A cysteine selenosulfide redox switch for protein chemical synthesis

Vincent Diemer, Nathalie Ollivier, Bérénice Leclercq, Hervé Drobecq, Jérôme Vicogne, Vangelis Agouridas*, Oleg Melnyk*

University of Lille, CNRS, Institut Pasteur de Lille, UMR CNRS 8204, INSERM U1019, Centre d'Immunité et d'Infection de Lille, F-59000 Lille, France

Abstract

The control of cysteine reactivity is of paramount importance for the synthesis of proteins using the native chemical ligation (NCL) reaction. We discovered that this goal can be achieved in a traceless manner during ligation by appending a simple *N*-selenoethyl group to cysteine. While in synthetic organic chemistry the cleavage of carbon-nitrogen bonds is notoriously difficult, we found that *N*-selenoethyl cysteine (SetCys) loses its selenoethyl arm in water under mild conditions upon reduction of its selenosulfide bond. Detailed mechanistic investigations uncover a novel mode of reactivity for Cys. Its implementation in a process enabling the modular and straightforward assembly of linear or backbone cyclized polypeptides is illustrated by the synthesis of biologically active cyclic hepatocyte growth factor variants.

Introduction

In recent years, the study of protein function has made tremendous advances thanks to the development of chemical synthetic tools and strategies for producing peptides and proteins. The vast majority of proteins obtained this way are assembled using native chemical ligation (NCL[1], Figure 1a) or derived methods .[2, 3, 4, 5] NCL involves the reaction of a peptide thioester with a Cys peptide to produce a native peptide bond to Cys. The synthesis of complex protein scaffolds requires the control at some point of the reactivity of Cys for orienting the order by which the peptide bonds connecting the various peptide segments are produced (Figure 1a).[6] Therefore, designing new strategies for modulating Cys

reactivity is a contemporary concern and stimulates the creativity of protein and organic chemists worldwide.[7, 8, 9, 10, 11]

One hallmark of the Cys residue is its involvement in the formation of disulfide or selenosulfide bonds (Figure 1b),[12] which often play a critical role in protein folding. Nature also exploits the redox properties of Cys thiols to control the activity of some enzymes featuring a Cys residue at their catalytic site.[13] Indeed, the conversion of a catalytic Cys thiol into a disulfide is a powerful means for shutting down enzymatic activity because disulfides are poor nucleophiles compared to thiolates. Thioredoxin-reductase or glutathione reductase are typical examples where the enzymes become active upon reduction of a disulfide bond.[13] In synthetic organic chemistry, the redox properties of the thiol group also offer a simple means for controlling its reactivity.[14] Unfortunately, acyclic dichalcogenide derivatives of Cys are labile or in fast exchange under the reducing conditions used for performing NCL. Consequently, such a bioinspired control of NCL by using Cys thiol as a redox switch has not so far proved achievable. In practice, Cys reactivity is instead masked during protein assembly by introducing classical alkyl- or acyl-based protecting groups on the α-amino group, on the side-chain thiol or both (for a recent review see reference [2]).

To circumvent the high lability of Cys acyclic disulfides during NCL and to use Cys thiol as a redox switch for controlling protein assembly, we sought to embed the Cys thiol in a cyclic dichalcogenide as such species are known to be significantly more oxidizing than their linear counterparts.[15] In this work, we explored the properties of SetCys, the cyclic selenosulfide obtained by introducing a selenoethyl appendage on the α-amino group of Cys (Figure 1c). We discovered that the products of NCL with SetCys peptides vary with the strength of the reducing agent. Importantly, SetCys spontaneously loses its selenoethyl arm in water at neutral pH in the presence of popular disulfide bond reductants such as dithiothreitol (DTT) or *tris*(2-carboxyethyl)phosphine (TCEP). This chemical behavior contrasts with the known difficulty in breaking carbon-nitrogen bonds, a process that usually requires harsch conditions,[16, 17] metal catalysis[18] or radical reactions.[19, 20] In contrast, the detailed mechanistic investigations reported here point toward an anionic mechanism that depends on the ionization state of SetCys in its ring-opened and reduced form. In this respect, SetCys uncovers a novel mode of reactivity for Cys and provides a useful means for accessing complex protein scaffolds as illustrated by the total

one-pot synthesis of biologically active linear or backbone cyclized variants of the hepatocyte growth factor (HGF) kringle 1 (K1) domain.

Results

SetCys peptides display an array of reactivities depending on the reducing environment

The NCL reaction is classically performed in the presence of aryl thiol catalyts,[21] of which 4-mercaptophenylacetic acid (MPAA) is considered as the gold standard.[22] In addition to its catalytic abilities, the latter also contributes to the maintenance of the reactants in a weakly reducing environment. MPAA can possibly be complemented by DTT and TCEP, two powerful reductants that are popular additives for NCL. Thus, MPAA and MPAA/DTT or MPAA/TCEP additive cocktails define two extremes in reductive power applied to ligation mixtures.

We first examined the behavior of the SetCys residue in the presence of MPAA alone, i.e., weakly reductive conditions, in the search for conditions where it could be silent. Exposure of a model SetCys peptide to a large excess of MPAA at neutral pH led to no apparent change (Figure 2a, property 1). In a second experiment, incubation of the SetCys peptide with a peptide thioester in the presence of MPAA furnished a ligated peptide featuring an internal SetCys residue (Figure 2a, property 2). Although we could not detect any reduced SetCys in the presence of MPAA alone (Figure 2a, property 1), perhaps due to its oxidation by molecular oxygen during workup and analysis, the formation of the SetCys amide product in this experiment shows that this species is likely present under these conditions. However, the rate of ligation was more than 10 fold lower than the rate observed for NCL with a Cys peptide. This observation prompted us to run a competitive reaction in which a peptide thioester and an equimolar mixture of SetCys and Cys peptides were reacted in the presence of MPAA (Figure 2a, property 3). Interestingly, this experiment resulted in the exclusive formation of the ligation product with the Cys peptide. We further showed that the SetCys peptide does not interfere with NCL even when the thioester component features a sterically demanding amino acid at its C-terminus, typically a valine residue (see Supporting Information). We also verified that internal Cys residues are unable to activate SetCys residue, which is therefore useful for the production of Cys-rich peptides (see Supporting Information).

Thus, the background NCL observed for a SetCys peptide in the presence of MPAA is unable to perturb a regular NCL involving a Cys peptide.

