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New chemistries for chemoselective peptide ligations and the total synthesis of proteins

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The identification of fast, chemoselective bond-forming reactions is one of the major contemporary challenges in chemistry. The requirements of the native chemical ligation — an *N*-terminal cysteine and *C*-terminal thioesters — have encouraged a search for alternative amide-forming ligation reactions. Among successful alternatives to native chemical ligation, are the α-ketoacid–hydroxylamine ligation with 5-oxaproline and, serine/threonine ligation, and potassium acyltrifluoroborate (KAT) ligation. In addition, the KAT ligation, along with the non-amide forming alkyne–azide ligation, is very useful for synthetic conjugations. All of these recent ligation methods were applied to synthesize different proteins, and have allowed chemists to incorporate unnatural amino acids, or to modify the peptide backbone.

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Introduction

The chemical synthesis of peptides and proteins is increasingly important for two major reasons. First, chemical protein synthesis allows the scientist to incorporate unnatural amino acids, posttranslational modifications, or labeling agents, thereby opening new opportunities for the understanding of protein molecules and controlling their biological mechanism of action. Second, the chemical synthesis of peptides and proteins can avoid contamination and other issues that can arise from their production from animal or recombinant sources.

Solid phase peptide synthesis (SPPS) developed by Merrifield [1] is a powerful method for the synthesis of homogeneous protein segments. Although some proteins have been made using exclusively SPPS [2–5] it is often difficult to synthesize protein fragments longer than 40–50 amino acid residues. In order to access larger peptides and proteins it is necessary to employ chemoselective segment assembly reactions, commonly called chemical ligations. In these reactions two mutually reactive functional groups undergo a chemoselective reaction independent of all the unprotected functional groups borne by the amino acid side chains. Ideally, these reactions would proceed in aqueous buffers, at low concentrations and under mild conditions (Figure 1).

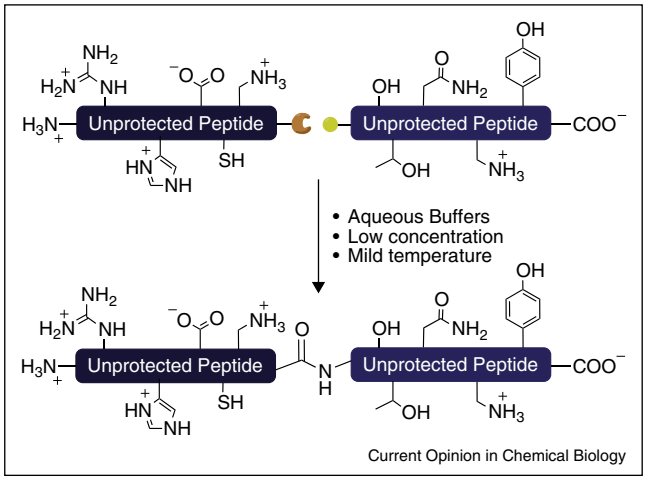
The development of the native chemical ligation (NCL) reaction by Dawson *et al.* [6] in 1994 has been a remarkable breakthrough, allowing an unprotected segment bearing a thioester function at the *C*-terminus to ligate with another unprotected segment bearing an *N*-terminal cysteine residue, under mild aqueous conditions, and in a highly chemoselective way. The reaction typically takes only a few hours at room temperature. NCL has allowed the synthesis of a large number of important proteins [7–10] and has been very well documented in recent years [11–14].

The major limitations of NCL are the difficulty of generating the thioester moiety, especially with Fmoc SPPS, and the low abundance of cysteine residues in protein sequences. Although desulfurization approaches to overcome the second limitation are expanding the available ligation sites, their application in multi-segment ligations remains challenging. Furthermore, there is great desire to have multiple ligation reactions that can be performed orthogonally to one another for the rapid assembly of proteins. In this review we will focus on the recent developments that has have made in the chemical ligation of peptides using reactions that are mechanistically distinct from the native chemical ligation.

