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Article

Förster Resonance Energy Transfer Assay for Investigating the Reactivity of Thioesters in Biochemistry and Native Chemical Ligation

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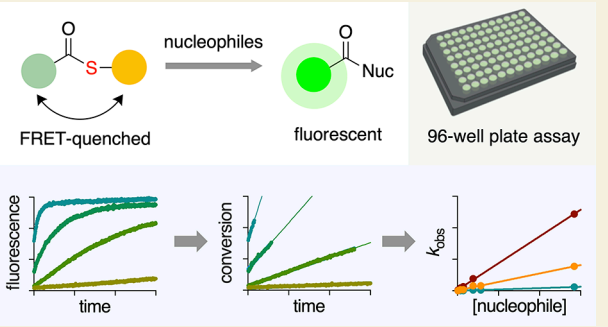
ABSTRACT: Thioesters are considered to be "energy-rich" functional groups that are susceptible to attack by thiolate and amine nucleophiles while remaining hydrolytically stable at neutral pH, which enables thioester chemistry to take place in an aqueous medium. Thus, the inherent reactivity of thioesters enables their fundamental roles in biology and unique applications in chemical synthesis. Here, we investigate the reactivity of thioesters that mimic acyl-coenzyme A (CoA) species and *S*-acylcysteine modifications as well as aryl thioesters applied in chemical protein synthesis by native chemical ligation (NCL). We developed a fluorogenic assay format for the direct and continuous investigation of the rate of reaction between thioesters and nucleophiles (hydroxide, thiolate, and amines) under various conditions and were able to recapitulate previously reported reactivity of thioesters. Further, chromatography-based analyses of acetyl- and succinyl-CoA mimics revealed striking differences in their ability to acylate lysine side chains, providing insight into nonenzymatic protein acylation. Finally, we investigated key aspects of native chemical ligation reaction conditions. Our data revealed a profound effect of the tris-(2-carboxyethyl)phosphine (TCEP) commonly used in systems where thiol−thioester exchange occurs, including a potentially harmful hydrolysis side reaction. These data provide insight into the potential optimization of native chemical ligation chemistry.



KEYWORDS: *thioesters, hydrolysis, acyl transfer, FRET, NCL, acyl-coenzyme A, lysine acylation*

INTRODUCTION

The thioester is an essential functional group in biology with unique chemical properties.[1] The large size and limited extent of delocalization of electrons from the sulfur atom to the carbonyl group render the thioester more reactive than amides and esters. However, thioesters remain stable toward hard oxygen nucleophiles under physiological conditions resulting in a long half-life in water at neutral pH values[2] while being highly reactive against softer thiolate nucleophiles.[3−6] As a result, thioesters enable efficient acyl transfer at neutral pH and take part in numerous biological reactions. The most abundant class of thioesters in biological systems is coenzyme A (CoA) species, which play central roles in metabolism and lysine acylation. The sulfhydryl group of CoA can be acylated by a wide variety of carboxylic acids; perhaps, most notably is the acetylated version (Ac-CoA, Figure 1), which feeds into the TCA cycle and is the product of β-oxidation of fatty acids among other sources. Ac-CoA is also essential for the posttranslational modification of the ε-amino groups of lysine residues, serving as a substrate for histone acetyltransferase (HAT) enzymes.[7,8] In addition, many other acyl groups have been found to modify lysine residues in our proteome,[9−11] which are also believed to involve their corresponding acyl-

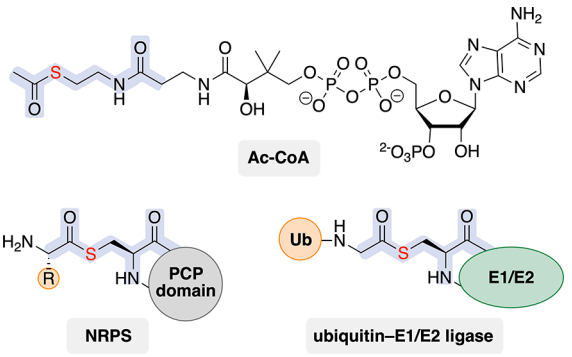


Figure 1. Examples of thioesters found in biology. Thioester motifs are highlighted by purple shading. PCP = peptidyl carrier protein; NRPS = nonribosomal peptide synthetase.

