Peptides

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Preformed Selenoesters Enable Rapid Native Chemical Ligation at Intractable Sites**

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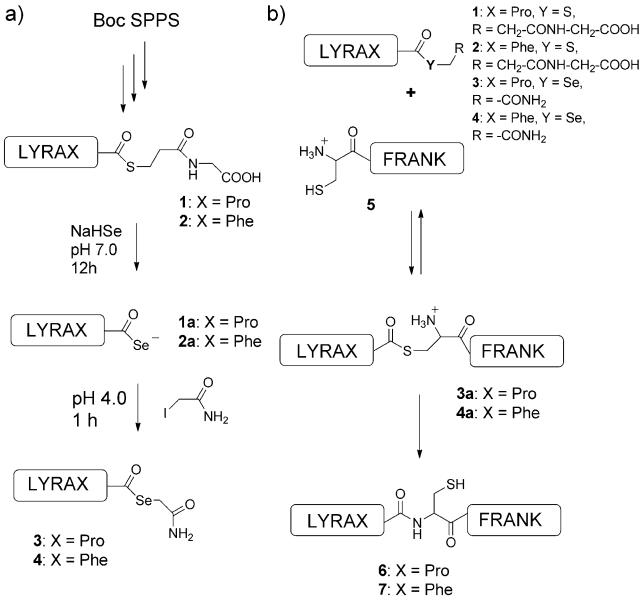
The discovery of native chemical ligation (NCL) by Kent and co-workers in 1994 was a critical step, enabling researchers to chemically access medium-sized proteins through total or semi-synthesis.[1] Virtually hundreds of proteins have now been prepared by the NCL method ranging in size from small polypeptides to medium-sized proteins of more than 200 amino acids.[2] The original conceptual framework of NCL is based on the reaction between an unprotected peptide with a C-terminal thioester and another unprotected peptide carrying an N-terminal cysteine (Scheme 1b).[2b] The sulfhydryl



Scheme 1. a) One-pot synthesis of peptidyl selenoesters from the corresponding thioesters. b) Mechanism of NCL.

group of the N-terminal cysteine residue undergoes a transesterification with the C-terminal thioester, which is generally believed to be the rate-determining step of the overall reaction. The resulting thioester-linked intermediate spontaneously and rapidly rearranges through an intramolecular S→N acyl shift to yield a native peptide bond.

Several parameters have been shown to affect the rate of an NCL reaction, including pH, the chemical structure, and the reactivity of exogenous thiol catalysts and the thioester leaving group, as well as the identity of the C-terminal amino acid of the thioester segment.[3] Systematic analysis has shown that specific amino acid thioesters—notably Ile, Val, Thr, and Pro—react prohibitively slowly.[3b] As long NCL reaction times (> 24 h) may lead to significant side reactions (notably thioester hydrolysis/direct aminolysis, tris(2-carboxyethyl)-phosphine HCl (TCEP) mediated desulfurization) these residues are generally unsuitable for Xaa–Cys ligation, and traditionally have been avoided. Peptidyl prolyl thioesters are by far the least reactive of all amino acids in a NCL reaction.[3b,4] This lack of reactivity of the prolyl thioesters appears to be the result of an orbital interaction of the carbonyl oxygen atom of the adjacent amino acid residue and the carbonyl carbon atom of the thioester functionality. Such an interaction effectively reduces its electrophilicity.[4] This led us to consider strategies that would increase the low inherent reactivity of prolyl thioesters and consequently allow them to undergo facile NCL. We reasoned that peptidyl prolyl selenoesters would be more effective as acyl donors than the corresponding thioesters because of the better leaving group characteristics of a selenolate versus a thiolate. While the use of selenocysteine (Sec) in NCL is well documented,[5] preformed peptidyl α-selenoesters have not been described nor has their potential in NCL reactions been investigated.