The most striking property of SetCys was observed when the SetCys peptide was subjected to the strong reducing conditions imposed by DTT or TCEP (Figure 2a, reaction 4). In this case, the reaction cleanly furnished the Cys peptide. We further documented that the reaction of a SetCys peptide with a peptide thioester in the presence of TCEP furnished a ligation product featuring a native Cys residue at the ligation junction (Figure 2a, reaction 5). In contrast, the loss of the *N*-alkyl substituent was not observed when the sulfur analog of SetCys, featuring a 2-mercaptoethyl group on the α-nitrogen, was treated similarly, even after extended reaction times (Figure 2b).[23, 24] The reactivity observed for SetCys depends specifically on the presence of selenium in its structure and, in that respect, SetCys is a novel illustration of the high difference in reactivity than can exist between thiol and selenol compounds.[25]

Insights into the conversion of SetCys to a Cys residue

From a mechanistic point of view, the loss of the selenoethyl group from the SetCys residue seems unlikely to involve radical intermediates since the reaction proceeds well in the presence of a large excess of sodium ascorbate and MPAA,[26, 27] two reagents known to be powerful quenchers of alkylselenyl or alkylthiyl radicals. Omitting ascorbate during the treatment of SetCys peptide **1** by TCEP yields the deselenized peptide Et-CALKEPVHGV-NH_2 as the major product, whose formation competes against the loss of the selenoethyl arm (see Supporting Information). Furthermore, the loss of the selenoethyl limb is also observed when dithiothreitol is used as a reducing agent, definitely ruling out the possibility that the reaction might involve a classical TCEP-induced dechalcogenation process.[28, 29] Further insights into the species involved in the reaction come from the data shown in Figure 3b, which presents the effect of pH on the rate of selenoethyl limb removal from a model SetCys peptide **2**. The pH-rate profile of the conversion of SetCys peptide **2** into cysteinyl peptide **3** shows a maximum at pH 6.0 ± 0.04 and two inflexion points at pH 4.8 and 7.3, which likely correspond to the pK_as of the SetCys selenol and ammonium groups respectively. These values are in agreement with the pK_a values reported for simple 2-selanylethylamines[30] and Cys derivatives[31] or estimated by calculation (Figure 3c). The fact that the pH-rate profile of the reaction corresponds to the predominance zone for the

selenoate/ammonium zwitterionic intermediate $\mathbf{2}^{+-}$ led us to propose that the decomposition of SetCys proceeds through the intramolecular substitution of the ammonium group by the selenide ion.

This mechanism results in the formation of an episelenide, which is known to be extremely unstable at room temperature and spontaneously decomposes into ethylene and selenium (Figure 3a).[17] While selenium can be captured by TCEP in the form of the corresponding selenophosphine, whose formation was indeed observed in these reactions, detection of ethylene gas was made difficult by the small scale of synthesis.

The proposed mechanism is reminiscent of the cleavage of alkylamines by phenylselenol, albeit such reactions usually require elevated temperatures and/or assistance by metals.[16, 17, 32] Intrigued by the ease of SetCys to Cys conversion, we sought to determine if the SetCys thiol participates to the departure of the 2-selanylethyl limb. To this end, a *N*-(2-selanylethyl)-alanyl (SetAla) peptide analogue was prepared and treated with MPAA/TCEP/ascorbate at the optimal pH for the SetCys to Cys conversion, i.e., pH 6.0 (Figure 3d). LC-MS analysis of the mixture showed the conversion of the SetAla residue into Ala, but at a rate considerably lower (~ 8.5 fold) than those measured for the SetCys to Cys conversion. This experiment shows that the departure of the 2-selanylethyl limb is greatly facilitated by the nearby SetCys thiol, perhaps by allowing an intramolecular proton transfer as depicted in Figure 3a.

Insights into the mechanism of SetCys-mediated ligation

Having scrutinized the mechanism of SetCys conversion to a Cys residue under strong reductive conditions, we next examined the species involved during ligation with a peptide alkyl thioester under the same redox conditions. The monitoring of the reaction between SetCys peptide **1** and peptide thioester **4** indicated that a first ligation product **5,** containing an internal SetCys residue, accumulated within the first minutes and then slowly disappeared over two days in favor of peptide **6** featuring a native peptide bond to Cys (Figure 4a,b).

Regarding the mechanism of SetCys-mediated ligation under strong reducing conditions, we hypothesized that the early formation of intermediate **5** is due to the interception of the reduced SetCys unit **2** by the thioester component. The latter is likely to be present in the form of the aryl thioester **7**, produced in situ from peptide alkyl thioester **4** by thiol-thioester exchange with the MPAA catalyst

(Figure 4c). Of the two nucleophilic sites present in reduced SetCys unit, the selenol moiety is probably the more reactive due to its lower pK_a and higher nucleophilicity. The formation of tertiary amides of type **5** is known to be reversible in the conditions used for the ligation through their capacity to undergo an intramolecular nitrogen to selenium or sulfur acyl group migration.[23, 24, 33] Therefore, SetCys peptide **2** is constantly present in solution and escapes the SetCys/SetCys amide equilibrium by irreversibly losing its *N*-selenoethyl limb as discussed above. The Cys peptide **3** produced this way is expected to undergo a classical NCL reaction with aryl thioester **7** to yield ligated Cys peptide **6**. Although the proposed mechanism arises from the properties of the SetCys unit described in Figure 2, we sought to confront it to kinetic data for validation. In addition, the model also tests the possibility of a direct conversion of SetCys amide **5** into final product **6**, being fully aware that the cleavage of the selenoethyl appendage from compound **5** through an ionic mechanism is unlikely due to the poor leaving group ability of imido nitrogens.

We first determined the rate constants associated with the thiol-thioester exchange process involving peptide thioester **4** and MPAA (k_{+2}, k_{-2}), with the conversion of SetCys peptide **2** into Cys peptide **3** (k_1) and with the reaction of peptide aryl thioester **7** with Cys peptide **3** (k_4), from model reactions run separately (see Supplementary Information). These rate constants were used to determine the remaining kinetic parameters k_{+3}, k_{-3} and k_5 by fitting the experimental data of the SetCys-mediated ligation (circles and triangles in Figure 4b) to the mechanistic model. The quality of the fit (dashed lines in Figure 4b, $p = 0.003$) strongly suggests that the conversion of intermediate **5** to peptide **6** exclusively occurs through SetCys peptide **2** since the kinetic constant for the direct process **5** $\rightarrow$ **6** is at least 100 fold below the value measured for the decomposition of the SetCys into the Cys residue, i.e. **2** $\rightarrow$ **3**. The second piece of information provided by the model is that the SetCys peptide in its reduced form reacts almost as fast with the peptide thioester component as does the Cys peptide. Finally, the model tells us that the loss of the *N*-selenoethyl sidechain from SetCys peptide **2** is the rate limiting step of SetCys-mediated ligation.