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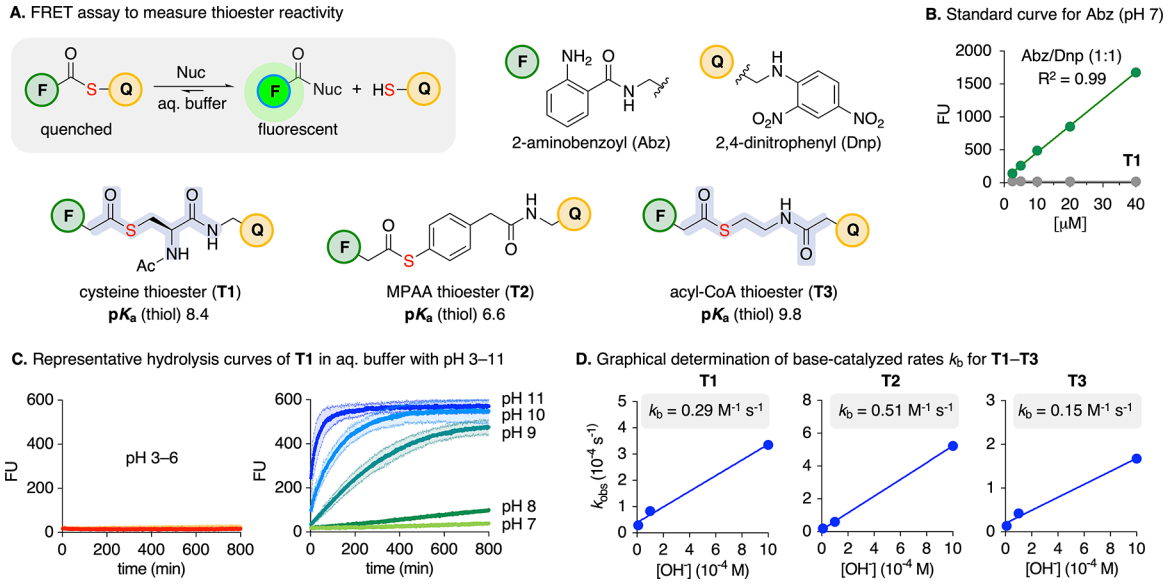

Figure 2. FRET assay design and hydrolysis data. (A) FRET assay principle and structures of designed FRET substrates (**T1**–**T3**). (B) Representative fluorescence–concentration standard curve for Abz-β-Ala-OH–Ac-Cys-Dnp (1:1) at pH 7. (C) Representative hydrolysis curves for **T1** in aq. buffer at pH 3–11. (D) Graphical determination of the base-catalyzed hydrolysis rates k_b for **T1**–**T3** by plotting k_{obs} of pH 9–11 against [OH^-]. Determined k_b (95% confidence interval [95% CI]) for **T1**–**T3**: k_b (**T1**) = 0.29 (0.18–0.44) M^{-1} s^{-1}, k_b (**T2**) = 0.51 (0.48–0.55) M^{-1} s^{-1}, and k_b (**T3**) = 0.15 (0.09–0.22) M^{-1} s^{-1}. FRET substrates **T1**–**T3** were used at a concentration of 20 μM in 100 mM aq. buffers–MeCN (9:1).

CoA species.[11] Acylation of the β-sulfhydryl group of cysteine gives rise to another prevalent group of thioesters in biology, which are often involved as intermediates in prominent acyl transfer reactions. Examples of such transformations are protein splicing,[12,13] sortase-mediated crosslinking,[14,15] the ubiquitylation of proteins by E2/E3 ligases,[16,17] and the synthesis of peptides by nonribosomal peptide synthetase (NRPS) enzyme cluster assembly lines[18−20] (Figure 1). Furthermore, thioesters have been proposed to play central roles in the chemistry preceding the origins of life; for example, as intermediates in amino acid synthesis,[21] "protopeptide" synthesis,[22] and as energy-rich molecules in prebiotic chemistry.[4,23]

The reactivity of thioesters and reversible nature of the thiol–thioester exchange reaction in an aqueous medium have been exploited in numerous applications ranging from dynamic combinatorial chemistry,[24−26] template-directed self-assembly of peptide nucleic acids,[27] oscillatory networks,[28] and chemical protein synthesis by the "native chemical ligation" (NCL) reaction,[29−31] which takes further advantage of an intramolecular S → N acyl shift at N-terminal cysteine residues.

The reactivity of thioesters has been extensively studied for decades.[1,32−34] Seminal work by the groups of Jencks and Bruice has laid the ground for our understanding of the electrophilic nature of thioesters.[2,3,35−39] Early work has focused on reactions with activated thioesters[38−44] and fewer studies have investigated the hydrolytic stability and reactivity of nonactivated thioesters in the pH-buffered aqueous medium.[45] Notably, Whitesides and coworkers conducted a detailed study of the rates of thiol exchange and stability of thioesters in the aqueous medium.[4]

The techniques previously applied to study the reactivities of thioesters include UV spectrophotometric analyses, high-performance liquid chromatography (HPLC), and/or nuclear magnetic resonance (NMR) spectroscopy.[4,35−39] We envisioned that the development of a Förster resonance energy transfer[46] (FRET)-based system for evaluation of thioester reactivity would enable ready adaptability to a given thioester motif of interest, versatility regarding test conditions, easy data collection, and high throughput (Figure 2A). Thus, enabling the broad investigation of conditions relevant to biochemistry and synthetic organic chemistry.

RESULTS AND DISCUSSION

Development of a FRET Assay To Measure Thioester Reactivity

FRET is widely used in biochemical assays and studies of biomolecular interactions[47,48] as well as in chemical sensors.[49] However, FRET-based applications have been more sparsely used for interrogation of chemical reactivity with few reports on ligation chemistry.[50−53] In contrast, protease substrates with quenched FRET pairs are widely used,[54,55] which inspired us to apply the same strategy to investigate the reactivity of various thioesters. Thus, we designed three quenched-FRET substrates that incorporate thioesters. The first substrate mimics *S*-acylcysteine thioesters present in NRPSs and ubiquitin–E2-ligase (**T1**), the second is a *p*-mercaptophenylacetic acid (MPAA)-derived thioester, which is extensively used in NCL (**T2**),[56] and the third resembles the thioesters found in acyl-CoA (**T3**) (Figure 2A). As fluorophore (**F**) and quencher (**Q**) pair, we chose 2-aminobenzoyl (Abz) and 2,4-dinitrophenylamine (Dnp), respectively (Figure 2A and Supplementary Scheme S1). We envisioned that this **F**/**Q** pair would not considerably influence the reactivity of the thioesters, give stable fluorescence output over a large pH range, and enable the screening of reaction conditions in the low micromolar range.