KAHA ligation with 5-oxaproline

In an effort to develop a general peptide forming ligation, the Bode group reported the α-ketoacid–hydroxylamine (KAHA) ligation as a chemoselective coupling of large, unprotected peptide segments [15]. The KAHA ligation can be classified into two mechanistically distinct reactions: the Type I ligation with *O*-unsubstituted hydoxylamines and the Type II ligations with *O*-substituted variants [16]. They have demonstrated that the KAHA

Figure 1



General concept of peptide chemical ligation reactions.

ligation is compatible with the synthesis of small peptides such as GLP-1 using the type I ligation [17], however this variant is not well suited to reactions in aqueous media generally employed for peptide solubilization and handling.

More recently, the Bode group designed 5-oxaproline as a monomer ideally suited for protein synthesis via type II KAHA ligations. The incorporation of 5-oxaproline on the *N*-terminus of one peptide segment gives clean ligation reactions with *C*-terminal peptide α-ketoacids under aqueous conditions [18,19••]. 5-Oxaproline has proved to be a powerful *N*-hydroxyamino acid, sufficiently reactive in aqueous solvent, and at the same time stable to ligation conditions and solid phase peptide synthesis. After further investigations and preliminary mechanistic studies (TG Wucherpfennig *et al.* [45]), we have recently determined that the primary products of KAHA ligations with 5-oxaproline are esters, which readily rearrange to the amides in basic buffers, leading to a homoserine at the ligation site (Figure 2a,b). The ligation reaction proceeds cleanly for the assembly of peptide segments with acceptable rates.

The low solubility of some peptide segments has created a particular interest in finding methods in which peptide solubility can be enhanced in order to facilitate the purification of peptide segments and chemical ligations. The utilization of depsipeptides during SPPS has shown great efficiency in overcoming this problem because of the high solubility compared to their amide counterparts [20]. Incorporation of isoacyl dipeptides is often used to improve the biophysical properties, preparation, and purification of hydrophobic peptides [21,22]. The isoacyl peptide esters can be converted to the native peptide amides by an *O*-to-*N* acyl shift in basic aqueous buffers. Many syntheses of depsipeptides employ solid phase peptide synthesis by coupling α-hydroxy acids onto protected α-amino acids. KAHA ligation with 5-oxaproline may have similar advantages in altering the properties of ligation products by the generation of depsipeptides inaccessible by expression or other ligation methods.

The versatility of the KAHA ligation was showcased by the chemical synthesis of Pup [19••] (1 ligation), CspA [19••] (1 ligation), UFM1 [18] (2 ligations), SUMO2 (3 ligations), and SUMO3 (2 ligations) proteins (TG Wucherpfennig *et al.* [45]) (Figure 2c); the preparation of several other proteins with >100 residues is currently in progress. For the synthesis of proteins by multiple KAHA 5-oxaproline ligations, we have shown that the 5-oxaproline moiety can be easily masked with an N^{α}-Fmoc-protecting group that is retained throughout resin cleavage, HPLC purification, and KAHA ligation(s). N^{α}-Fmoc-protecting group can be removed by brief treatment with an amine base in DMSO, providing a facile approach to the synthesis of larger proteins by multiple segment ligations. In our work to date, the mutation of 2–3 amino acids to a homoserine residue has not influenced the ability of the described proteins to form the expected tertiary structures, as assayed by CD spectroscopy and biochemical assays. At the moment we anticipate that the homoserine residue could serve as a surrogate for threonine, serine, methionine, aspartic acid, and asparagine residues. Unless these residues play a structural or functional role we assume to have multiple choices for the ligation sites.

The KAHA ligation with 5-oxaproline provides direct access to depsipeptides and creates a new class of clean chemoselective, ester-forming ligation which may present possible advantages in protein total synthesis by increasing the solubility of the ligated products, glycoproteins, and therapeutic peptides [23,24].

Serine/threonine ligation (STL)

Tam *et al.* demonstrated in 1994 that an unprotected peptide segment bearing a glycolaldehyde ester at the carboxyl terminus was able to ligate to another unprotected segment if there was a serine, threonine or cysteine residue at the *N*-terminus. The ligation proceeds in pyridinium acetate buffer in a chemoselective manner and results in the formation of a pseudoproline at the site of ligation [25–27]. Unfortunately the reaction was rather slow and they did not find a way to convert this pseudoproline into a native peptide bond.

In 2010 Li *et al.* reported a modified version of this aldehyde by using an *O*-salicylaldehyde ester (SAL) at the *C*-terminus. After ligation with another unprotected fragment bearing a terminal threonine or serine they were