SetCys redox-switch enables the straightforward synthesis of cyclic proteins in one-pot

Having characterized the differential reactivity of the SetCys unit under mild and strong reducing conditions, we further sought to develop a simple process enabling the synthesis of cyclic

proteins using SetCys as a redox switch (Figure 5). The motivation for this particular application comes from the observation that although a few studies pointed out the potential of protein cyclization for improving protein thermal stability,[34, 35] resistance to proteolytic degradation[35] and potency,[36] this modification has not so far been widely used for the design of protein therapeutics. This situation contrasts with the success of small cyclic protein scaffolds such as cyclotides used as platforms for drug design,[37] and the frequent use of macrocyclization in the development of small peptidic drugs.[38] The fact that protein cyclization is seldom used for protein optimization cannot be ascribed to inappropriate N-C distances, because half of the protein domains found in the protein data bank (PDB) have their C and N extremities joinable by linkers made of a few amino acids.[39] Rather, this situation reflects the paucity of tools for building cyclic proteins in a modular approach that facilitates the optimization of the linker joining the N- and C-termini.[40]

Classical methods leading to cyclic proteins involve the macrocyclization of a bifunctional and linear precursor,[41] primarily by using the native chemical ligation reaction (NCL[1]) between a C-terminal thioester group and an N-terminal cysteine (Cys) residue (Figure 5a).[2, 42] Following this strategy, the optimization of the linker requires the production of a library of extended precursors of varying length and composition, an approach that inevitably makes the production of cyclic analogs cumbersome.

In this work, we sought to develop a modular one-pot method enabling the grafting of the linker to a unique linear protein precursor (Figure 5b). This can be achieved by exploiting the silent properties of the SetCys residue under mild reducing conditions for performing the first NCL (see property 3, Figure 2a), and then by using it as a redox switch for triggering the second cyclative NCL (see property 5, Figure 2a). Regarding the acyl donors, the process utilizes the good reactivity of alkyl thioesters in the presence of MPAA for the first ligation step. The second acyl donor is introduced as the *bis*(2-sulfenylethyl)amido (SEA)[43] latent thioester surrogate, which nicely complements the SetCys unit since it can also be activated in the presence of TCEP.[44, 45] The Cys / SEA couple of functional groups is located on the linear protein precursor, while the SetCys / alkyl thioester functionalities are on the linker peptide. In the presence of MPAA alone, the Cys-mediated NCL with the peptide alkyl thioester (Step 1, Figure 5b) exclusively yields a bifunctional polypeptide intermediate, which is activated at both ends by the addition of TCEP in one-pot (Step 2) to produce the backbone-cyclized polypeptide (Step 3). The

process is highly tolerant of polypeptide length as similar isolated yields were obtained for the production of medium to large cyclic peptides (30-93 AAs, ~27-37% overall yields). The examples include the synthesis of two cyclic and biotinylated variants of hepatocyte growth factor / scatter factor (HGF/SF) kringle 1 domain (K1), i.e., **cK1-1** and **cK1-2**, from a unique 78 AA linear precursor. These cyclic polypeptides differ by the length of the linker joining N and C-termini of the K1 protein (10 and 14 residues respectively).

SetCys chemistry proved equally useful for the C-to-N one-pot assembly of linear polypeptides from three peptide segments (Figure 5a,c). The peptide segments could be added sequentially (Figure 5c, Method A) or mixed altogether from the beginning of the assembly process (Figure 5c, Method B) with equal selectivity and efficiency.

Folding and biological activity of biotinylated K1 cyclic analogs

The signaling of the HGF/SF/MET system plays a crucial role in the regeneration of several tissues such as the liver or the skin, while its deregulation is often observed in cancer. The HGF/SF K1 domain contains the main HGF/SF binding site for the MET tyrosine kinase receptor and thus constitutes an interesting platform for designing future drugs based on this couple of proteins.[46] In this study, we sought to produce cyclic analogues of the K1 domain to investigate the tolerance of the K1/MET signaling system to this modification. The X-ray crystal structure of the K1 protein shows that its tertiary structure is made up of a series of loops stabilized by three disulfide bonds (Figure 6a).[47] The N- and C-terminal cysteine residues are on the opposite side of the MET binding loop and are linked by a disulfide bond. The N- and C termini are thus close in space and can be joined by a peptide linker made of a few amino acid residues which include a biotinylated lysine residue. The latter is used to multimerize the ligand using streptavidin (**S**) due to the observation that multivalent presentation of the K1 domain is important for achieving high binding and agonistic activities.[46]

The successful synthesis of the cyclic K1 polypeptides **cK1-1** and **cK1-2** set the stage for the folding step. **cK1-1** and **cK1-2** were folded into **cK1-1f** and **cK1-2f** respectively using the glutathione/glutathione disulfide redox system (Figure 6b,c).[45] The folding mixtures converged to a major form after 24 h and were purified by dialysis (Figure 6c, see also Supplementary Information).

Extensive proteomic analysis of the folded proteins **cK1-1f** and **cK1-2f** showed the exclusive formation of the native pattern of disulfide bonds between Cys128-206, Cys149-189 and Cys177-201 as shown in Figure 6b (see Supplementary Information). Thus the cyclization does not perturb the correct pairing of the Cys residues.

cK1-1f and **cK1-2f** proteins were first analyzed for their capacity to bind to the recombinant MET extracellular domain. The competitive AlphaScreen® assay with recombinant NK1 protein showed that the backbone cyclized proteins **cK1-1f** and **cK1-2f** were ~10 fold less potent in binding the MET receptor than the biotinylated analog **K1B** (Figure 6d). This result was unexpected because the cyclization site is diametrically opposite the MET binding site. The capacity of the cyclic K1 proteins to activate the MET receptor was further examined using human HeLa cells (Figure 6e). MET phosphorylation induced by the cyclic K1 proteins was found to be less than that observed with the reference **K1B** analog. However, the tested K1 analogs triggered downstream signaling pathways, i.e., phosphorylation of AKT and ERK, with almost equal potency. Because previous studies showed marked differences between MET phosphorylation levels and the strength of MET specific phenotypes induced by HGF or HGF mimics,[48] we further analyzed the capacity of the different K1 proteins to trigger the scattering of human cells in vitro (Figure 6f). In this assay using human Capan 1 cells, the cyclic proteins behaved similarly to the reference protein **K1B** in the concentration range tested (10 pM-100 nM) by their capacity to induce a mesenchymal-like phenotype and cell scattering. To summarize, cyclization in this case results in a significant loss of affinity, although the backbone cyclization site is opposite to the receptor binding site. However, this loss of affinity does not translate into the cell scattering activity. This work highlights the need for simple synthetic methods toward cyclic proteins to rapidly investigate the interest of backbone cyclization for improving protein properties.

In conclusion, the cyclic selenosulfide derivative of cysteine, i.e. SetCys, shows an array of reactivities in water depending on the reducing power of the solution. A striking property of SetCys is its conversion to a native Cys residue by cleavage of a nitrogen-carbon bond under extremely mild conditions if a strong reducing agent is present in the solution. This transformation also occurs in the presence of a peptide thioester component and thus leads to the production of a native peptide bond to Cys by native chemical ligation. In contrast, SetCys remains silent during NCL if it is conducted under

mild reducing conditions. The redox-switch properties of SetCys are particularly adapted for flexible synthetic designs that involve nothing more than common additive that are routinely used in NCL reaction.