To validate the feasibility of a FRET-based assay that could be performed in a 96-well plate format, we determined the hydrolytic stability and the base-mediated rate constants (k_b) for the hydrolysis of **T1**–**T3** at pH 3–11 (for rate equations, see Supplementary Figure S1). In the first step, we measured a standard curve for a solution, containing a 1:1 mixture of Abz-

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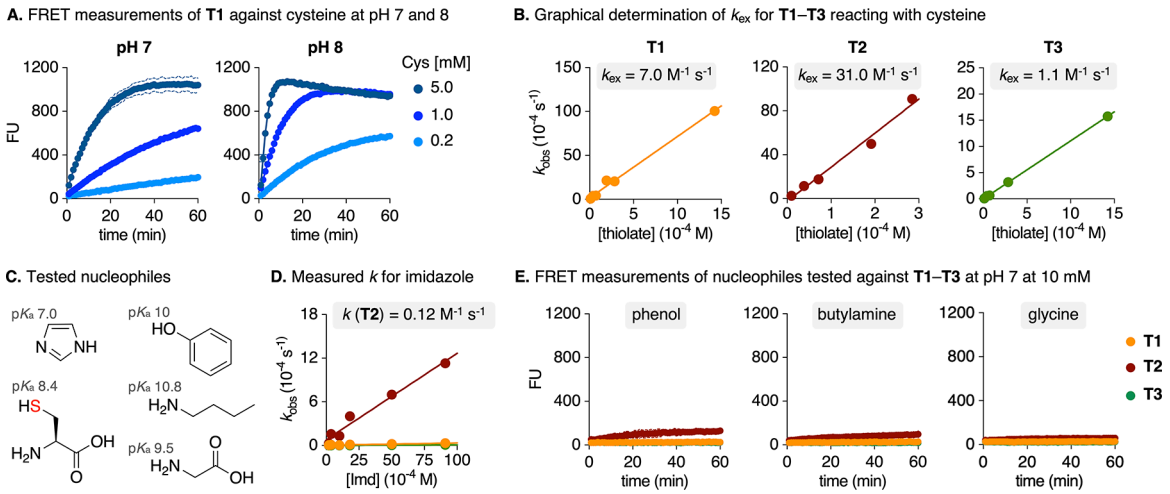

Figure 3. Thioester reactivity against nucleophiles. (A) Representative curves for the reaction of cysteine with **T1** at pH 7 and pH 8. (B) Graphical determination of rate constants k_{ex} for **T1**–**T3** by plotting k_{obs} (assuming pseudo-first-order reaction kinetics: $-\ln([\mathbf{T1}]_t/[\mathbf{T1}]_0)$ vs time) against [thiolate], which is pH-dependent on the θ coefficient. Determined k_{ex} (95% CI) for **T1**–**T3** reacting with cysteine: k_{ex} (**T1**) = 7.0 (6.2–7.8) M^{-1} s^{-1}, k_{ex} (**T2**) = 31 (24–38) M^{-1} s^{-1}, and k_{ex} (**T3**) = 1.1 (1.08–1.14) M^{-1} s^{-1}. (C) Tested nucleophiles and their pK_a values. (D) Determined k (95% CI) for **T2** reacting with imidazole: k (**T2**) = 0.12 (0.09–0.15) M^{-1} s^{-1}. (E) FRET measurements of nucleophiles tested at 10 mM at pH 7 against **T1**–**T3** with insufficient reactivities to determine k_{ex}. FRET substrates **T1**–**T3** were used at a concentration of 20 μM in 100 mM aq. buffers–MeCN (9:1) including 250 μM of TCEP.

β-Ala-OH (**F**) and Ac-Cys-Dnp (**Q**), resembling the hydrolysis products of **T1** at pH 3–11, which showed excellent correlation between concentration and fluorescence units (FU). Further, the fluorescence of Abz was fully quenched in the substrate **T1** (Figure 2B and Supplementary Figure S2). Next, we conducted continuous fluorescence measurements of solutions of **T1**–**T3** (20 μM) in aqueous buffers (pH 3–11) (Figure 2C and Supplementary Figures S3–S5). For all three thioesters, the curves remained flat at mildly acidic to neutral pH (Figure 2C) with observed rates (k_{obs}) in the ~$1–2 \times 10^{-7}$ s^{-1} range ($t_{1/2}$ = 80–40 days), but the very low levels of hydrolysis did not enable precise measurements on the timescale performed.

