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Thiodepsipeptides

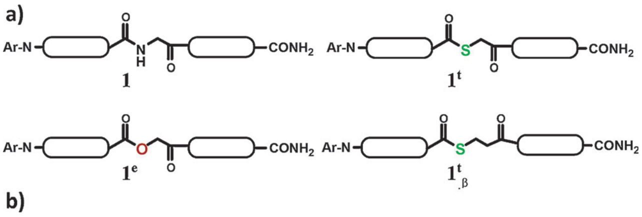
A High-Resolution Structure that Provides Insight into Coiled-Coil Thiodepsipeptide Dynamic Chemistry**

*Zehavit Dadon, Manickasundaram Samiappan, Anat Shahar, Raz Zarivach, and Gonen Ashkenasy**

Chemical synthesis and cell-based expression methods can afford incorporation of non-natural entities into proteins. New structures are obtained, and new functions gained, by attachment of amino acids with non-native side-chains,[1,2] modified backbones such as β- and γ-amino acids,[3–5] or peptide bond analogues such as peptoids, esters, and thioesters.[6–9] Backbone modification with longer and flexible amino acids allows expansion of the conformational space occupied by proteins, while introduction of ester (depsipeptide) and thioester (thiodepsipeptide) bonds enhances their reactivity towards hydrolysis and other nucleophilic attacks. The durability of thioester bonds in neutral aqueous solutions and their reactivity in thiol–thioester exchange reactions make them a relevant choice for performing dynamic chemistry in water.[10–15] Particularly interesting is the possibility of utilizing such transformations for exchanging domains between different protein molecules, owing to sequence mutations or in response to chemical and physical changes.

Self-organization of molecular networks has been extensively studied by scientists interested in systems chemistry.[16,17] When studying protein-based networks, it was demonstrated that the network connectivity and overall topology can be dictated by the sequence-specific information embedded in coiled-coil architectures,[18–20] and that careful design of the interhelical recognition interface can be used to affect the network in a predictable manner.[14,20,21] It is suggested here that the adaptive behavior of such networks, namely their rewiring in response to external triggers, can be greatly expanded if the coiled-coil proteins are formed within dynamic networks in which domain exchange readily takes place. Towards this end, we utilize coiled-coil protein analogues that contain thioester bonds within their sequences. We predict that to be mechanistically relevant for domain exchange, the thioester bond should be isostructural with the peptide bonds to maintain the 3D structure, and it should also be kept exposed and reactive towards small-molecule thiols and/or thiol-containing proteins. To highlight these characteristics, we provide here the first high-resolution structure (1.35 Å) of a thioester coiled-coil protein, and compare it to the structure of the native and depsipeptide analogue proteins. The integrity of the thioester bonds and their accessibility to other molecules are revealed by analysis of the crystal structure, as well as from complementary thiol-exchange assays. We then show using a set of mutants that the thioester stability can be correlated with the backbone regularity and the coiled-coil unfolding stability. Finally, a small library formed of these thioester mutants is screened for domain exchange in the absence and presence of an external template molecule, revealing significant template effect and exchange-product amplification.

The sequence of the key thioester peptide (**1**^{t}) was designed by replacing a glycine residue (G18) in a previously studied (GCN4-based) coiled-coil peptide **1**[21] with a thioglycolic acid (Figure 1). Peptides **1**, **1**^{t}, and their ester analogue **1**^{e}, containing a glycolic acid in position 18, were synthesized and purified (Supporting Information, Figures S1 and S2).



b)

Ar-RVARLEKKVSALEKKVAGLEXEVARLKKLVGE-CONH$_2$ (1)
Ar-RVARLEKKVSALEKKVAZLEXEVARLKKLVGE-CONH$_2$ (1^t)
Ar-RVARLEKKVSALEKKVABLEXEVARLKKLVGE-CONH$_2$ (1^e)
Ar-RVARLEKKVSALEKKVAZ$_\beta$LEXEVSRLKKLVGE-CONH$_2$ (1$^t_\beta$)
Ar-RVRALERKVSELARKVAZLEXEVARLKKLVGE-CONH$_2$ (1$^t_{E13A}$)
Ar-RVASLEKKVSALKKKVAZLEXEVARLKKLVGE-CONH$_2$ (1$^t_{E13K}$)
Ar-RVASLEKEVSALEKKVAGLEKEVARLKKLVGE-CONH$_2$ (2)

