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A fluorogenic native chemical ligation for assessing the role of distance in peptide-templated peptide ligation

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ABSTRACT

Protein-templated reactions have been used for fragment-based drug discovery as well as for covalent labeling, detection and manipulation of proteins. In spite of the growing interest in protein-templated reactions, little is known about the design criteria. Herein we present a systematic study on the effects of proximity in peptide-templated reactions. To facilitate reaction monitoring at low concentrations we developed a fluorogenic native chemical ligation that is based on the integration of a fluorescence quencher in the thiol leaving group. The reaction system provided up to 39-fold increases of emission from a fluorescein unit. By using templates based on coiled coils as models we investigated the effect of misalignments. The distance-reactivity pattern for remotely aligned peptides was remarkably different to reaction scenarios that involved seamlessly annealed peptides with overhanging functional groups.

1. Introduction

Templates facilitate chemical reactions by bringing functional groups into proximity. As a result, templated reactions proceed under dilution conditions when traditional bimolecular reactions are inefficient. Templating is the hallmark of conditional reaction systems in which bond formation (or bond cleavage) is under the control of a third party molecule i.e. the template. Nucleic acids have frequently been used for the design of templated reactions.[1–4] Noteworthy examples include studies of chemical replication[5–7], gene synthesis,[8,9] DNA-encoded libraries[10–15] and nucleic acid detection with chemical amplification.[16–23] Recently, there is increased interest in the development of protein-templated peptide ligations.[24,25] The pioneering studies were focused on explorations of peptide-based replicators.[26–29] The current developments include templated reaction systems for fragment-based drug discovery[30] as well as covalent labeling,[31–35] detection[36] and manipulation of proteins.[37]

Despite the growing evidence for the usefulness of protein-templated chemistry – and in stark contrast to research in nucleic acid-templated chemistry[38–40] – there is little information about the factors that influence the extent to which peptide ligation rates increase upon recognition of peptide templates. First, the rate enhancement induced by the template depends on the concentration of reactants and templates, the rate of the non-templated background reaction and the stability of the reactant-template complex. In addition, templating may affect the activation barrier of the chemical reaction. A key requirement is that the affinity of the reactants for the template is high enough to increase the effective molarity of the functional groups at concentrations too low for reactions in absence of template. There is yet another factor that is difficult to grasp. For a given reaction, the template must align the functional groups in an arrangement that leads to increases in effective molarity. Herein we present a systematic investigation of proximity effects in peptide-templated reactions (Fig. 1) enabled by the first fluorogenic native chemical peptide ligation.

2. Results and discussion

2.1. Reaction design

Coiled-coil formation has previously been used to replicate α-helical peptide from the leucine zipper domain of the yeast-transcription factor GCN4 upon templated native chemical ligation.[26] In our layout of a templated reaction that proceeds without replication we took recourse to the de novo designed coiled-coil peptides $(KIAALKE)_n$ and $(EIAALEK)_n$.[41] This recognition system has allowed the covalent and non-covalent labeling of purified proteins and proteins on live cells.[31] To probe the reactivity of misaligned functional groups we considered, on the one hand, a stepwise extension of the templates T, which contain 3–12 spacer amino acids (X in Fig. 1) between the two binding sites. On the other hand, we envisioned the use of reactive peptides **A** and **B** that seamlessly anneal with the template, yet contain flexible

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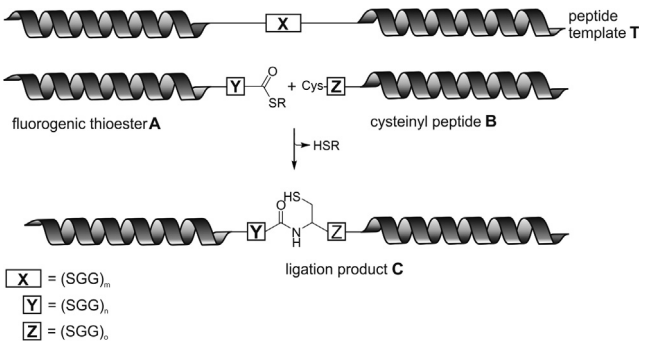

Fig. 1. Assessing proximity effects in peptide (**T**) templated native chemical ligation of peptide thioester **A** and cysteinylpeptide **B** by variation of spacers X, Y and Z.

extensions (Y and Z, Fig. 1) that increase the distance between the functional groups on the template.

2.2. Coiled coil-templated native chemical ligation

The native chemical ligation between reactive peptides **2** and **3** (Fig. 2) was investigated by HPLC analysis of aliquots. Formation of product **4a** proceeded in 59% yield (180 min) and was still not completed at the highest 5 mM concentration tested (Fig. 2B). As expected, the non-templated native chemical ligation proceeded inefficiently at submillimolar concentration. At 50 μM, the non-templated reaction provided only 5% yield after 180 min. The addition of template **1** to reactive peptides **2** and **3** led to a dramatic increase of reactivity. Already after 60 min product **4a** was formed in 40% yield (Fig. 2C). Near maximal 50% conversion was observed after 180 min (Fig. S2A). This shows that the achievable yield is governed by the amount of template (50% template load in this case) and is indirect proof for an existing template effect. The observed rate enhancement conferred by the template showed