The curves for pH 8–11 displayed a pH-dependent increase in fluorescence due to hydrolysis and by plotting k_{obs} against [OH^-], we were able to obtain k_b values for **T1**–**T3** (Figure 2D; for details regarding the determination of k_b, see Supplementary Figure S1). Our measurements are in good agreement with previously reported k_b values by Whitesides and co-workers, which were obtained using NMR spectroscopy.[4] The value for **T2** (k_b = 0.51 M^{-1} s^{-1}) matches the value of a phenyl thioester (k_b = 0.64 M^{-1} s^{-1}) and **T3** (k_b = 0.15 M^{-1} s^{-1}) behaves very similar to *S*-methyl thioacetate (k_b = 0.14 M^{-1} s^{-1}).[4] The value of **T1** (k_b = 0.29 M^{-1} s^{-1}) lies between that of **T2** and **T3**, which was expected based on the pK_a values of the departing thiols.

Reactivity of Thioesters in Acyl Transfer Reactions

Having validated the reproducibility for k_b, we determined the reactivity of **T1**–**T3** against a selection of nucleophiles that represent common functional groups present in the cell (thiol, imidazole, phenol, and primary amines) (Figure 3C). We performed experiments at pH 7 and 8 and with a large excess of nucleophiles to achieve pseudo-first-order reaction kinetics. The effective concentration of reactive nucleophiles at given pH was calculated, taking into account their respective pK_a values (thiolate/phenolate or unprotonated nitrogen; for details, see Supplementary Figure S1). The first nucleophile we investigated was cysteine, which facilitated the measurement of the thiol–thioester exchange reaction constant (k_{ex}), due to the rapid and irreversible S → N acyl shift following the initial thiol–thioester exchange[57,58] (Figure 3A,B and Supplementary Figure S6). We determined k_{obs} at three different concentrations at pH 7 and pH 8, resulting in six data points, which were plotted against the effective concentration of thiolate (pK_a 8.4 for cysteine)[59] to obtain k_{ex} for **T1**–**T3** (Figure 3B). We validated the reaction products (Supplementary Figure S9) and neglected the rates of hydrolysis at pH 7 and 8 because they are several orders of magnitude lower than the rates of the exchange reactions. The k_{ex} values for **T1**–**T3** were lower than k_{ex} values reported by Whitesides and co-workers but the overall trend remained the same.[4] For the alkyl thioester **T3** (k_{ex} = 1.1 M^{-1} s^{-1}), the exchange constant is similar to that of *S*-methyl thioacetate (k_{ex} = 1.7 M^{-1} s^{-1}), while the exchange constant was 31 M^{-1} s^{-1} for **T2** and 120 M^{-1} s^{-1} for the phenyl thioester used by Whitesides and co-workers. These data are not fully comparable because the previous study used sodium 2-mercaptoethanesulfonate (MESNa), which has a higher pK_a value than cysteine (pK_a 9.5 for MESNa).[4]

Next, we tested the reactivity of selected nitrogen and oxygen nucleophiles against **T1**–**T3** (Figure 3C–E). Imidazole was the only nucleophile in our series that displayed reactivity at pH 7 and only against **T2** with a measured rate constant of 0.12 M^{-1} s^{-1} (Figure 3D and Supplementary Figure S7), which is 258-fold lower than k_{ex} for cysteine. However, we could only identify the carboxylate and free thiol, arising from imidazole-catalyzed hydrolysis of **T2** (Supplementary Figure S9), which strongly suggests that the measured rate constant refers to this hydrolysis reaction.[38,60] The low reactivity recorded for **T1** and **T3** might be due to the pK_a value of the released thiols. The other nucleophiles we tested (phenol, butylamine, and glycine) have pK_a values above 9.5 and would form ester or amide bonds after the acyl transfer at pH 7, but none of the nucleophiles displayed any measurable reaction with **T1**–**T3** at concentrations up to 10 mM (Figure 3E and Supplementary Figure S8). At pH 8, a reaction of **T2**

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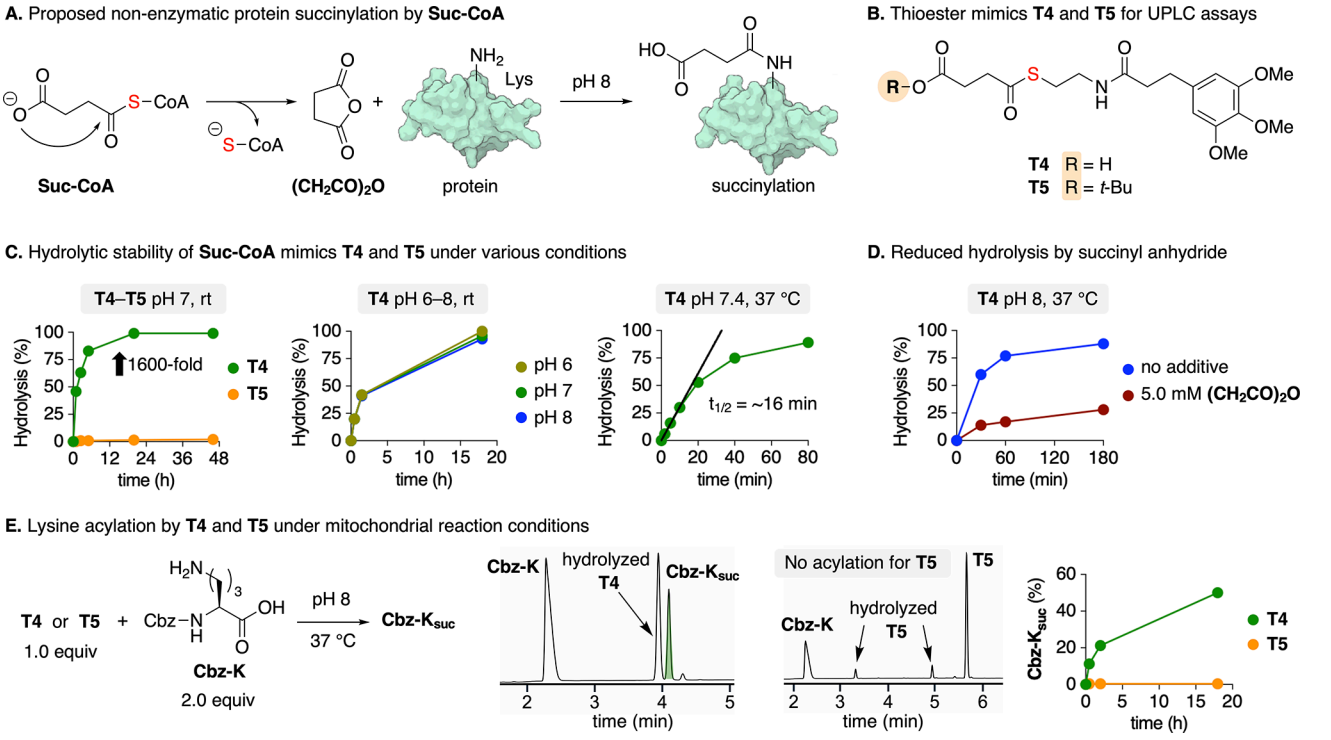