Acknowledgements

We thank ANR for financial support (CyProt, ANR-19CE07-0020).

Author information

Affiliations

University of Lille, CNRS, Institut Pasteur de Lille, UMR CNRS 8204, INSERM U1019

Vincent Diemer, Nathalie Ollivier, Bérénice Leclercq, Hervé Drobecq, Jérôme Vicogne, Vangelis Agouridas, Oleg Melnyk

Author contributions

VD performed the experiments and wrote the manuscript. NO prepared the linear K1 precursor. HD performed the proteomic experiments. BL and JV performed the AlphaScreen® and the cell-based assay. VA performed the modelization study and wrote the manuscript. OM conceived the study and wrote the manuscript.

Corresponding authors

Correspondence to Oleg Melnyk or Vangelis Agouridas.

Ethics declarations

Competing interests

The authors declare no competing interests.

Supplementary information

Experimental details, materials and methods, kinetic model for SetCys-mediated ligation, LC-MS data and NMR spectra.

Data Availability

The source data underlying Figs 6d-f are provided as a Source Data file.

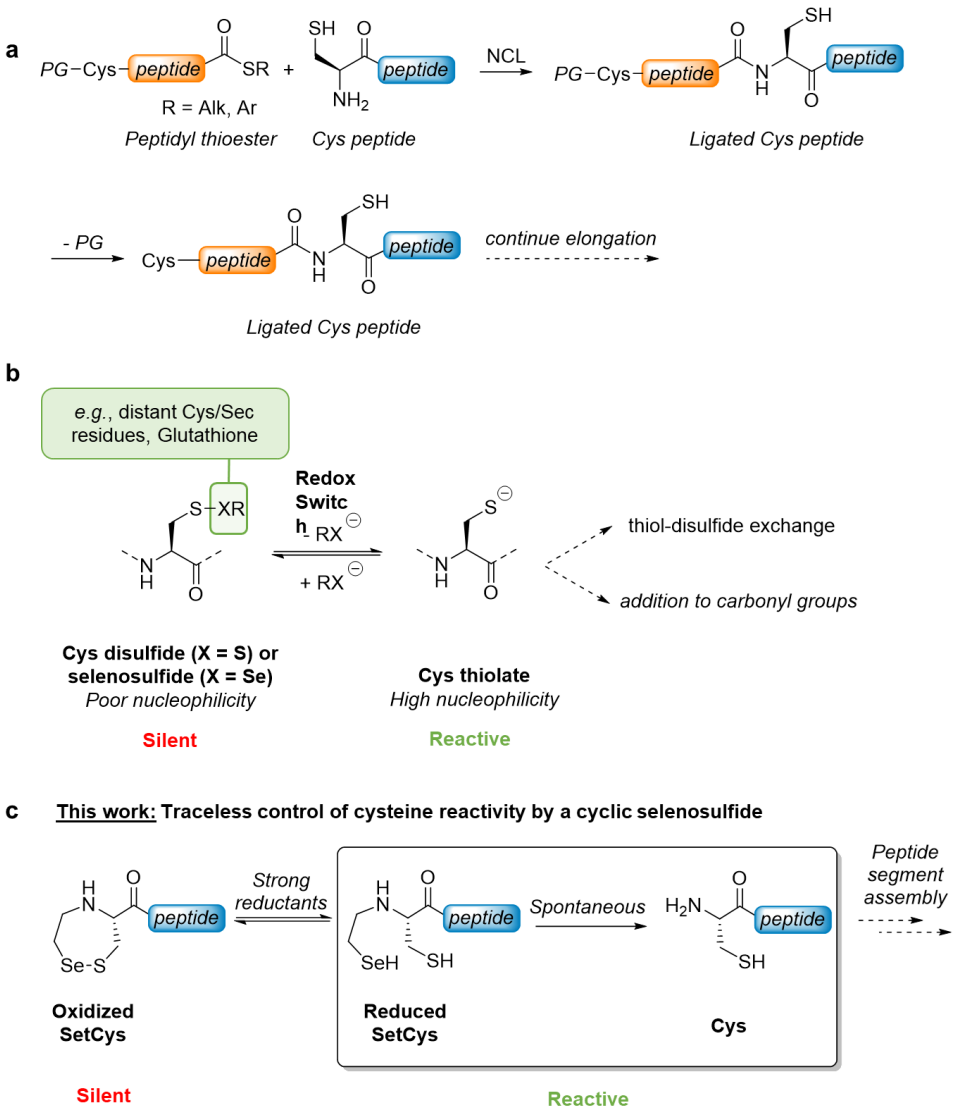


Figure 1. a) Principle of the native chemical ligation (NCL) between a C-terminal peptide thioester and a cysteinyl (Cys) peptide. The control of the site of ligation requires masking any N-terminal Cys residue present in the mixture. b) The reversible formation of disulfide or diselenide bonds is a hallmark of Cys thiol chemistry in proteins and is used by nature as a redox switch to control Cys thiol reactivity. c) The *N*-(2-selanylethyl) group of SetCys shuts down the nucleophilic properties of Cys thiol by formation of a cyclic selenosulfide bond. It is removed spontaneously in water at neutral pH under reductive conditions.