Figure 1. a) Peptide-bond analogues and b) peptide sequences. Ar = 4-acetamidobenzoate, Z = -SCH$_2$CO, Z$_\beta$ = -SCH$_2$CH$_2$CO, B = -OCH$_2$CO, X = Lys-Ar. **1**, **1**^{t}, and **1**^{e} sequences are identical, except for having different peptide-bond analogues at position 18. Minor mutations introduced to non-informal solvent-exposed positions of **1**$^t_\beta$ and **1**$^t_{E13A}$, relative to **1**^{t} sequence, to facilitate better HPLC separation in the network experiments (Figure 4).

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[*] Z. Dadon, Dr. M. Samiappan, Prof. G. Ashkenasy
Department of Chemistry, Ben Gurion University of the Negev
Beer Sheva, 84105 (Israel)
E-mail: gonenash@bgu.ac.il
Prof. G. Ashkenasy
Ilse Katz Institute for Nanoscale Science and Technology
Ben Gurion University of the Negev
Beer Sheva, 84105 (Israel)
Dr. R. Zarivach
Department of Life Sciences, Ben Gurion University of the Negev
Beer Sheva, 84105 (Israel)
Dr. A. Shahar, Dr. R. Zarivach
Institute of Biotechnology in the Negev
Ben Gurion University of the Negev
Beer Sheva, 84105 (Israel)

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The coiled-coil fold characteristics and the three-dimensional structures of these analogues were analyzed and compared. CD measurements (Supporting Information, Figure S3) showed the typical coiled-coil spectrum for all three of the compounds, albeit with high helix content for **1** (87 %), and somewhat compromised helicity for $\mathbf{1}^{t}$ and $\mathbf{1}^{e}$ (67 % and 68 % helix, respectively). Peptides **1**, $\mathbf{1}^{t}$, and $\mathbf{1}^{e}$ were then crystalized from circa 10 mg mL^{-1} solutions, under similar crystallization conditions, and into the same space group ($P2_12_12_1$). The crystal structures were solved to 2.1, 1.35, and 1.40 Å, respectively (Supporting Information, Table S1). All three peptides assembled into trimer structures of parallel and highly regular α-helices (Figure 2; Supporting Information, Figures S4 and S5). Interestingly, we have not observed dimeric coiled-coils, even though a close variant of **1**, also containing valine and leucine residues in the hydrophobic core, was crystalized earlier in both the dimer and trimer forms.[22] The effect of incorporating a thioester into the backbone of $\mathbf{1}^{t}$ is further highlighted by crystal structure alignment with **1**. Only minimal differences were observed along the entire structure (RMSD = 0.37 Å), except for some deformation of $\mathbf{1}^{t}$ structure around the thioester bond (Figure 2b).

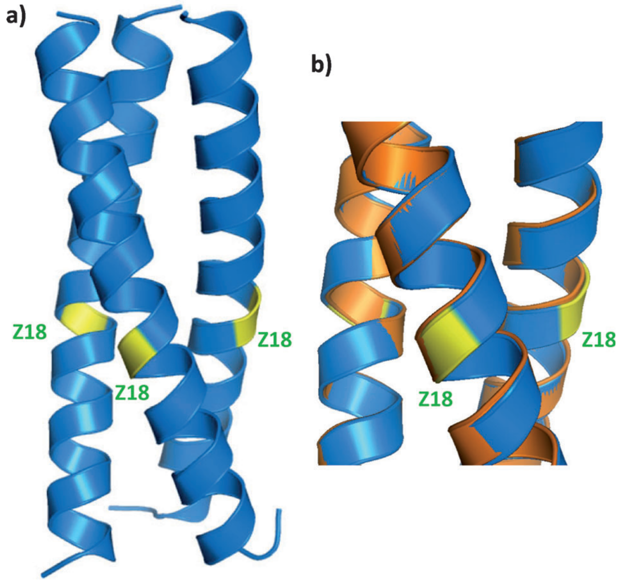


Figure 2. a) Side view of the crystal structure of $\mathbf{1}^{t}$; the yellow ribbon marks the thioester thiol position. b) Crystal structure alignment of $\mathbf{1}^{t}$ (marine blue) and **1** (orange), showing almost identical structures with some deformation around the thioester site of $\mathbf{1}^{t}$. The crystal structure of $\mathbf{1}^{e}$, additional views of $\mathbf{1}^{t}$ structure, and crystallization procedure, conditions, and collected data are given in Supporting Information.