Figure 4. Succinyl-CoA reactivity and stability. (A) Proposed mechanism for non-enzymatic protein succinylation by **Suc-CoA** via formation of succinyl anhydride [$(CH_2CO)_2O$]. (B) Structures of thioester substrates **T4** and **T5**. (C) Hydrolysis of **T4** and **T5** under various conditions. (D) Reduced hydrolysis of **T4** in the presence of $(CH_2CO)_2O$ (5 mM). (E) Acylation reaction of Cbz-lysine (**Cbz-K**) (2 mM, 2.0 equiv) with **T4** or **T5** (1 mM, 1.0 equiv) to give *α*-*N*-Cbz-*ε*-*N*-succinyllysine (**Cbz-K$_{suc}$**) under conditions found in the mitochondria (pH 8, 37 °C).

with n-butylamine was observed, and the n-butylamide product was confirmed (Supplementary Figure S9). Only very limited reactions occurred with **T1** and **T3** (Supplementary Figure S8). This result is interesting in the context of nonenzymatic acylation of proteins since n-butylamine can be considered a mimic of the *ε*-amino group of lysine side chains and the thioester motif of **T3** has structural similarity to an acyl-CoA species.

Lysine Succinylation and Stability of Succinyl-CoA Mimicking Thioesters

It has been shown that posttranslational modification (PTM) to lysine residues in the proteome may occur by nonenzymatic acyl transfer from acyl-CoA and *S*-acylglutathione species.[61−65] However, our data for **T1** and **T3** vide supra suggest that direct acyl transfer reactions from thioesters to amines are prohibitively slow under physiological conditions, even at pH 8 in the mitochondria. Thus, these data provide further chemical evidence for nonenzymatic acylation to happen via a two-step mechanism in which a protein's cysteine side chain undergoes thiol−acyl-CoA thioester exchange, followed by intramolecular acyl transfer to an *ε*-amino group of lysine residues.[66] Further, the special cases of acyl-CoA species, based on dicarboxylic acids that are able to form 5- or 6-membered anhydrides, have been argued to proceed via anhydride formation, followed by lysine acylation in a non-specific manner (Figure 4A).[67] Because of the diverse roles of succinyl-CoA (**Suc-CoA**) in the cell[68] and its high intracellular concentration,[69] we investigated the fundamental reactivity of this thioester in more detail.

We prepared two thioester mimics to study the hydrolytic stability and reactivity of **Suc-CoA**; **T4**, containing a free carboxylic acid capable of forming succinyl anhydride and **T5**, which is masked as a *tert*-butyl ester that cannot form succinyl anhydride (Figure 4B and Supplementary Scheme S2). The presence of the free carboxylate prohibited the use of our FRET assay and we therefore performed all kinetic experiments by ultra-performance liquid chromatography (UPLC). First, we incubated both thioesters at pH 7 and observed striking differences in their stability, with **T4** undergoing hydrolysis in minutes, while **T5** behaved like the above-studied thioesters, leading to a 1600-fold difference in rate (Figure 4C and Supplementary Figures S10 and S11). We assumed that the increase in hydrolysis arises from the formation of succinyl anhydride, which should therefore be independent of pH as long as the carboxylic acid is deprotonated. Indeed, the hydrolysis rate of **T4** was identical at pH 6−8 (Figure 4C) and the addition of succinyl anhydride significantly reduced the extent of hydrolysis, which explains the decreasing rate of hydrolysis over time due to the increasing concentration of released succinyl anhydride, as observed in all experiments (Figure 4D). Our findings are in agreement with early reports of the fast hydrolysis rate of **Suc-CoA**,[45,70] and we found the very short-lived nature of **T4** ($t_{1/2}$ ∼ 16 min) to be in stark contrast to other acyl-CoA species represented by **T3** and **T5** ($t_{1/2}$ ∼ 18 days). Next, we compared the ability of **T4** and **T5** to acylate *α*-*N*-Cbz-protected lysine (**Cbz-K**) at the pH value found in the mitochondria (pH 8, 37 °C), because many proteins in the mitochondria have been reported to be succinylated[71−73] (Figure 4E and Supplementary Figure S12). In accordance with the results obtained for **T3** and butylamine, **T5** did not react with lysine via direct acyl transfer. In contrast, **T4** led to nearly 50% conversion based on the thioester. Thus, our collective findings provide experimental evidence—based on fundamental chemical reactivity—to support the claim that