To probe their feasibility for NCL we undertook the synthesis of the peptidyl α-selenoesters **3** and **4**. The α-thioester peptides **1** and **2** were prepared by the standard in situ neutralization protocol for Boc (*tert*-butoxycarbonyl) solid-phase peptide synthesis (SPPS; Scheme 1a),[6] and after cleavage from the solid support and purification, substitution of the thioester moiety with a selenoester functionality was achieved by a two-step procedure. First, the peptide thioesters were converted into the corresponding selenoacids **1a** and **2a** by treating the unprotected peptide (5 mM) with NaHSe (300 mM) in aqueous buffer at pH 7 for 10 hours. As the resulting selenoacids appeared to be susceptible to hydrolysis under conditions commonly employed for HPLC purification of peptides (TFA/acetonitrile/water, pH 1–2; TFA = trifluoroacetic acid), they were converted without purification in one pot into the corresponding α-selenoesters **3** and **4** by alkylation with 2-iodoacetamide at pH 4 for 2 hours, and purified by HPLC (see the Supporting Information). This simple procedure allows preparation of peptide α-selenoesters from readily available peptide α-thioesters in near quantitative yield and appears to be compatible with most side-chain functionalities commonly found in complex unpro-

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tected peptides (see Figures 1 and 2 in the Supporting Information).

We next set out to compare the reactivity of the peptides **1**–**4** in NCL reactions with peptide **5** under standard NCL conditions (6 M guanidine HCl, 0.2 M sodium phosphate, 50 mM 4-mercaptophenylacetic acid (MPAA), 100 mM TCEP, pH 6.8, each peptide at a concentration of 1 mM; Scheme 1 b).[3a,7] Aliquots were removed regularly and were quenched by adding an equal volume of a solution of 20% acetonitrile, 5% TFA, and 75% water. The aliquots were subsequently analyzed by HPLC, which allowed us to monitor both product formation (Figure 1) and various reaction intermediates (see the Supporting Information). Second-order rate constants (Table 1) were derived from the integrated rate equation by following the consumption of the starting material (CFRANK, **5**) as described recently.[4]

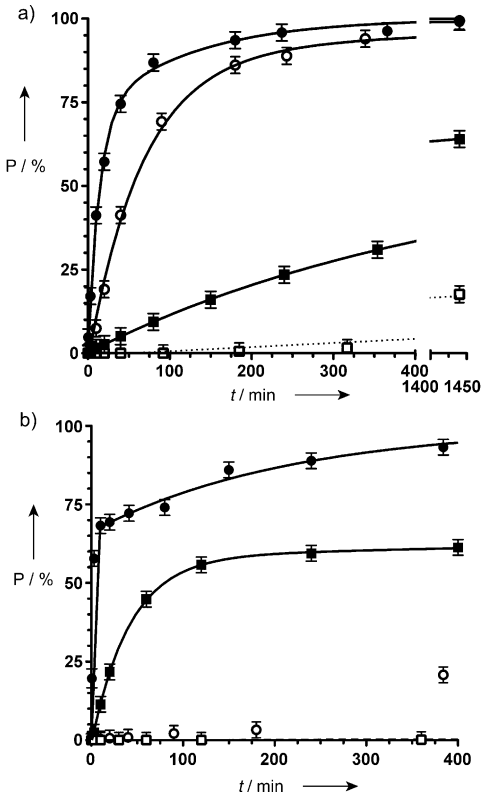

Figure 1. Product formation (P, as percent complete) as a function of time for NCL of **1** (□), **2** (○), **3** (■), and **4** (●) with CFRANK (**5**). Reaction conditions: a) 6 M guanidine HCl, 0.2 M sodium phosphate, 50 mM 4-mercaptophenylacetic acid (MPAA), 100 mM TCEP, pH 6.8 or b) 6 M guanidine HCl, 0.2 M sodium phosphate, 28 mM DPDS, 100 mM TCEP, pH 6.2. Peptide concentration: 1 mM. Data were fitted to a double exponential equation.

Our data (Figure 1 a, Table 1) show that the LYRAP-thioester **1** reacts at almost three orders of magnitude slower than the LYRAF-thioester **2**, results that are in agreement with results previously obtained by the groups of Dawson and Kent.[3b,4] Gratifyingly, both **3** and **4** reacted significantly faster than the corresponding α-thioesters. Detailed analysis (see the Supporting Information) however showed, that both

Table 1: Second-order rate constants for the NCL of peptide thioesters and selenoesters.