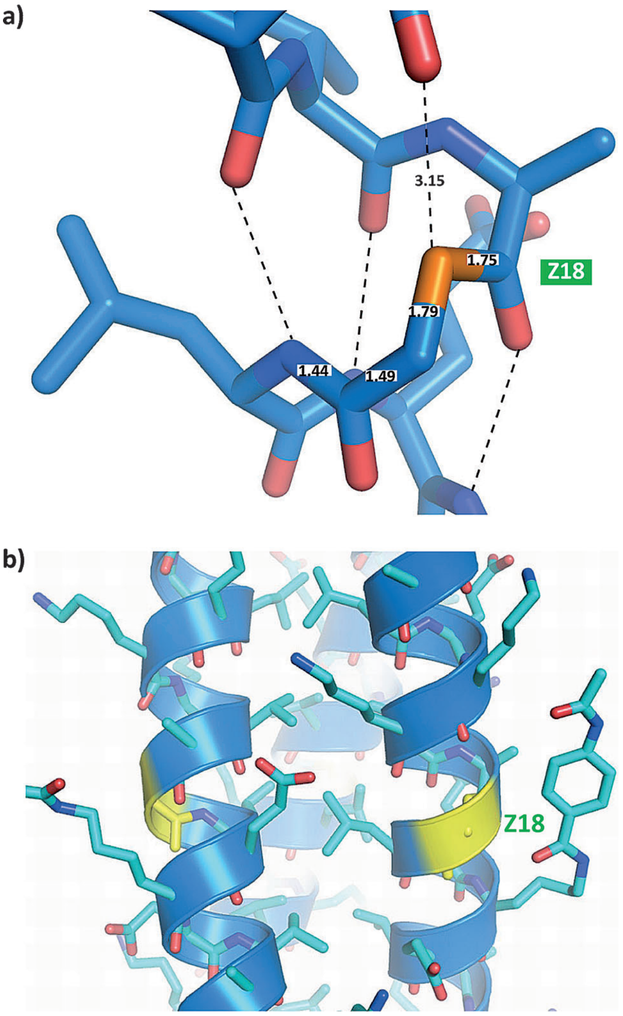
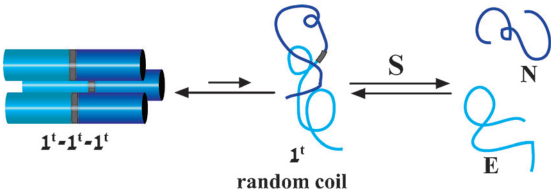


Figure 3. Close-up view of the thioester site of $\mathbf{1}^{t}$. a) Bond and interatom distances in the thioester vicinity. The bond and interatom distances found in the other two chains of $\mathbf{1}^{t}$ and in the structures of **1** and $\mathbf{1}^{e}$ are given in the Supporting Information, Figure S6. b) Stick model highlighting the side-chains that engulf the thioester and may interfere with reacting thiol molecules.

Analysis of the high-resolution structure of $\mathbf{1}^{t}$ allows detailed characterization of the thioester bond, formed between the thioglycolic acid sulfur atom and Ala 17 carbonyl carbon, and its environment, including assignment of bond lengths (Figure 3 a; Supporting Information, Figure S6) and identification of close-by side-chains (Figure 3 b; Supporting Information, Figure S5 b) that are likely to interfere with incoming molecules during the thiol–thioester exchange reactions. For example, the loss of one hydrogen bond per chain in $\mathbf{1}^{t}$ and $\mathbf{1}^{e}$ (versus **1**) can be directly deduced from the crystal structures. These reveal 0.2–0.4 Å longer distances between the thioester S or ester O atoms and the K14 carbonyl oxygen atom (Supporting Information, Figure S6). GnHCl-dependent denaturation experiments have shown that the coiled-coil structure of $\mathbf{1}^{t}$ is less stable than that of **1** by 1.1 kcal mol^{-1},[14] namely implying about 0.4 kcal mol^{-1} structure destabilization for each thioester bond. The relatively minor destabilizing effect is similar to that observed earlier for removing a solvent exposed hydrogen bond,[7] and can be explained by water molecules solvation of the sulfur (and oxygen) atoms, which reduces repulsion between them and the K14 carbonyl oxygen lone pair.[7]

