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Revisiting ligation at selenomethionine: Insights into native chemical ligation at selenocysteine and homoselenocysteine

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ABSTRACT

Selenomethionine (Sem) has been incorporated recombinantly into proteins many times to elucidate their structure and function. In this paper, we revisit incorporation via chemical protein synthesis to shed light on the mechanism of native chemical ligation. The effect of chalcogen position on ligation is investigated, and selenium-containing peptide ligation is optimized. Additionally, selective methylation is performed on selenolates in a peptide in the presence of unprotected thiols.

1. Introduction

In protein chemistry, certain amino acids serve uniquely useful roles in elucidating the structure, and hence the function, of the proteins in which they reside. One such example is selenomethionine (Sem), the selenium-containing analog of methionine (Met). A product of plants' processing selenium in minerals, selenomethionine is an essential source of selenium for many forms of life.[1,2] Besides its nutritional value in nature, selenomethionine has been used by scientists for years as a tool in both NMR[3] and crystallographic studies[4] in order to uncover the structure and function of proteins. In addition, a Sem and *p*-cyanophenylalanine pair was recently used to probe protein structure, wherein Sem quenches the fluorescence of *p*-cyanophenylalanine via electron transfer, providing a sensitive fluorescent probe to uncover helical structures in proteins.[5] A recent study[6] utilizes a biocompatible, redox-based approach to apply chemoselective modifications to Met; Se's even lower redox potential makes Sem an interesting candidate for future applications.

Sem is undoubtedly a useful tool for protein study, and it is generally introduced into the protein sequence through recombinant expression. However, such an approach replaces all Met residues in the protein sequence with Sem. In addition, not all proteins are easily expressed recombinantly. For these cases, chemical protein synthesis (CPS) can be advisable. CPS, which relies heavily on solid-phase peptide synthesis (SPPS),[7] allows for the building of proteins amino acid by amino acid, and easily enables the chemist to swap out any amino acid in the sequence with natural or unnatural moieties. For shorter peptides and proteins, SPPS can be the sole method employed. For longer sequences, however, ligation of unprotected protein segments using methods such as native chemical ligation (NCL)[8] can be utilized.

NCL allows for the joining of two unprotected peptide segments under mild conditions. In its proposed mechanism, the sulfur of an N-terminal Cys on one peptide attacks the C-terminal, labile thioester of the other segment. A subsequent, irreversible S-to-N acyl shift yields a native peptide bond (Scheme 1). Cys's close relative, selenocysteine (Sec, U)[9–11] is equally capable of participating in NCL; this discovery has since been used in the synthesis of natural and unnatural selenoproteins to shed light on protein folding[12,13] and other aspects of protein chemistry.[14] As a response to Sec and Cys's relatively low occurrence in protein sequences, a variation on NCL was developed in which the thiol of Cys was removed following ligation to yield Ala.[15,16] Similar strategies were used to enable NCL using removable, thiol-containing auxiliaries[17–23] or the use of moieties with thiolated side chains.[24–34] More complex, multistep syntheses employing thiazolidine[35] or selenazolidine[36] "masked precursors" have also been employed to access difficult-to-synthesize proteins.

In a similar vein, NCL was expanded to methionine.[37,38] The initial ligation was performed at homocysteine (hCys, hC) rather than Cys, and subsequently methylated to give the native methionine at the ligation site (Scheme 1). However, this method was not useable

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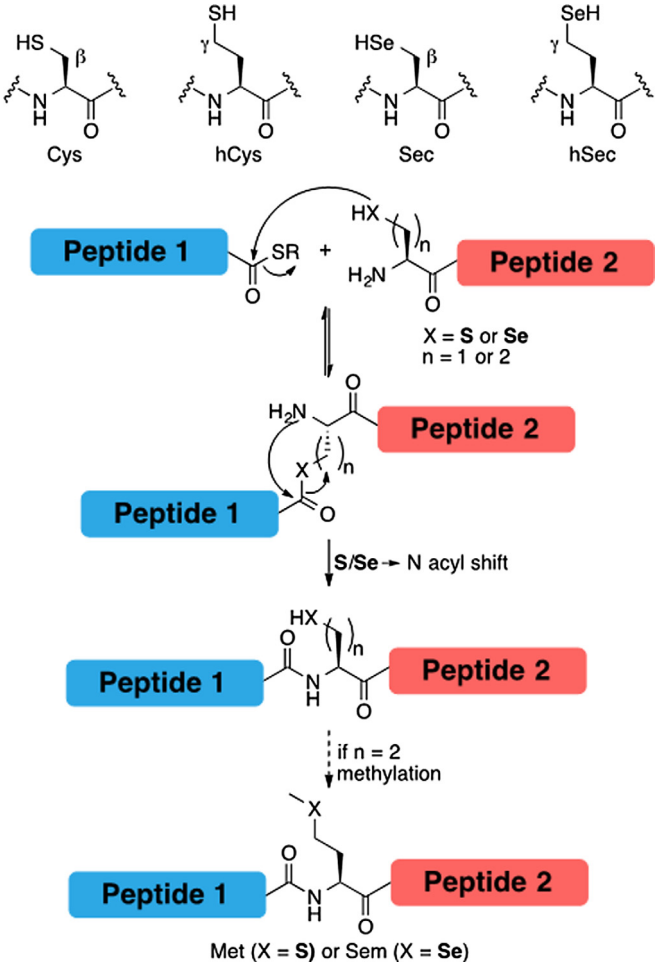

Scheme 1. NCL at Cys, hCys, Sec, and hSec. Methylation after NCL at hCys and hSec will provide Met and Sem.

on proteins containing Cys residues, as Cys's lower pK_a meant it would surely undergo unwanted methylation under the described conditions. In 2003, Roelfes and Hilvert reported successful NCL at Sem using a homoselenocysteine (hSec, hU) precursor,[39] and since that time, to the best of our knowledge, these findings were not utilized in CPS. However, we believe that with optimization, ligation at Sem can provide a much-needed tool in contemporary protein study.