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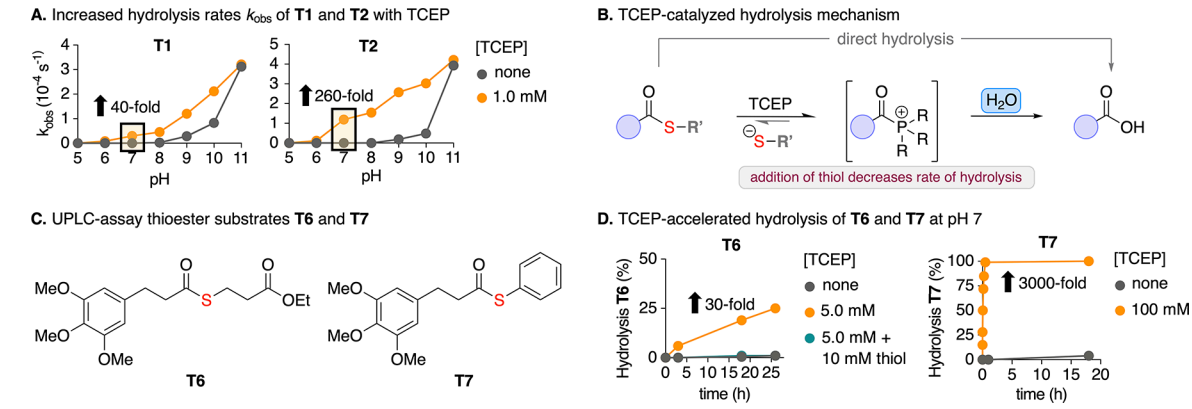

Figure 5. TCEP-accelerated hydrolysis of thioesters. (A) Observation of increased hydrolysis rates k_{obs} of **T1** and **T2** in the presence of tris(2-carboxyethyl)phosphine (TCEP; 1 mM) in our FRET assay. (B) Proposed pathway for the acceleration of hydrolysis of thioesters by TCEP. (C) Structure of thioester substrates **T6** and **T7**. (D) Hydrolysis of **T6** and **T7** (200 μM) in the presence of TCEP with and without added thiol. R = 2-carboxyethyl; R′ = alkyl or aryl.

