split OmpF could also be reassembled by virtue of fused Pol intein fragments under these conditions. Overexpressed, intact OmpF was previously found in the inclusion bodies of *E. coli* and could be solubilized in and refolded from 6 M urea. Importantly, the Pol intein is also compatible with our cysteine labeling procedure because it employs serine as a nucleophilic resi-

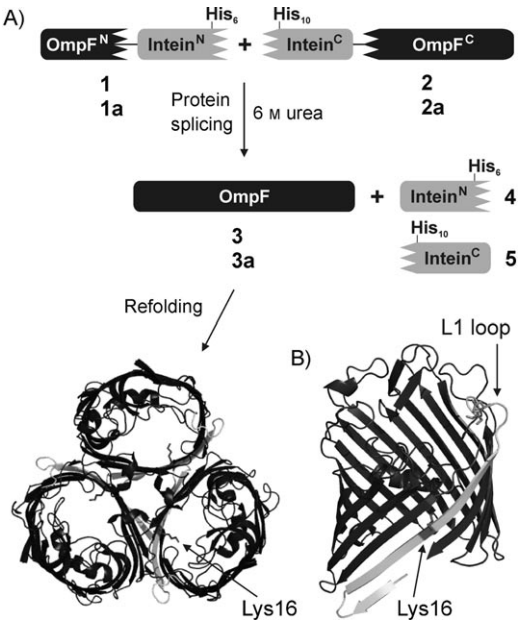

Figure 1. Schematic representation of intein fusion constructs, protein splicing reactions, and OmpF structure. A) Assembly of OmpF by fragment ligation employing the *Psp*-GBD Pol intein and top view of the OmpF homotrimer. See Table 1 for protein sequence details. B) Side view on one OmpF monomer. OmpF^N(1–30) is shown in light grey; OmpF^C(31–340) is shown in black; Lys16, Asn27, and Ser31 are represented as sticks. OmpF structure representations were generated using PyMol and PDB file 2OMF.[4]

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due at both splice junctions. Thus, the split intein constructs are not inactivated through alkylation of a cysteine that is essential for protein splicing.[8]

OmpF intein fusion constructs **1** and **2** were designed with the split position in the flexible L1 loop region of OmpF following the first pore-forming β-strand between Asn30 and Ser31 (see Figure 1 and Table 1).[4] Since the intein's Ser(+1) nucleophile is the only amino acid remaining at the ligation site, protein splicing would produce the native 340 amino acid OmpF sequence **3**. Alternatively, in a second set of constructs, we split OmpF in the same loop region at position 26 and included the intein flanking sequences LEA and SGL at the N- and C-terminal splice junctions, respectively, to give constructs **1a** and **2a** (Figure 1 and Table 1). These sequences were previously shown to result in efficient protein *trans*-splicing with this intein.[7] The constructs were separately expressed in a porin-deficient *E. coli* strain,[9] purified as inclusion bodies and solubilized in buffer with 6 M urea (Figure 2A, lanes a and b).[10] For the assembly of OmpF, constructs **1** and **2** were incubated in splice buffer containing 6 M urea at 37 °C for 18 h. Protein splicing was observed by the appearance of a new band in a Coomassie-stained SDS gel at 37 kDa, which corresponds to the monomeric OmpF reaction product **3** (Figure 2A, lane c). The percent conversion was estimated to be 10–15%. Alternative constructs **1a** and **2a** gave ligation product **3a** more efficiently with about 30–40% conversion; this indicates a beneficial influence of the extra residues that flank the intein (see Figure S1 in the Supporting Information).[10] The identities of the splice products as full length OmpF were also demonstrated by comparison with a truncated OmpF control protein to rule out the possibility of C-terminal cleavage, which is a well-known side-reaction (see Figure S3). As all starting materials and reaction products except for the OmpF splice product contained histidine tags, they could be removed to a large extent by using Ni-NTA chromatography. Subsequently, the OmpF trimer was refolded by insertion into small unilamellar vesicles composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and dodecyl-β-D-maltoside in equimolar amounts.[11] The trimer is stable under conditions of SDS-PAGE as long as the sample is not boiled. Indeed, we observed a new band at the calculated molecular weight of 111.3 kDa, which disappeared upon heating of the sample to 95 °C (Figure 2A, lanes d and e). This finding thus provided the first evidence for the correct structure of the OmpF splice product **3**. The refolding to the trimer proceeded with an efficiency of ~70% as estimated from SDS-gels. Further purification of the refolded trimer was achieved by gel extraction.[10] However, not all of the OmpF-intein starting material **2** could be removed by this procedure (Figure 2A, lanes f and g), probably because of a tendency of this protein to stick to the vesicles used for refolding. Importantly, however, a control experiment showed that pure **2** was not able to refold into a homo-oligomeric structure under these conditions (data not shown). The structural integrity of spliced OmpF **3** was further confirmed by comparison of its circular dichroism (CD) spectrum with that of a recombinantly prepared OmpF reference sample (Figure 2B). The CD spectra showed a minimum at 219 nm and a maximum in the far UV range at 197 nm, which is typical for high β-sheet content and consistent with reported spectra of OmpF.[12]