Upon revisiting the paper, we chose to use it as a doorway to further insight in CPS. Firstly, we noted that in ligations at Met and Sem, the chalcogen attacking the thioester is in a γ, rather than β, position on the amino acid side-chain. We wanted to investigate further the effect this imposed on the ligation reaction, and what light it shed on the proposed mechanism of NCL. Secondly, under Roelfes and Hilvert's reported conditions, the ligation of hSec and a thioester was complete after five days.[39] In all likelihood, this can be attributed to the sensitivity of selenols to air-oxidation and formation of stable diselenides.[40] We believed that this time could be improved using recent discoveries made by our group. Thirdly, after noting the different pK_a's of selenols and thiols,[41,42] we wanted to show selective methylation of hSec to Sem was possible in the presence of unprotected thiols. This chemoselective methylation is not possible when methylating hCys to Met, making the case for performing NCL at Sem rather than Met even more powerful and useful.

In this paper, we revisit ligation at selenomethionine to address these points and better understand the nature of NCL.

2. Results and discussion

In order to investigate the effects of β- vs. γ-chalcogens on the overall rate of NCL, a series of small peptides was designed. ZRAFS (Z = Cys, hCys, Sec, hSec) peptides were synthesized to allow analysis of the effect of the β- vs. γ-chalcogen position on ligation as well as to see how thiol and selenol ligations differed under our selected conditions. In addition, LYRAX-COSR (X = Gly, Leu, Val) peptides were synthesized to see how rate of reaction varied with steric hindrance of the thioester.[43] In accordance with standard protocol, all ligations were performed with 3 mM peptides at pH slightly above 7 and in the presence of thiol catalyst (250 mM MPAA).[44] Normally, TCEP is also present in reactions with Cys in order to avoid oxidation of the thiols and encourage faster reactions. However, TCEP is known to remove selenium in Sec,[9,45,46] so TCEP is not normally used in Sec-ligations. This reason, combined with Sec's comparatively low redox potential[40] in comparison to Cys, and its tendency to form stable diselenides under ambient conditions, mean that Sec ligations proceed rather slowly in ambient conditions.[9–11] Luckily, in a recent paper,[46] we reported that sodium ascorbate, a mild radical quencher, successfully hinders the deselenization reaction. For this reason, we included 50 mM TCEP and 100 mM sodium ascorbate in all ligations.

Not surprisingly, ligations involving the sterically unencumbered LYRAG-COSR proceeded the fastest, with all reactions over 90% complete in under two hours (Figs. 1a, S1–S4, S17). No discernible difference in rate was apparent in any of the Gly ligations; β- and γ-chalcogens appeared to ligate at similar rates. Remarkably, both selenol and thiol containing peptides ligated at identical rates, showing that the provided conditions can successfully reduce ligation times at the previously sluggish Sec and hSec.[39]

Ligations performed at the bulkier LYRAL-COSR did take longer – most ligations were complete between two and four hours after beginning (Figs. 1b, S5–S8, S18). While overall rates did seem similar, we noted that ligation at hCys was only 70% complete at two hours when compared to all other ligations, which were over 80% complete. This small but significant difference cannot be explained by different energetics of a five- vs. six-membered transition state of the reaction. Rather, the small difference in rates here is more likely due to differences in pK_a's. Cys is known to have a pK_a of 8.3,[41] whereas hCys has a slightly higher pK_a of 8.9.[42] At ligation pH of 7, a larger percentage of Cys (5.6%) compared to hCys (1.2%) will be deprotonated, allowing it to perform the necessary first step of the reaction, nucleophilic attack at the thioester, slightly faster. In this vein, Sec and hSec ligations should be significantly faster due to their low pK_a's.[41] However, their lower redox potential,[40] and resulting sensitivity to oxidation and formation of diselenides, as explained below, serves to slow their ligation rate significantly.

Our final set of ligations, performed at the quite sterically hindered, β-branched Val in LYRAV-COSR, took significantly longer (Figs. 1c, S9–S12, S19).[43] Both ligations involving thiols were complete within 24 h, and, as a result, our selenol-containing peptides were left to react for similar periods of time. However, at 8 h, some degree of deselenization was observed at Sec and hSec ligations (URAFS and hURAFS, respectively), and at 24 h an even greater amount of deselenization was seen. Taking this as an indication that some amount of sodium ascorbate had decomposed over the longer course of the reaction, we increased the concentration of this radical quencher to 200 mM, a fourfold excess over the reductant and deselenization reagent, TCEP. We were pleased to note that this large excess of sodium ascorbate was capable of inhibiting deselenization to a satisfactory degree for 24 h. Still, selenol-containing peptides were significantly slower in ligating when compared to thiol-containing peptides, with the selenol-containing