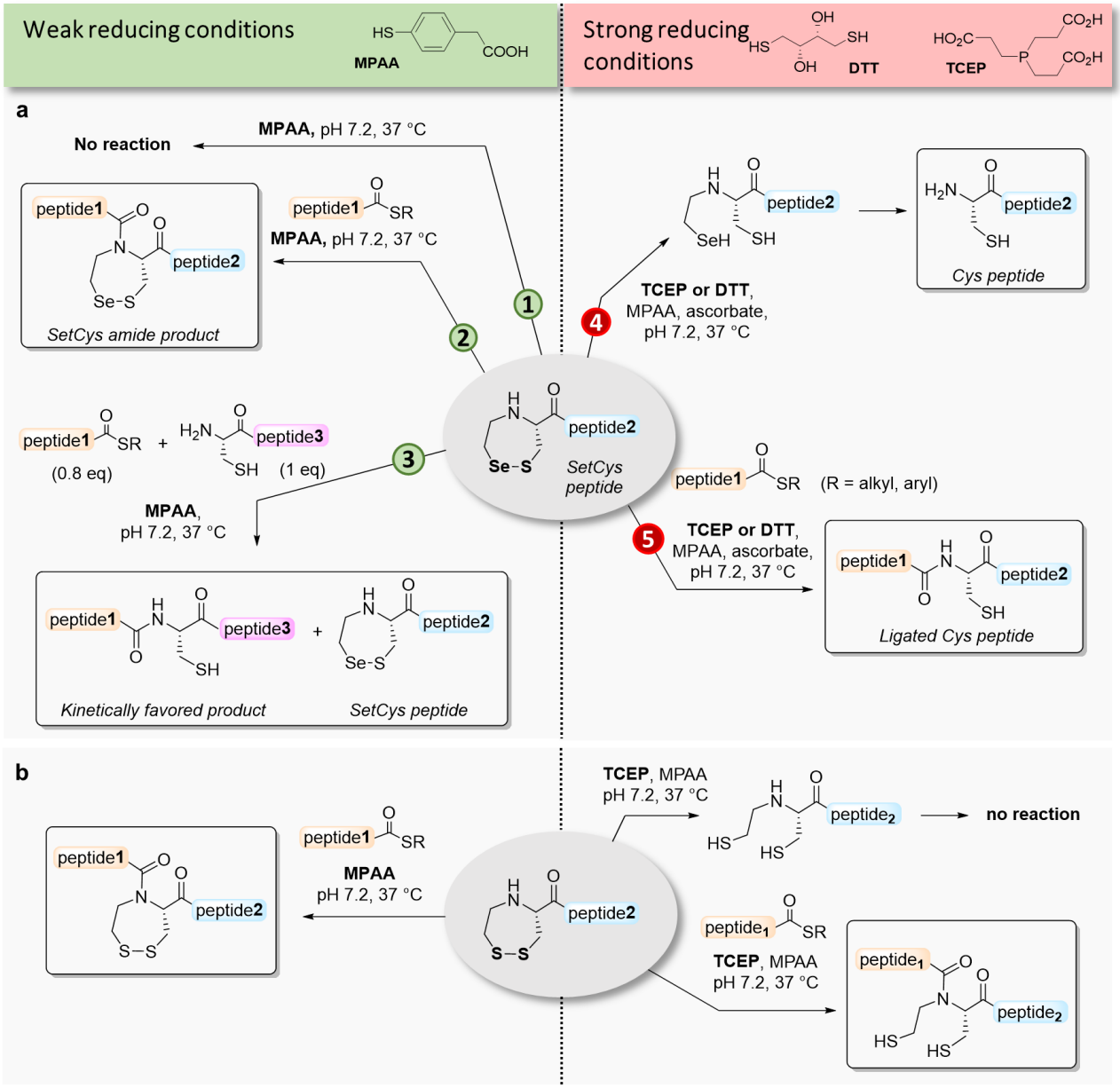


Figure 2. a) The reactivity of *N*-(2-selanylethyl)cysteine (SetCys) peptides is controlled by the reducing strength of the mixture. The numbers on the arrows indicate different experimental conditions. Condition 1-3: weakly reducing conditions, typically in the presence of an excess of 4-mercaptophenylacetic acid (MPAA) at neutral pH. Conditions 4 and 5: strong reducing conditions, typically in the presence of dithiothreitol (DTT) or *tris*(2-carboxyethyl)phosphine (TCEP) at neutral pH. b) Ligation of *N*-(2-sulfanylethyl)cysteine (SutCys) peptides with peptide alkyl thioester under weak or strong reducing conditions. Peptide sequences: peptide 1 = RLKEPVHGA-, peptide 2 = ALKEPVHGV-NH_2, peptide 3 = ILKEPVHGV-NH_2.