Figure 3 b shows that the thioester bond is solvent-exposed, but it is also engulfed by the side-chains of residues K15, X21 (X = aryl-modified Lys; Figure 1), and E22 of the same chain, and E20 from the opposing helix. The regular helix structure of the thiodepsipeptide coiled-coils and this shielding of the thioester bond render **1^t** stable against hydrolysis and thiol-dependent decomposition. Indeed, the thioester was only hydrolyzed to a limited extent (ca. 5%) during two days in pH 7 aqueous solution. We have further evaluated the decomposition of **1^t** in the presence of molar excess of external small-molecule thiols (S), to yield a thioester (E) and *n*-terminal thioglycolic (N) fragments (Scheme 1). Equilibration of **1^t** (100 μM) in the presence of the alkyl thiol 2-mercapto ethyl sulfonate (1.0 mM) in pH 7 showed some decomposition (Supporting Information, Figure S7), revealing an equilibrium constant $K_{eq} = ([E] \times [N]/[1^t] \times [S])$ (Scheme 1) of 1.5×10^{-3}. The same experiments in unfolding conditions (3 M GnHCl) resulted in fast and efficient decomposition into E and N ($K_{eq} = 2.1$) and more significant hydrolysis. Furthermore, shielding of the thioester was evidenced from equilibration in the presence of *tert*-butylthiol (1.0 mM) that resulted in slow decomposition for which no equilibrium could be reached after 2 days.



Scheme 1. Equilibration of a thiodepsipeptide in the presence of excess thiol molecule (S) to yield a thioester (E) and thiol-containing (N) fragments.

In recent studies, Gellman, Woolfson, and their co-workers have shown that the extent of thioester formation in loop positions of helix–loop–helix structures can be correlated with the stability of the coiled-coils.[23–26] To probe whether such correlation exists for thioesters located within the helix itself, we have related the unfolding stability of **1^t** and its mutants (**1^t_{E13A}**, **1^t_{E13K}**, and **1^t_{β}**; Figure 1) to their equilibrium in thiol-dependent decomposition (Scheme 1). **1^t_{E13A}** and **1^t_{E13K}** mutants consist of Ala and Lys residues, respectively, in place of Glu 13, which constitutes part of the helix–helix interaction interface. These mutations are expected to have minor (**1^t_{E13A}**) and considerable (**1^t_{E13K}**) destabilizing effects on the formed structures, relative to **1^t**. Peptide **1^t_{β}** consists of 3-mercaptopropanoic acid in position 18 readily incorporated to form a thioester bond with an extra backbone methylene as a β-amino acid analogue (Figure 1 a). The CD measurements (Supporting Information, Figure S3) showed that **1^t_{E13A}** forms a stable coiled-coil structure, with a helix content similar to **1^t** (63%); **1^t_{β}** can also form coiled-coils but with lower helix content (51%), and **1^t_{E13K}** cannot form coiled-coils under the studied conditions. This unfolding stability trend was found indeed to correlate well with the thiol-dependent decomposition, where we found K_{eq} values of 3×10^{-3}, 4.2×10^{-3}, and 1.0 for **1^t_{E13A}**, **1^t_{β}**, and **1^t_{E13K}**, respectively.

The reversible thiol–thioester exchange reactions can drive domain replacement between proteins in response to environmental changes, such as changes in folding/denaturation conditions and/or introduction of external template molecules. Dynamic exchange reactions have been described before[27] for small molecules[27] or libraries of short peptides.[15,28–31] We have followed such a process within a small dynamic library made of the four thiodepsipeptides **1^t**, **1^t_{E13A}**, **1^t_{E13K}**, and **1^t_{β}** (95 μM each). These were equilibrated together in presence of their respective electrophilic fragments, **E** (200 μM), **E_{E13A}** (100 μM), and **E_{E13K}** (100 μM), and excess of 2-mercaptoethyl sulfonate (Figure 4; Supporting Information, Figure S8). The effect of external template on domain exchange was evaluated when the mixture was seeded with peptide **2** (Figure 1) that forms stable coiled-coils with the most unstable thioester **1^t_{E13K}**.[14] The concentrations of the four peptides at equilibrium ($t \geq 30$ h) were calculated from HPLC chromatograms of the complex mixture (Figure 4 b; Supporting Information, Figure S8 a). Figure 4 c shows that in the absence of template, the library equilibrated such that **1^t_{E13K}** concentration decreased significantly (−80%), while **1^t_{E13A}** and **1^t_{β}** concentrations stayed almost unchanged and the concentration of **1^t** almost doubled. This is explained by degradation of **1^t_{E13K}**, which produces **N** molecules that then