Table 1. Primary sequences of protein constructs and splice products.

Construct	Sequence	MW [kDa]
1	OmpF(1–30)-*Psp* Pol intein(1–440)-His_6	54.8
1a	OmpF(1–26)-LEA-*Psp* Pol intein(1–440)-His_6	54.7
2	His_{10}-*Psp* Pol intein(441–537)-OmpF(31–340)	47.8
2a	His_{10}-*Psp* Pol intein(441–537)-SGL-OmpF(27–340)	48.5
3	OmpF (native sequence)	37.1
3a	OmpF(1–26)-LEASGL-OmpF(27–340)	37.7

To determine the functionality of the OmpF porin, we performed single channel conductance measurements in a black lipid membrane (BLM). Figure 3A (black line) shows a typical current trace obtained for the purified native OmpF splice product **3**, which exhibits the characteristic properties of the OmpF trimer.[13] Simultaneous opening of all pores in the trimer is followed by closing events of the single monomers with current decrements of 54.5 ± 3.4 pA (Figure 3B). For comparison, we extracted native OmpF trimers from *E. coli* cell envelopes and recorded very similar current traces with current alterations of 51.2 ± 3.0 pA due to monomer closings (Figure 3B and Figure S4A). This represents trimer conductances of 1.17 ± 0.07 nS and 1.09 ± 0.06 nS, respectively, which are in good agreement with previously reported values.[14] Thus, the OmpF reassembled from two fragments by protein *trans*-splicing is a fully active channel protein. Similar activity was also measured for the OmpF splice product **3a**; this indicates that the insertion of six amino acids (LEASGL) into the L1 loop did not affect trimer formation or conductance properties.

Having shown that the integral membrane protein OmpF could be prepared from two

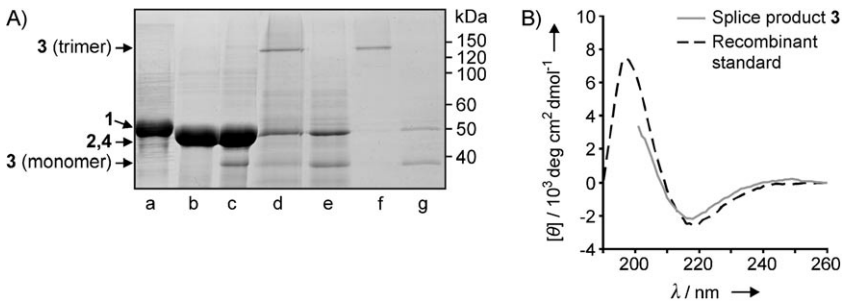

Figure 2. Reconstitution of native OmpF by protein *trans*-splicing and refolding. A) Coomassie-stained SDS-PAGE gel of OmpF preparation (lane a: **1**; b: **2**; c: protein splicing reaction of **1** and **2**; d: refolding mixture of purified **3**; e: sample shown in d after heat denaturation; f: OmpF trimer extracted from SDS-gel, g: sample shown in f after heat denaturation). B) CD spectra.