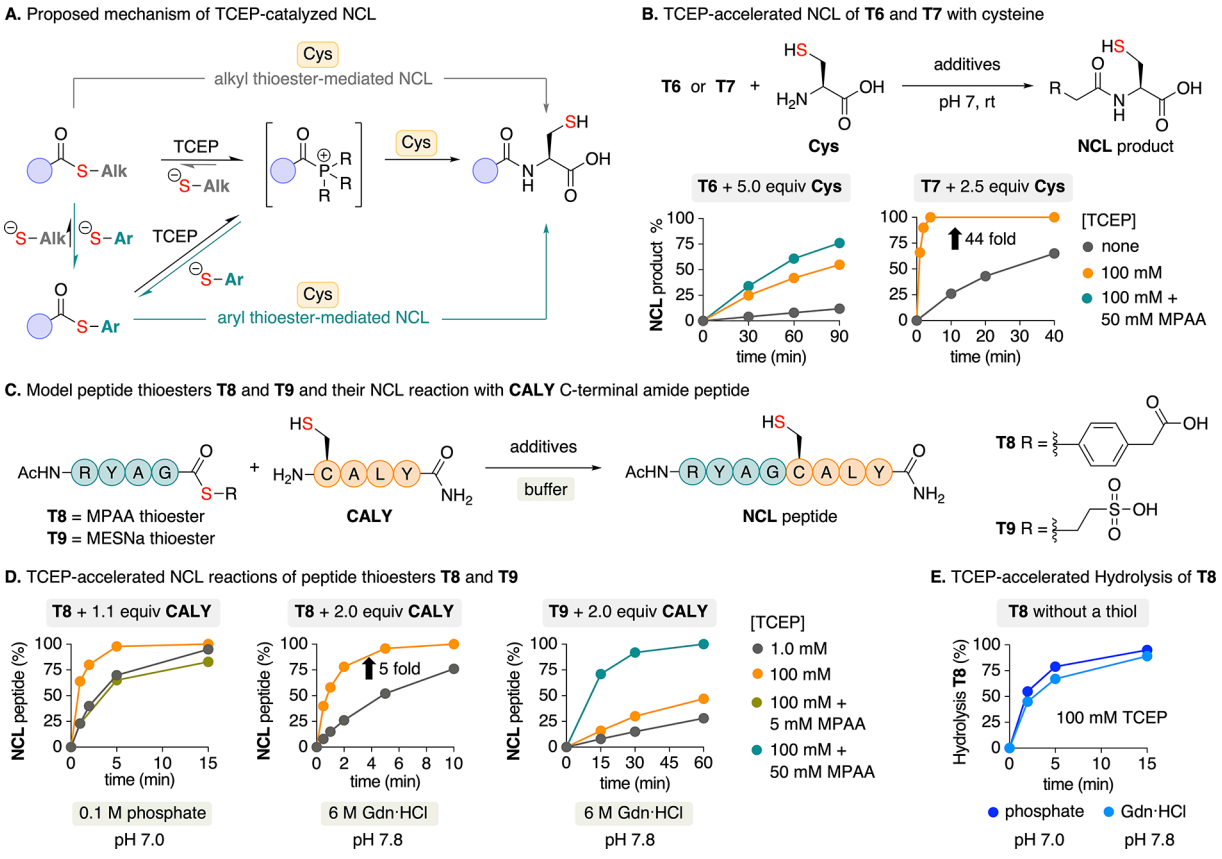

Figure 6. TCEP-accelerated native chemical ligation. (A) Proposed pathways for the acceleration of NCL by TCEP. (B) NCL reaction of **T6** and **T7** (200 μM) with cysteine in the presence of TCEP with and without MPAA at pH 7 in phosphate buffer. (C) Structures of peptide thioesters **T8** and **T9** and N-terminal cysteine-containing peptide **CALY**. (D) NCL reaction of **T8** and **T9** (200 μM, 1.0 equiv) with peptide **CALY** (220 or 400 μM, 1.1 or 2.0 equiv) in the presence of TCEP (1 mM or 100 mM) and MPAA (none added, 5 mM or 50 mM) performed in phosphate buffer (0.1 M, pH 7) or Gdn·HCl (6 M) in phosphate buffer (0.1 M, pH 7.8). (E) Accelerated hydrolysis of **T8** in a buffer in the presence of TCEP (100 mM).

nonenzymatic protein succinylation could happen via succinyl anhydride formation.

TCEP Is a Nucleophilic Catalyst for Thioester Hydrolysis and Native Chemical Ligation

During the optimization of our FRET assay, we observed an increase in the hydrolysis rates of **T1** and **T2** in the presence of the commonly used reducing reagent tris(2-carboxyethyl)-phosphine (TCEP) (Figure 5A and Supplementary Figure S13). The acceleration was most pronounced around neutral pH (40- and 260-fold increase for **T1** and **T2**, respectively). We found this observation to be concerning because TCEP is a common additive used in high mM concentrations in NCL reactions and other in vitro experiments involving C-terminal thioester peptides.[74] Two previous works have reported on increased thiol−thioester exchange in the presence of

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TCEP[75,76] and in the first study, Tam et al. argued for the formation of a reactive acyl-phosphonium intermediate.[75] In support of this hypothesis, phosphines are widely used as nucleophilic catalysts for acylation reactions in organic chemistry,[77] including examples with aryl thioesters.[78] Therefore, we propose that the phosphorous atom in TCEP (pK_a 7.66) reacts with the thioester to form an acyl-phosphonium species, which readily hydrolyzes in the absence of other external nucleophiles (Figure 5B). The proposed TCEP-accelerated hydrolysis pathway should be repressed if a better nucleophile than water is present, such as a thiol catalyst, which would lead to the reformation of a hydrolytically stable thioester.

To test this hypothesis and to address the impact of TCEP on hydrolysis, we synthesized two simple thioesters **T6** and **T7**, which are amenable to the assessment of the effect of external thiol additives by UPLC (Figure 5C and Supplementary Scheme S2). First, we incubated **T6** with and without TCEP (5 mM) at pH 7 or with TCEP (5 mM) and ethyl 3-mercaptopropionate (10 mM), corresponding to the leaving group of **T6** (Figure 5D and Supplementary Figure S14). The hydrolysis of **T6** was increased by 30-fold in the presence of TCEP alone, while no acceleration of hydrolysis was observed when the thiol was added as well, supporting our proposed TCEP-accelerated hydrolysis pathway. We next examined the effect of high concentrations of TCEP (100 mM) on aryl thioester **T7** and observed a 3000-fold increase in hydrolysis rates resulting in $t_{1/2} \sim 5$ min vs $t_{1/2} \sim 12$ days (Figure 5D and Supplementary Figure S15).

The significantly increased hydrolytic susceptibility of the adduct formed between **T7** and TCEP led us to investigate how this intermediate would affect the rate of NCL reactions (Figure 6A). NCL reactions involve a C-terminal peptide thioester and an N-terminal cysteine-containing peptide. Often, an aryl thiol "catalyst", such as MPAA, is added to accelerate the trans-thioesterification step and to prevent non-productive thioester adducts on internal cysteine residues.[74] The thiol catalyst also maintains a reducing environment; however, TCEP is often applied as an additional reducing reagent at concentrations ranging from 10 to 200 mM,[79] although protocols without the addition of TCEP have also been reported.[80] Lastly, aryl thiol additives prevent the unwanted desulfurization of the cysteine side chain caused by TCEP.[81] We sought to explore how the addition of TCEP would change the ligation rate in the presence and absence of thiol catalyst and if rate-acceleration would be observed as previously reported.[76,82,83]

The NCL reaction with nucleophilic additives involves more than one step, but we could only follow the first thiol−thioester exchange when using our FRET thioesters **T1**−**T3** and we used **T6** and **T7** to assess NCL reaction rates in the presence of TCEP. We first observed a moderate 6-fold acceleration by TCEP (100 mM) on NCL of **T6** with cysteine (**Cys**) while adding TCEP (100 mM) and MPAA (50 mM) increased the rate 11-fold (Figure 6B and Supplementary Figure S16). Despite the increase in rate, caused by the addition of thiol catalyst, the NCL reaction of **T6** with **Cys** was slower than the reaction of the preformed aryl thioester **T7** with **Cys**. The addition of TCEP (100 mM) to **T7** led to a 44-fold rate increase resulting in ∼an 800-fold difference between the uncatalyzed NCL reaction of **T6** and the TCEP-catalyzed NCL reaction of **T7** (Figure 6B and Supplementary Figure S17).