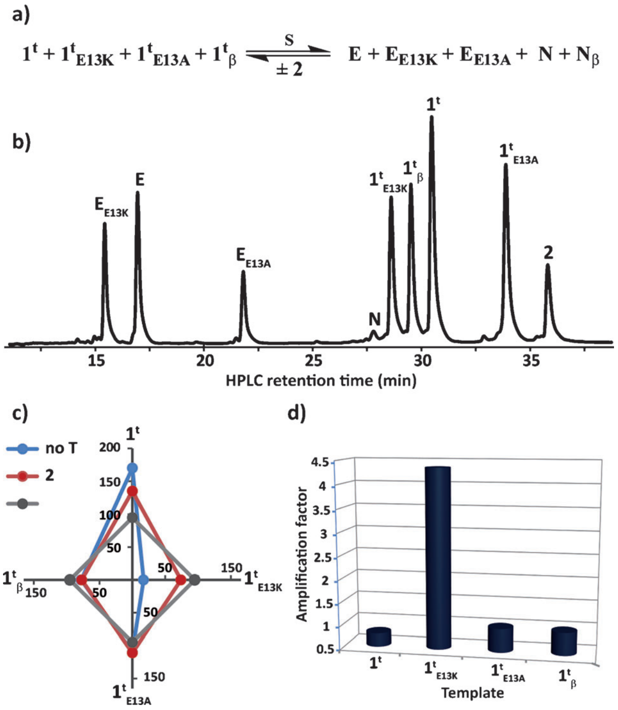


Figure 4. Network equilibration experiments. a) Network reaction formula. b) Representative HPLC chromatogram that was used to follow the network equilibration. c) Measured concentrations (μM) of thiodepsipeptides **1^t**, **1^t_{E13A}**, **1^t_{E13K}**, and **1^t_{β}** within the network medium before equilibration (gray), after equilibration without any external template (blue), or after equilibration in the presence of peptide **2** as a template (red). d) Calculated amplification factors, obtained by dividing the concentration of a thiodepsipeptide in template-seeded reaction by its concentration in the template-free reaction. Experimental details and further characterization of the network experiments are given in Supporting Information.

react with **E** to form primarily peptide $\mathbf{1^t}$, leading to formation of the more stable $\mathbf{1^t}$-containing coiled-coil structures. When the reaction was re-equilibrated in the presence of **2**, the product distribution was significantly different, revealing degradation of $\mathbf{1^t}_{\mathbf{E13K}}$ to a much lower extent and accordingly lower formation of $\mathbf{1^t}$. The template amplification effect on product distribution in the library is manifested in Figure 4d, showing exclusively the amplification of $\mathbf{1^t}_{\mathbf{E13K}}$ (amplification factor = 4.4), no meaningful effect on $\mathbf{1^t}_{\mathbf{E13A}}$ and $\mathbf{1^t}_{\beta}$ concentrations, and an amplification effect of less than unity as an indirect result of lower degradation of $\mathbf{1^t}_{\mathbf{E13K}}$.

In summary, we have shown that detailed characterization of the thioester bond integrity and its environment can be valuable for explaining and predicting dynamic behavior of thioester peptides and proteins. While the coiled-coil motif is very abundant in nature (appearing in about 5% of all proteins), and as ester and thioester bonds are frequently used by chemists in research and industry, it was interesting to find out that no high-resolution structure was available for coiled-coil depsipeptides or thiodepsipeptides. We suggest that the thiol–thioester-driven exchange in our model can be relevant for studying the dynamic chemistry of other technologically and pharmaceutically important proteins.

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