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fragments by protein *trans*-splicing, we wished to test if this preparation could be combined with the cysteine labeling strategy outlined above. In particular, we were interested in incorporating synthetic groups into the water-filled pore of each OmpF subunit. These synthetic groups could serve to modulate the properties of the OmpF pore, which displays only very little cation selectivity. For example, they could be used to achieve altered selectivity and conductance or ligand binding. Such engineering of protein ion channels represents an exquisite goal with manifold potential applications in nano- and biotechnology, neurobiology and medicine.[15] Prominent examples in this field include the chemical engineering of the pores in hemolysin[16] and the mechanosensitive channel MscL,[17] as well as the light-dependent gating of different potassium channels.[18] Curiously, all of these proteins form a single pore or channel from multiple subunits in a homo-oligomeric fashion. In contrast, each pore of the OmpF trimer is made up of one single polypeptide chain. Therefore OmpF should provide easier access to stoichiometrically defined conjugates with distinct properties due to their asymmetrical composition. Other groups have previously reported the constriction of the OmpF pore using site-directed mutagenesis and by linking small molecules to appropriately positioned cysteine residues through disulfide bridges.[19]

The native OmpF sequence is devoid of cysteines. Only the homing endonuclease part of the Pol intein contains a single cysteine that was mutated to alanine (C351A) in construct **1a** to generate a cysteine-free $OmpF^{N}$-Int^{N} construct. (**1a** and **2a** were used in the following because of higher splicing activity). The side chain of Lys16 in the $OmpF^{N}$ fragment was chosen for modification to modulate the conductance of the channel because it points towards the pore lumen at the site of its largest constriction (see Figure 1).[4] Finally, a K16C mutation was introduced to give construct **1b** (Scheme 1) and this intein fusion protein was prepared as described above. The iodoacetamide building block **6**, which bears an [18]crown-6-derived moiety that should fit into the 7×11 Å constriction zone of the OmpF pore, was synthesized as described in the Supporting Information.[10] Construct **1b** was then reacted with **6** and the resulting modified protein **1b-crown** was then subjected to the protein splicing reaction with protein **2a** to give

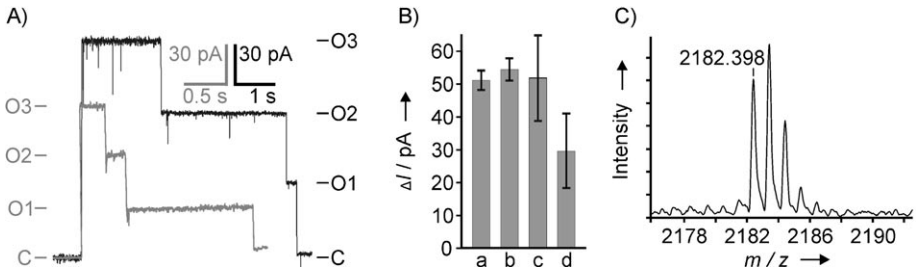
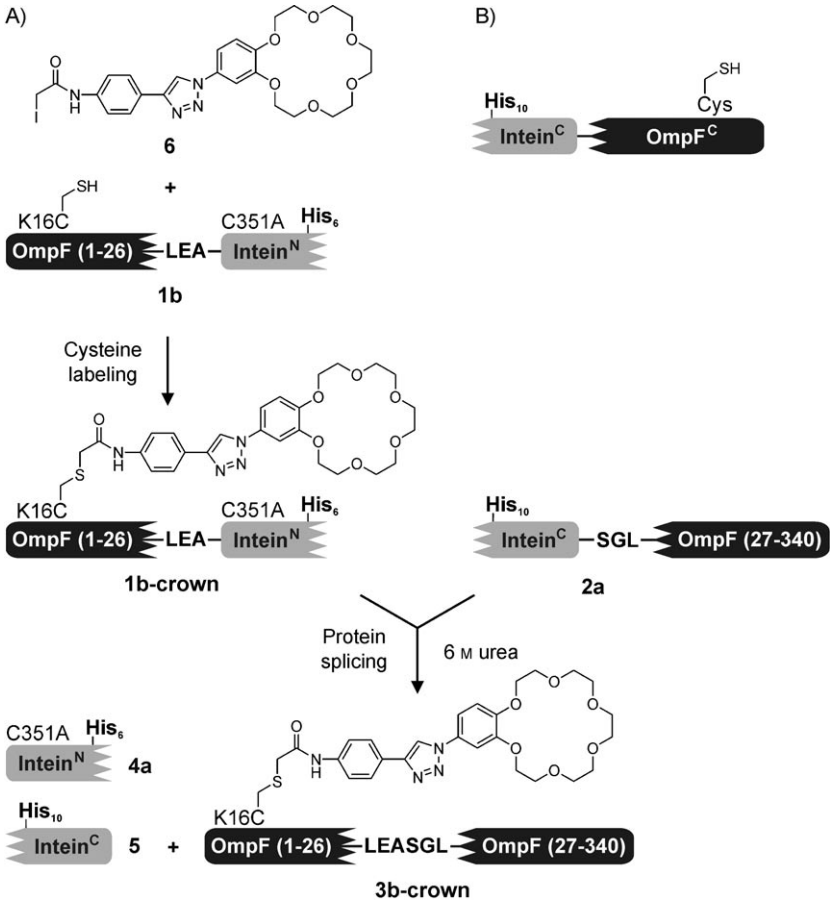