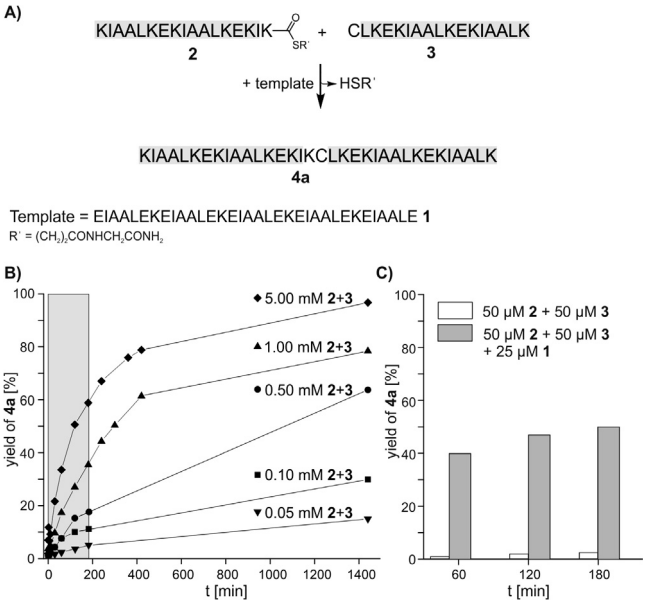

Fig. 2. A) NCL between alkyl thioester **2** and **3** in absence and presence of peptide template **1**. B) Yields of **4 a** determined by HPLC–MS analysis of the NCL between **2** and **3** at peptide concentrations of 0.05 mM, 0.1 mM, 0.5 mM, 1 mM and 5 mM over 24 h (reaction buffer: 100 mM NaH_2PO_4, 2 mM TCEP, pH 7.0). The gray frame indicates the interval of interest in subsequent reactions. C) Yields of **4a** at 60, 120 and 180 min of the NCL between **2** and **3** at 50 μM concentration in presence of 25 μM **1**.

that a 50 μM peptide concentration was sufficiently low to reduce the rate of the background reaction and, at the same time, high enough to permit formation of the split coiled coil complex.

2.3. Synthesis of fluorogenic peptide thioesters

Assessing the various combinations of peptides **A** and **B** in reactions on templates **T** (see Fig. 1) by UPLC analysis would be an arduous task. Fluorescence measurements combine high sensitivity with ease of operation, and are amenable to high throughput. We therefore set out to develop a fluorogenic native chemical ligation. We envisioned a signaling system in which the C-terminal amino acid of the peptide thioester carries both the fluorophore and the quencher (Fig. 3). While the fluorophore remains in the product the quencher is part of the thiol leaving group. As a result, native chemical ligation will lead to enhancements of fluorescence. To avoid an undesired thiol-thioester exchange, resulting in unwanted fluorescence signaling, the native chemical ligation should be performed in absence of thiol additives.[42]

In preparation for the synthesis of fluorescence-quenched peptide thioesters such as **8** we connected Trt-3-mercaptopropionic acid with Disperse Red (**5**) by means of an ester bond (Scheme 1A). Treatment with trifluoroacetic acid and excess triisopropylsilane afforded the quencher thiol component **HSQ**. For the synthesis of peptide thioester **8** we took recourse to the Fmoc-based synthesis of peptide thioesters with self-purification effect previously developed by us.[43,44] The advantages of this method comprise: i) the possibility of modifying the peptide side chains at the C-terminus of the resin-bound peptide at a stage where the orientation of the peptide switches and the C-terminus is readily accessible, ii) full flexibility as far as the choice of fluorophore and quencher is concerned and iii) ease of purification because truncation products as well as excess thiol component can readily be washed away from the resin-bound thioester after thiolysis. The synthesis was commenced with a Fmoc-Gly-loaded polystyrene resin **9** to which Fmoc-Glu(Ph*i*Pr)-OH and, subsequently, 4-sulfamoylbutyric acid was coupled (Scheme 1B). The resulting safety-catch resin **10** was loaded with Fmoc-Lys(Alloc)-OH which provides an orthogonally protected side chain for introduction of fluorescein at a later stage of the synthesis. The peptide chain was elongated by the classic means of solid phase peptide synthesis following the Fmoc-strategy achieving **11**. The cyclization linker **CL** was introduced at the last step of chain assembly. The very acid labile trityl- and Ph*i*Pr-groups were removed under mild acidic conditions and cyclization was induced upon PyBOP activation of the side chain-unprotected glutamic acid residue. After cyclization, the resin-bound acylsulfonamide **12** was treated with trimethylsilyldiazomethane. The resulting *N*-methylated acylsulfonamide is activated for thiolysis, which was induced by treatment with the quencher thiol **HSQ** in presence of sodium thiophenolate. Subsequent washing removed excess thiol as well as capped truncation products which were excluded from macrocyclization and are,

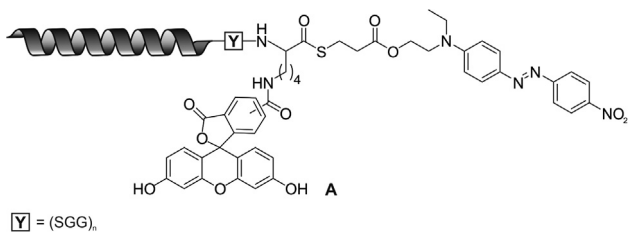

Fig. 3. Fluorogenic peptide thioester **A** contains a thiol component that integrates Disperse Red as quencher of fluorescence emission from the lysine side-chain appended fluorescein.