Peptide ester	Standard NCL conditions[a]		Selenol NCL conditions[b]	
	k [$M^{-1}s^{-1}$]	Factor	k [$M^{-1}s^{-1}$]	Factor
LYRAP-[COSR^1]	0.00057 ± 0.00066	1	n.d.	n.d.
LYRAF-[COSR^1]	0.437 ± 0.032	766	n.d.	n.d.
LYRAP-[COSeR^2]	0.019 ± 0.002	33	0.198 ± 0.007	347
LYRAF-[COSeR^2]	0.827 ± 0.039	1450	7.7 ± 0.1	13 500

[a] 6 M guanidine HCl, 0.2 M sodium phosphate, 50 mM MPAA, 100 mM TCEP, pH 6.8; [b] 6 M guanidine HCl, 0.2 M sodium phosphate, 28 mM DPDS, 100 mM TCEP, pH 6.2. n.d. = not determined; $R^1 = -CH_2-CH_2-CONH-CH_2-COOH$; $R^2 = -CH_2-CONH_2$.

3 and **4** rapidly transesterified with the thiol catalyst MPAA to form the corresponding thio(4-carboxymethyl)phenyl ester: transesterification of the phenylalanyl selenoester was quantitative in less than 1 minute, whereas the prolyl selenoester reacted quantitatively within 10 minutes. In contrast, the thioalkyl esters **1** and **2** were very slow to form the activated thioaryl ester intermediate, which did not accumulate significantly over time and was rapidly consumed (see the Supporting Information). It seems plausible that the rate enhancement observed for α-selenoesters versus α-thioesters is primarily due to significantly faster formation of the activated thioaryl ester. Thus, in the case of **3** and **4**, the rate-determining step is likely to be trans-thioesterification of the (rapidly formed) thioaryl ester with the cysteine sulfhydryl, whereas for **1** and **2** the overall reaction rate is limited by formation of the thioaryl ester. Hence, similar rate enhancements should be achievable by using preformed MPAA thioesters.[8] Our data indicates that peptide selenoesters are superior acyl donors for thiol nucleophiles in NCL, a finding that is consistent with the exceptional leaving group ability of selenolates. The observation that transesterification of selenoesters with the MPAA catalyst leads rapidly and quantitatively to the formation of the corresponding thio(4-carboxymethyl)phenyl ester and selenol, suggests a highly exergonic process that is kinetically favored. Hence, this energetically favorable (downhill) reaction leads in fact to deactivation of the peptide α-selenoester and loss of acylation power, which led us seek alternative ways to preserve its original reactivity.

In view of these findings we next turned our attention to the NCL catalyst. As mentioned above, the thiol catalyst plays a critical role during NCL. It serves as a mild reducing agent helping to keep cysteine sulfhydryls in the reduced state and during trans-thioesterification activates the less reactive thioalkylesters as well as reverses unproductive thioesters.[3a,c] We anticipated that a selenol catalyst such as selenophenol (generated in situ from diphenyldiselenide (DPDS) and TCEP) could substitute effectively for MPAA in NCL. To this end, ligations of **1**–**4** with CFRANK (**5**) were repeated in 6 M guanidine HCl, 0.2 M sodium phosphate, 28 mM DPDS, 100 mM TCEP, pH 6.2 with each peptide at a concentration of 1 mM (Figure 1 b, Table 1). Under these reaction conditions both **1** and **2** reacted sluggishly, thus demonstrating both the poor reactivity of thioalkyl esters in NCL as well as the

inability of the selenophenol catalyst to efficiently activate them through transesterification.

In contrast, the α-selenoesters **3** and **4** rapidly and quantitatively trans-selenoesterified with selenophenol (**4**: <30 s, **3**: <10 min) to form the corresponding peptidyl selenophenyl esters. Inspection of the kinetic data revealed pronounced biphasic product formation[*] (Figure 1b): a fast phase up to about 55–65% completion at $t=5$ min [**4**, $k=(0.69\pm0.10)$ min^{-1}] followed by a slower phase [**4**, $k=(4.8\pm0.8\times10^{-3})$ min^{-1}]. This behavior coincided with the appearance of a new peak in the HPLC chromatograms and was identified as LYRAXC(LYRAX-[COS-])FRANK. Evidently, this branched thioester originates from a side reaction, in which the product cysteine sulfhydryl is additionally acylated by the selenoester. Hence it seems plausible, that for the fast phase the rate-determining step is transesterification between the selenoester and CFRANK to form the desired ligation product LYRAXCFRANK. As the reaction progresses, the product will compete with CFRANK for the selenoester, which is essentially consumed within 20 minutes. Thus, the rate-determining step for the slow phase is likely the dissolution of the branched thioester by transesterification with selenophenol and re-activation of the acyl donor.