To further address the impact of TCEP on the rates of NCL reactions, we next turned to model peptide thioesters **T8** and **T9**, a cysteine-containing peptide amide [H-Cys-Ala-Leu-Tyr-NH_2 ("**CALY**")], and two commonly used thiols, MPAA and MESNa, respectively (Figure 6C). First, we performed the NCL reaction between **T8** and **CALY** in the presence of TCEP (1 mM or 100 mM) in phosphate buffer and observed a ∼5-fold rate increase with the higher TCEP concentration. When we added MPAA (5 mM) together with TCEP (100 mM), the rate dropped to the level observed for the lower concentration of TCEP (1 mM) (Figure 6D left panel and Supplementary Figure S18).

Next, we performed the reaction in the more commonly used buffer for NCL reactions [guanidinium chloride (Gdn·HCl) (6 M) in phosphate buffer (0.1 M), pH 7.8] with excess of **CALY** (2 equiv), where the addition of TCEP (100 mM) also furnished a rate acceleration of ∼5-fold (Figure 6D and Supplementary Figure S19). These data for the NCL reaction of **T8** suggest that the reactivity of the adduct formed by the reaction of **T8** with TCEP surpasses the reactivity of the MPAA thioester itself in this model system.

Then, we compared the rate of NCL reactions between peptide alkyl thioester **T9** and **CALY**. The reaction rate only increased 2-fold with the addition of TCEP (100 mM), while using TCEP (100 mM) and MPAA (50 mM) led to a 15-fold increase compared to the reaction containing only 1 mM of TCEP (Figure 6D and Supplementary Figure S20). However, the rate of the NCL reaction of **T9** in the presence of MPAA and TCEP did not exceed that of the preformed MPAA thioester **T8** even without TCEP acceleration. Finally, we found that **T8** also undergoes rapid hydrolysis in the presence of TCEP with $t_{1/2} < 3$ min in the absence of other nucleophiles than water (Figure 6E and Supplementary Figure S21).

Taken together, our data show that the aryl thioesters are much more prone to hydrolysis and rate acceleration by reaction with TCEP than the alkyl thioesters tested, which, at least in part, may be explained by the pK_a value of TCEP being higher than the aryl thiols and lower than the alkyl thiols used in the study.

CONCLUSIONS

To conclude, we designed and synthesized three different quenched-FRET substrates for kinetic assays of the reactivity of thioester motifs found in biologically relevant processes and chemical protein synthesis by native chemical ligation. This new platform rapidly delivered rates of hydrolysis and acyl transfer to various nucleophiles at varying pH values, and derived k_b and k_{ex} (thiol) values for four of the examples were in excellent agreement with those reported by Whitesides and co-workers. Thus, the high stability of thioesters toward hydrolysis (OH^-) was recapitulated and the pK_a value of the thiol, corresponding to the leaving thiolate, versus the incoming nucleophile proved to dictate the rate of acyl transfer as would be expected.[1,3,45]

The high stability of the acyl-CoA-mimicking FRET substrate led us to design a model system to investigate the reactivity of succinyl-CoA toward the side chains of lysine residues, using a UPLC assay format. These experiments showed that posttranslational protein lysine acylation by direct acyl transfer from acyl-CoA species is unlikely. Thus, providing further evidence that lysine acylation may occur by chemical modification through vicinal cysteine side chain modification or by enzymatic catalysis.

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Finally, we found the commonly used phosphine-based reducing agent, TCEP, to have a pronounced effect on the rates of both hydrolysis and acyl transfer of the thioester-containing species. These effects were also dependent on the pK_a value of the corresponding acid of the leaving thiolate, relative to that of TCEP itself. Further, the hydrolysis reaction could be suppressed by the addition of external thiol which is common practice in NCL protocols, explaining why hydrolysis of the thioester is usually not observed in NCL reactions. Nevertheless, the order of addition of reactants in the NCL reaction might be important as premixing of peptide thioesters and TCEP without thiol catalyst could potentially lead to unwanted thioester hydrolysis. Further, our data emphasize potential for selecting thiol−phosphine pairs with optimally matching pK_a values as an avenue for future improvement of NCL reaction conditions.

We envision that the quenched-FRET assay format developed herein can be broadly adapted to other thioester motifs or to selenoesters, which have also recently been applied in the ligation of peptides.[84]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.3c00095.

Rate equations; FRET assay data; UPLC assay data; schemes depicting the synthesis of substrates and compounds used; experimental methods; chemical synthesis and compound characterization data; as well as copies of HPLC traces, ^{1}H and ^{13}C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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