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Communication

Mechanism of N-to-S acyl transfer of *N*-(2-hydroxybenzyl) cysteine derivatives and origin of phenol acceleration effect

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ABSTRACT

N-(2-Hydroxybenzyl)cysteine derivatives were recently disclosed to be efficient crypto-thioesters for native chemical ligation (NCL). To elucidate the mechanism of the relevant N-to-S acyl transfer process as well as the origin of the acceleration effect of the phenol substitutes, a density functional theory (DFT) study was performed. It was found that the N-to-S acyl transfer of *N*-(2-hydroxybenzyl)cysteine derivatives involve four major steps: concerted nucleophilic addition of thiolate/proton transfer, inversion of an amine moiety, water-assisted proton transfer and C—N bond cleavage. The phenol substitutes promote the nucleophilic addition of thiolate by protonating the carbonyl oxygen atom synergistically and the proton transfer from hydroxyl to amide nitrogen atom is the rate-determining step of the N-to-S acyl transfer. By contrast, changing the phenolic hydroxyl to methoxyl was found to significantly slow down the nucleophilic addition of thiolate and thus hinders the N-to-S acyl transfer overall. These computational results are consistent with the observation of previously reported control experiments, by which our proposed mechanism is further validated.

Chemical synthesis enables precise control on protein composition and incorporation of nonnatural functionality into proteins. Owing to these advantages, chemical synthesis of proteins found wide applications in biochemistry [1], such as protein ubiquitination [2], intein splicing [3], mirror image proteins [4] and all-$_L$ and all-$_D$ varients of proteins [5]. Meanwhile, chemical synthesis is also an important access to protein pharmaceuticals [6], *e.g.*, nonglycosylated human EPO [7], parathyroid hormone [8] and homogenously glycosylated human interferon-β [9].

The key to the success of chemical synthesis of proteins is the chemical ligation of unprotected synthetic peptides. Native chemical ligation (NCL), a method developed in 1994 and involving the reactions of C-terminal peptide thioesters and N-terminal cysteine-containing peptide [10], has now evolved into one of the most popular ligation methods [11–13]. *Tert*-butyloxycarbonyl (Boc) solid phase peptide synthesis (SPPS) approaches were originally used for the preparation of peptide thioester but had been gradually replaced by 9-fluorenylmethoxycarbonyl (Fmoc) SPPS methods due to the troublesome HF treatment [14]. Nevertheless, the piperidine used for the deprotection in Fmoc SPPS approaches also possible wrecks peptide thioesters. To solve this problem, the strategy that peptide thioesters are generated after Fmoc SPPS by treating more stable precursors, *e.g.*, *N*-acylureas [15,16], benzotriazole [17] and hydrazides [18,19] with thiophenol, was developed. On the other hand, intramolecular acyl transfer, which is advantageous in entropy effect, was also utilized to prepare peptide thioester [14]. In the scenario of N-to-S acyl transfer (Scheme 1), amino acids bearing protected mercapto groups are employed for peptide chain elongation. After that, the prepared crypto-thioesters transform into real thioesters *via* intramolecular N-to-S acyl transfer and react with *N*-terminal cysteine-containing peptide fragments to complete NCL.

In this context, various structurally different crypto-thioesters have been developed [20–26], aiming to realize rapid acyl transfer under mild conditions. Very recently, Aucagne and co-workers reported *N*-(2-hydroxybenzyl)cysteine thioesterification devices (Scheme 1), which rapidly rearrange into thioesters at neutral pH and thus one-pot reactions of thioester formation and NCL are allowed [27–29]. The NCL using Ala and Ser crypto- thioesters can be complete in 24 h at 37 °C with the apparent second order kinetic constants close to the values of standard NCL with thioesters [30]. This method was also successfully applied to synthesize the cysteine-rich peptides, and the reduced form of MT7 and Cg-BigDef1 were obtained with isolated yields of 14% and 18%, respectively. The appealing performance of *N*-(2-hydroxybenzyl)

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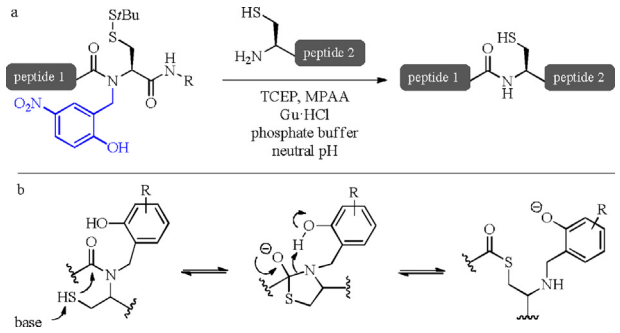

Scheme 1. (a) NCL of *N*-(2-hydroxybenzyl)cysteine crypto-thioesters and (b) previously proposed mechanism for the corresponding N-to-S acyl transfer.

cysteine thioesterification device was found to result from the phenol substitutes according to control experiments. Specifically, replacing the phenolic hydroxyl by methoxy makes the crypto-thioester unreactive. Meanwhile, the acyl transfer/NCL reaction was found to be slightly slower at lower pH than at neutral pH. Blocking the thiol group with stable protecting group leaded to a worse NCL yield, suggesting a N-to-S acyl transfer mechanism rather than a N-to-O shift mechanism. As to the role of the phenol, Aucagne proposed that the amide nitrogen atom is protonated by the phenol *via* a six-membered transition state during the acyl shift (Scheme 1b).