Figure 3. Analysis of chemically modified OmpF pores. A) Current traces of single trimeric pores of splice products **3** (black) and **3b-crown** (grey). BLM measurements were performed in 5 mM Hepes, pH 7.2, 150 mM KCl at 140 mV. Depicted on the right and left hand side are the numbers of open monomers. B) Mean values of current decrements due to monomer closing events within a single OmpF trimer (a: membrane-extracted OmpF standard; b: porin **3**; c: porin **3b**; d: porin **3b-crown**). C) MALDI-TOF-MS analysis of **3b-crown** confirmed the presence of the tryptic peptide fragment containing the modified Cys16 (calc. mass: 2182,039 Da).



Scheme 1. A) Generation of chemically modified OmpF pores. This reaction scheme illustrates the preparation of modified splice product **3b-crown**. B) Illustration of an additional cysteine in the $OmpF^{C}$ fragment that could be chemically modified in a fashion orthogonal to the cysteine in the $OmpF^{N}$ fragment.

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OmpF porin **3b-crown** (see Scheme 1). The latter could be reconstituted to give the modified trimeric porin with an efficiency of ~60% (see Figure S2).[10] Mass spectrometry analysis of a tryptic digest of **3b-crown** further confirmed the correct covalent attachment at Cys16 (Figure 3C). Thus, the protein splicing reaction could be successfully combined with the prior chemical modification at an internal position of the target protein. Finally, BLM measurements of **3b-crown** revealed an average decrement of 29.7±11.3 pA correlated to monomer pore closing events (Figure 3A grey line and Figure 3B, corresponding to an open trimer conductance of 0.64±0.24 nS). This value represents a reduction by ~43% as compared to the native OmpF and the control protein **3b** without modification (Figure 3B and Figure S4B; **3b** is produced by protein splicing of **1b** and **2a**). We assume that this effect is caused by a partial steric constriction of the pore. Synthetic molecules like the crown ether **6** could serve in the future as modulators and synthetic selectivity filters when appropriately anchored inside the pore.

In summary, we showed that the membrane protein OmpF could be assembled by the artificially split Pol intein under denaturing conditions. These results underline the robustness of the split Pol intein for the preparation of proteins from two fragments. We have demonstrated that this fragment ligation by protein *trans*-splicing can be combined with a selective cysteine modification in one protein fragment prior to the protein reassembly. While the product **3b-crown** described in this study also could have been prepared by conventional labeling of a single cysteine in a full-length OmpF, it is important to point out that our approach offers the additional possibility to selectively address a second cysteine in the OmpF pore that is located in the other OmpF fragment (see Scheme 1B; framed insert). By these means, an asymmetric and regioselective attachment of either one molecule by a two-point fixation or two synthetic moieties by one covalent linkage could be achieved in order to more precisely modulate the conductance properties of this channel protein. In general, this approach could serve to selectively attach two labels at two internal cysteines in a protein—for example two fluorophores that could be used to conduct intramolecular FRET experiments.

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Keywords: intein · ion channels · membrane proteins · protein modifications · protein splicing

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