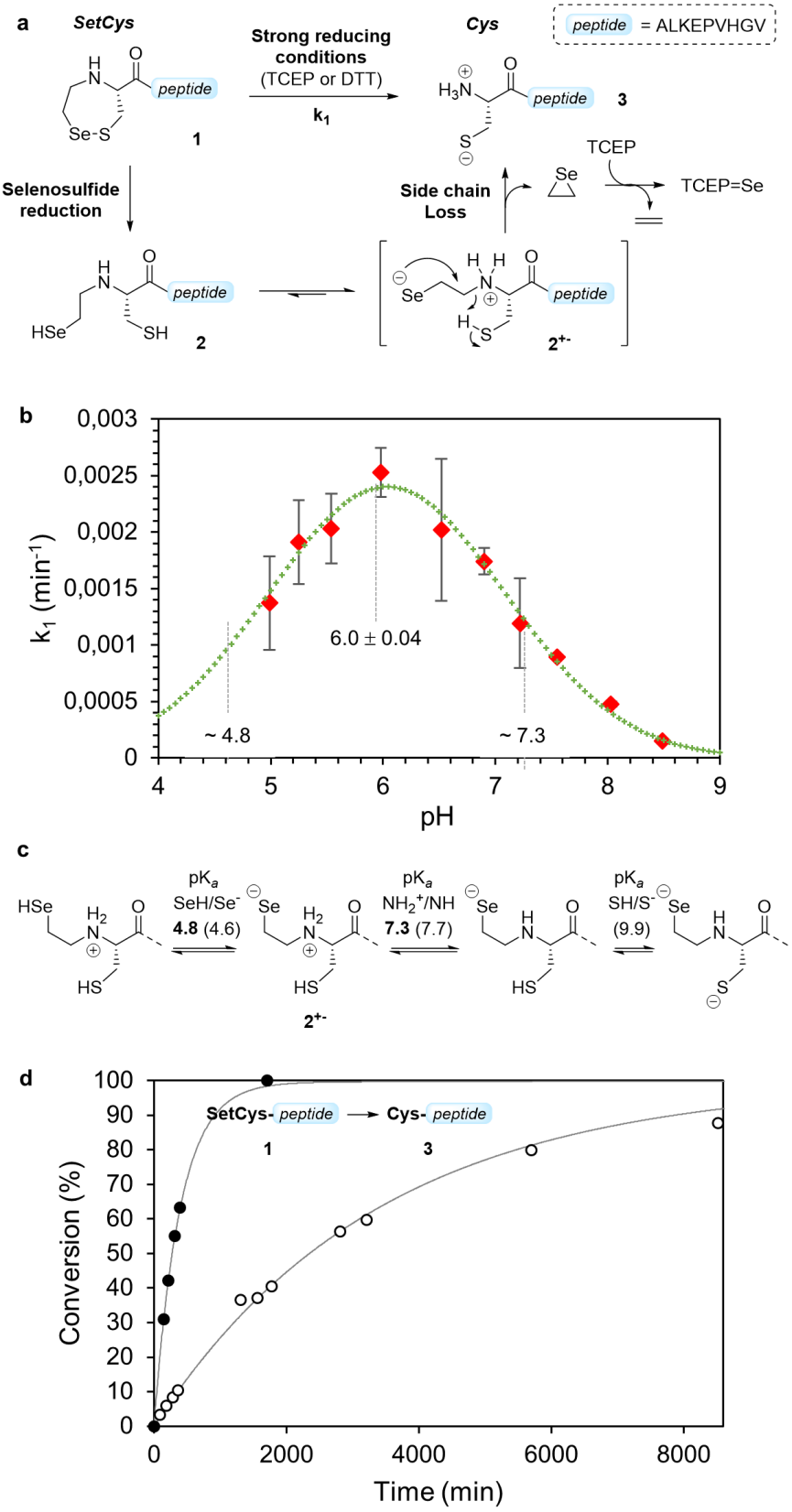
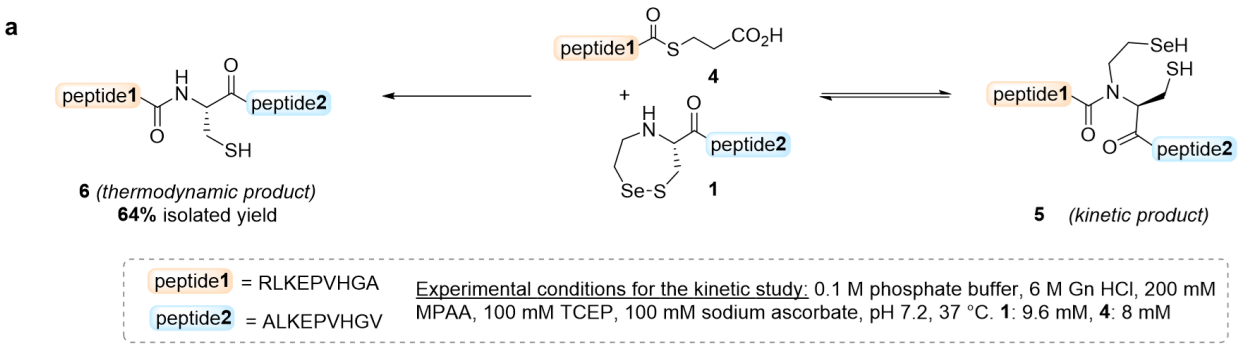
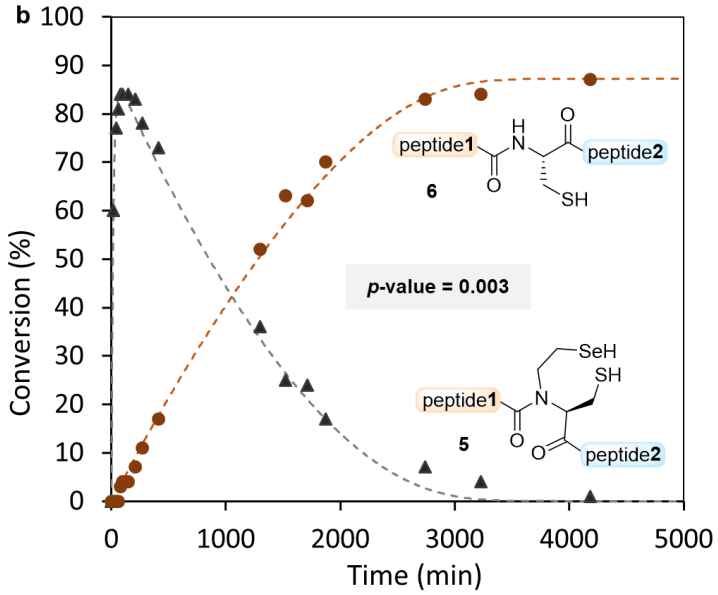
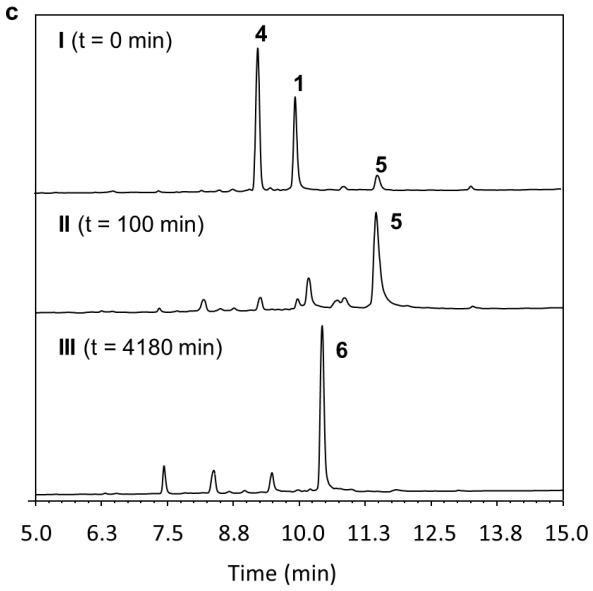


Figure 3. a) Proposed mechanism for the loss of the *N*-selenoethyl appendage of N-terminal SetCys peptides; b) Experimental pH-rate profile of the conversion of N-terminal SetCys peptides to the

corresponding N-terminal cysteinyl peptide (red diamonds). The data were fitted to a Gaussian (green curve, $p < 0.001$) to determine the pH values for the inflexion points (pH 4.8 and 7.3). c) Different ionization states for the SetCys residue in open form. The pK_a values in bold correspond to the inflexion points determined in b. The values in parentheses were calculated using ACDLabs® software. d) Rate of 2-selanylethyl limb cleavage in SetCys (●, $k = 2.53 \times 10^{-3}$ min^{-1}) and SetAla (O, $k = 2.96 \times 10^{-4}$ min^{-1}) model peptides at pH 6.


a
peptide1
peptide2
SH
6 (thermodynamic product)
64% isolated yield
4
1
Se-S
5 (kinetic product)
peptide1 = RLKEPVHGA
peptide2 = ALKEPVHGV
Experimental conditions for the kinetic study: 0.1 M phosphate buffer, 6 M Gn HCl, 200 mM MPAA, 100 mM TCEP, 100 mM sodium ascorbate, pH 7.2, 37 °C. 1: 9.6 mM, 4: 8 mM


b
Conversion (%)
Time (min)
p-value = 0.003
6
5


c
I (t = 0 min)
II (t = 100 min)
III (t = 4180 min)
Time (min)