To minimize this side reaction we reasoned that a simple solution would be to increase the ratio of **5** relative to the selenoester given that the starting material acyl acceptor **5** will more effectively outcompete the product for the acyl donor. This is indeed the case (Figure 2): when **3** (1.0 mM) is reacted with increasing concentrations of **5** (1.0–5.1 mM) the

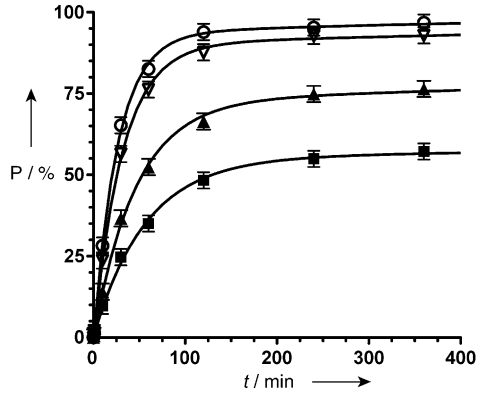

Figure 2. Product formation (P) as a function of time for NCL of **3** (1 mM) and increasing concentrations of CFRANK **5**: 1 mM (■); 1.5 mM (▲); 3.4 mM (▽); 5.1 mM (○). Data were fitted to a double exponential equation.

plateau characterizing the end of the fast phase is shifted considerably to higher product yields. Thus, by using peptidylprolyl selenoesters such as **3** and an excess of **5** under the conditions described above, near quantitative (>90%) NCL at proline is achieved in as little as 2 hours—a rate enhancement of about 350 times when compared to standard NCL reactions. In practical terms the requirement for an excess of **5** is acceptable, because excess peptide can be easily recovered during the customary HPLC purification step following a NCL.

Under similar reaction conditions **4** achieved near quantitative (>95%) conversion within a few minutes, thus bringing the NCL rates in line with more traditional procedures for amide formation (e.g. anhydrides or active esters as acyl donors in SPPS; see Figure 7 in the Supporting Information). Despite the superior reactivity of peptidyl selenoesters as acyl donors in NCL, the chemoselectivity is maintained, that is, no reaction was observed between **4** and peptide AFRANK, which lacks the essential β-mercapto group of cysteine (see the Supporting Information). Moreover, the selenoester-mediated NCL proceeded without epimerization at Phe or Pro, and the peptide configuration and stereochemistry were fully retained (see the Supporting Information).

The underlying second-order rate constants (Table 1) revealed that in the presence of selenophenol at pH 6.2 ± 0.1, the α-selenoesters reacted about one order of magnitude faster than when MPAA was used as a catalyst at pH 6.8 ± 0.1 (Table 1). This directly supports our initial hypothesis that α-selenoesters are superior acyl donors in NCL. In contrast, selenophenol as a catalyst is less potent than MPAA in reversing unproductive thioesters such as the branched thioester intermediates. This is exemplified by comparing the observed rate constants of the slow phase (Figure 1) for **4** under standard NCL conditions [$k=(8.1\pm1.7\times10^{-3})$ min^{-1}] to those under selenol NCL conditions [$k=(4.8\pm0.8\times10^{-3})$ min^{-1}]. These findings can be explained on the basis of the pioneering work of Hupe and Jencks on the mechanism and structure–reactivity relationships of acyl-transfer reactions.[9] Selenols are more acidic than structurally similar thiols, for example, the pK_a of the mercapto group of cysteine is approximately 8.3, whereas that of the selenol of selenocysteine has been determined to be around 5.4.[10] Consequently, selenolate is a weaker nucleophile but better leaving group than a structurally similar thiolate. Thus, in a simple transesterification reaction between a selenoester and a thiolate the equilibrium will favor the thioester and free selenolate—well in agreement with our observations.

In its current form the selenoester-mediated NCL is incompatible with unprotected nonligation site cysteines largely because of the inefficiency of the selenol catalyst to reverse the formation of unproductive thioesters. However, we anticipate that selenols equipped with electron-donating substituents leading to increased basicity/nucleophilicity of the selenol functionality will be more efficient catalysts in selenoester NCL than the selenophenol used herein; work in this area is currently underway. Finally, our findings should justify an examination of selenoacids and selenoesters in other thioester/thioacid-based coupling techniques, such as the direct aminolysis method.[11]

[*] This biphasic product-formation behavior is also evident in NCL reactions under standard conditions (Figure 1a), but much less pronounced.

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