In addition to experimental studies, the rapid growth of NCL and other peptide ligation methods in recent years has also brought much attention to investigating the relevant reaction mechanisms with theoretical methods [31–39]. However, the detailed mechanism of N-to-S acyl transfer of crypto-thioesters as well as the origin of acceleration effect of substitutes was rarely covered. To consummate the understanding of the thioester formation from crypto-thioesters, a case study on the N-to-S acyl transfer of C-terminal *N*-(2-hydroxybenzyl)cysteine derivatives was performed in this manuscript with the aid of DFT methods.

DFT calculations were performed at the level of M06-2x/def2-svp/SMD (solvent = water) (see Supporting information for more detailes) [40–42]. In the presence of tris(2-carboxyethyl)phosphine (TCEP), the disulfide bond can be broken to generate a thiol [43]. As discussed in previous studies [31–37], the thiol further ionizes to generate a thiolate to initiate the N-to-S acyl transfer at neutral pH. Therefore, we chose the thiolate **1** as the model reactant for mechanistic study (Fig. 1). In **1**, the phenol moiety forms a hydrogen bond with the thiolate moiety. We examined other conformations in which the phenol forms hydrogen bond with the amide nitrogen atom (**1-N**) or carbonyl oxygen atom (**1-O**), or no intramolecular hydrogen bond forms (**1-non**), but they are less stable than **1** (Scheme S1 in Supporting information). On the other hand, considering the N-to-S acyl transfer proceeds in aqueous solution, extra water was considered to stabilize the thiolate. The interaction of the thiolate with three water molecules (**1-3w**) is stronger than these with two water molecules (**1-2w**) or one water molecule (**1-1w**) (Scheme S1). This is understandable that the thiolate has three lone electron pairs. Although the presence of **1-3w** does not disturb the relative feasibility of the different mechanisms discussed after, it is important to explain the observation in the control experiment that changing the hydroxyl to methoxyl (*vide infra*).

Next, the nucleophilic addition of the thiolate to the carbonyl was investigated. Akin to the Aucagne's proposal (Scheme 1), the phenol was first considered to forms a hydrogen bond with the amide nitrogen to break the conjugation in the amide bond. In this case, the electron-donating effect of the lone electron pair of the nitrogen atom to the carbonyl is expected to be crippled, by which the eletrophilicity of the carbonyl is improved. The corresponding nucleophilic addition occurs *via* **TS1** with an elementary Gibbs free energy barrier of 21.5 kcal/mol referring to **1** (Fig. 1). After **TS1**, a five-membered-ring alcoholate **2** is formed. Then the direct C—N bond cleavage from **2** to form a thioester was considered but no transition state was located for this process. Relax energy surface scan of the C—N bond from **2** indicates that prior proton transfer is met before the C—N bond cleavage (Fig. S1 in Supporting information). Specifically, the proton transfer from the phenol to the nitrogen atom occurs smoothly *via* **TS2** and generates **3** with an energy decrease of 2.6 kcal/mol. Along with the nitrogen protonation, the C1—N1 bond significantly elongates (1.5082 Å in **2** and 1.6065 Å in **3**, Fig. 2) and the C1—O1 bond shortens (1.2800 Å in **2** and 1.2649 Å in **3**), suggesting that the proton transfer is conducive to the thioester formation. Indeed, the C—N bond cleavage occurs facilely *via* **TS3** to generate the thioester **4** with a low barrier of 2.6 kcal/mol referring to **3**. Note that the N-to-S acyl transfer is endogenic and similar results were also found in previous studies because thioester is less stable than amide [31–39], but the following NCL transforms thioester to amide and is able to drive the reaction forward.

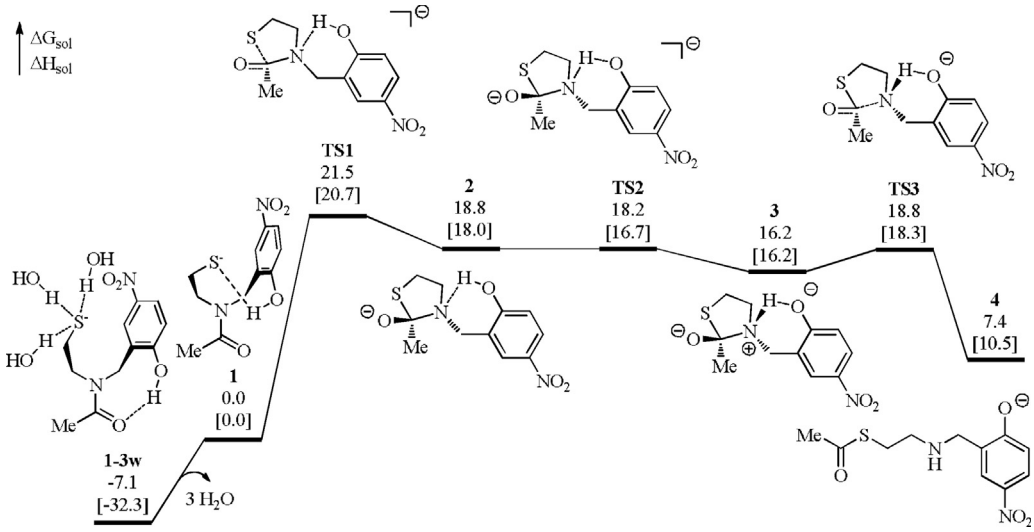

Fig. 1. Calculated energy profile of the N-to-S acyl transfer of **1** starting from the nucleophilic addition of thiolate with the presence of the hydrogen bond between the phenol and the amide nitrogen atom. Solution-phase Gibbs free energies and enthalpies in brackets are given in kcal/mol.

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