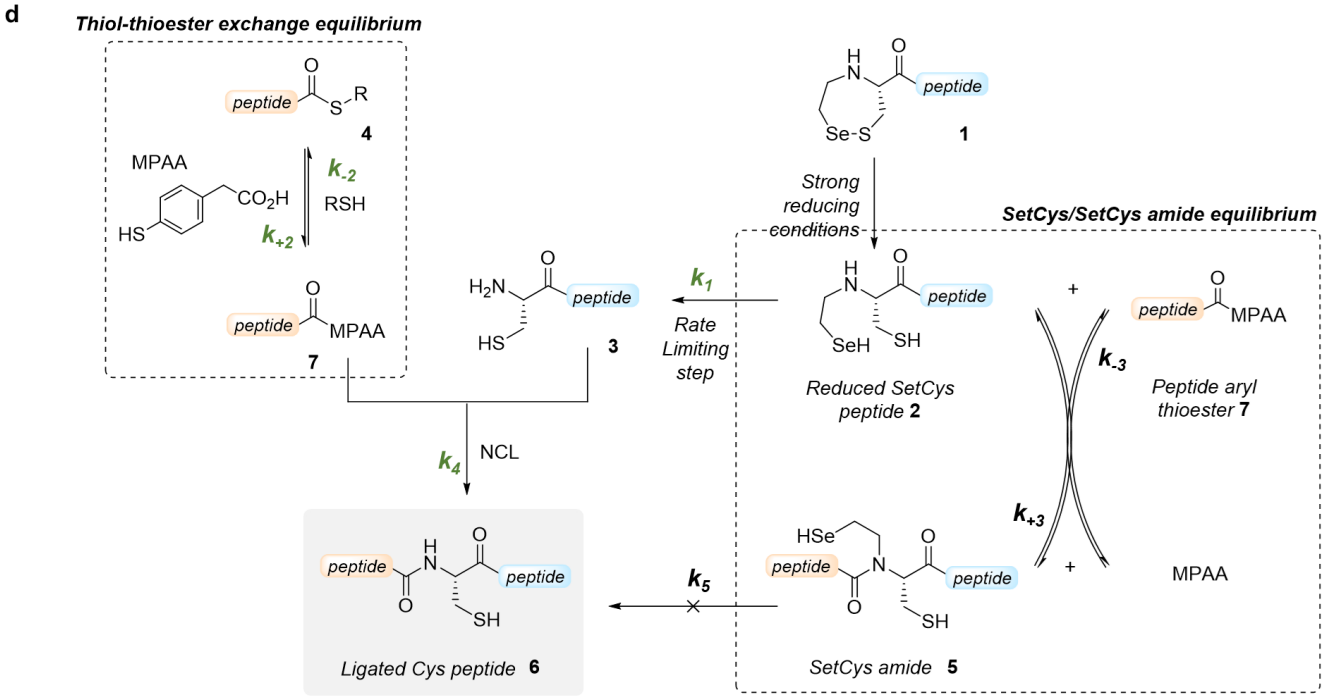
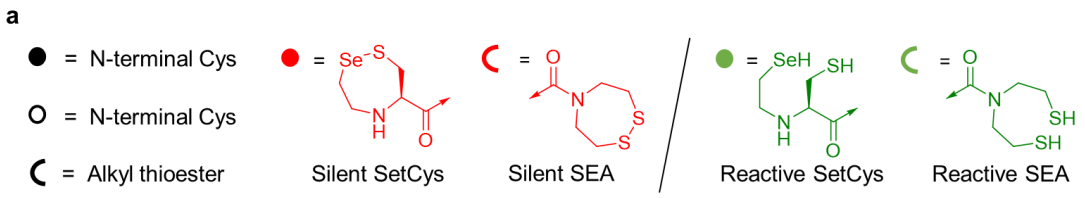
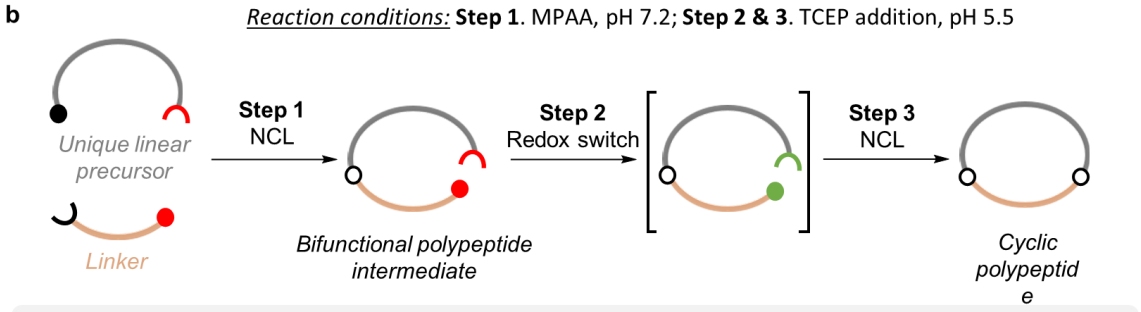

d
Thiol-thioester exchange equilibrium
MPAA
RSH
7
3
Strong reducing conditions
SetCys/SetCys amide equilibrium
Rate Limiting step
NCL
Reduced SetCys peptide 2
Peptide aryl thioester 7
SetCys amide 5
Ligated Cys peptide 6

Figure 4. a) Ligation of *N*-(2-selanylethyl)cysteine peptides with peptide alkyl thioester in NCL standard conditions yields a product featuring a native peptide bond to cysteine; b) RP-HPLC monitoring of the conversion of peptides **5** (black triangles) and **6** (brown circles) throughout the course of the reaction. Fitting curves for each compound are represented by dashed lines; c) Proposed mechanistic model for the ligation of *N*-(2-selanylethyl)cysteine peptides under strong reducing conditions. The rate constants were obtained by software-assisted numerical integration of rate equations (Kintek explorer^{TM}).





Linkers

8a ALKEPVHGA
8b $(GS)_2K(Biot)(GS)_2A$
8c $(GS)_3K(Biot)(GS)_3A$

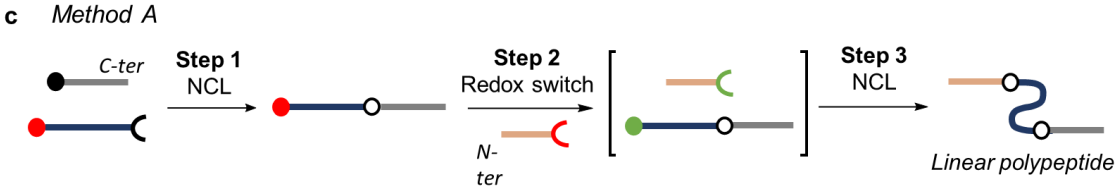
Unique linear precursors

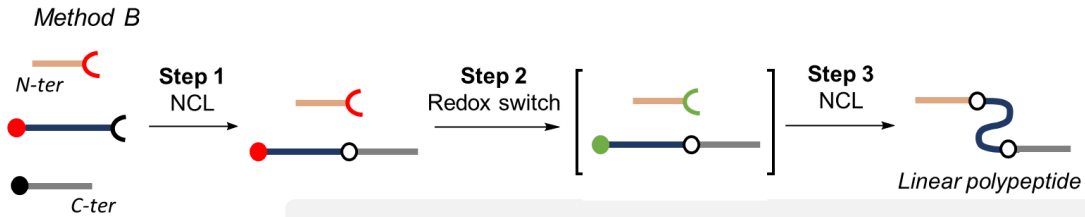
9a PDTRPAPGSTAPPAHGVTS
9b (K1 domain)
IIGKGRSYKGTVSITKSGIKCQPWSSMI
PHEHSFLPSSYRGKDLQENYCRNPRG
EEGGPWCFTSNPEVRYEVCDIPQ

Cyclic polypeptides

		Yield*	Nb of AAs			Yield*	Nb of AAs
model cyclizations				*K1 cyclizations*			
10	(**8a** + **9a**)	27%	30	**cK1-1**	(**8b** + **9b**)	31%	89
11	(**8b** + **9a**)	28%	31	**cK1-2**	(**8c** + **9b**)	37%	93

*Isolated yields





N-ter segment — *N-ter* — **12** IRNCIIGKGRSYKGTVSITKSGIK

Bifunctional central segment — **13** QPWSSMIPHEHSFLPSSYRGKDLQENY

C-ter segment — *C-ter* — **14** RNPRGEEGGPWCFTSNPE VRYEVCDIPQCSEVK(Biot)

Linear polypeptide

Biotinylated linear K1 domain **15** (**12** + **13** + **14**)
Method A **28%**
Method B **29%** isolated yields

Figure 5. a) Legend; b) One-pot grafting of a peptide linker to a unique linear precursor yields cyclic polypeptides. Application to the total synthesis of cyclic and biotinylated analogs of HGF/SF K1 domain (**cK1-1**, **cK1-2**); c) One-pot synthesis of linear K1 domain.

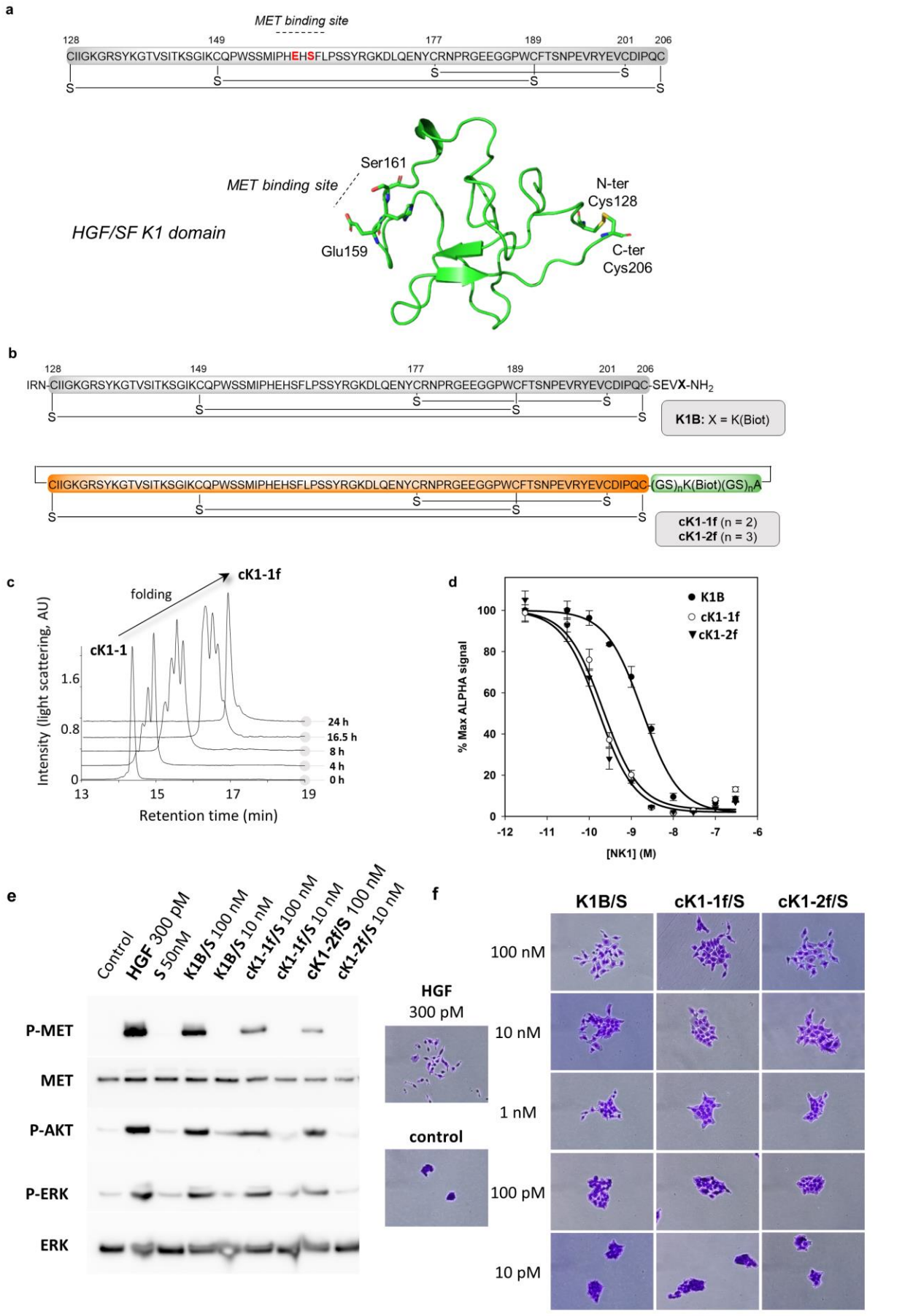

a
MET binding site
128 149 177 189 201 206
CIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEHSFLPSSYRGKDLQENYCRNPRGEEGGPWCFTSNPEVRYEVCDIPQC
HGF/SF K1 domain
Ser161
MET binding site
N-ter
Cys128
Glu159
C-ter
Cys206
b
IRN-CIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEHSFLPSSYRGKDLQENYCRNPRGEEGGPWCFTSNPEVRYEVCDIPQC-SEVX-NH2
K1B: X = K(Biot)
CIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEHSFLPSSYRGKDLQENYCRNPRGEEGGPWCFTSNPEVRYEVCDIPQC-(GS)nK(Biot)(GS)nA
cK1-1f (n = 2)
cK1-2f (n = 3)
c
folding
cK1-1f
cK1-1
Intensity (light scattering, AU)
24 h
16.5 h
8 h
4 h
0 h
Retention time (min)
d
K1B
cK1-1f
cK1-2f
% Max ALPHA signal
[NK1] (M)
e
Control
HGF 300 pM
S 50nM
K1B/S 100 nM
K1B/S 10 nM
cK1-1f/S 100 nM
cK1-1f/S 10 nM
cK1-2f/S 100 nM
cK1-2f/S 10 nM
P-MET
MET
P-AKT
P-ERK
ERK
f
K1B/S
cK1-1f/S
cK1-2f/S
HGF
300 pM
control
100 nM
10 nM
1 nM
100 pM
10 pM

Figure 6. a) Primary and tertiary structure of HGF/SF K1 domain (pdb entry 1BHT). b) Biotinylated K1 analogs tested for their capacity to bind MET receptor and induce MET-specific phenotypes. The pattern of disulfide bonds determined experimentally corresponds to the native pattern found in K1 domain X-ray crystal structures. c) LC-MS monitoring of the folding of **cK1-1** peptide into **cK1-1f**. d) Competitive AlphaScreen® assay with recombinant NK1 protein. **K1B** or **cK1-1f** or **cK1-2f** were mixed with increasing concentrations of NK1 and with extracellular MET domain fused with human IgG1-Fc (MET-Fc) and incubated with streptavidin AlphaScreen® donor beads and Protein A acceptor beads. e) HeLa cells were treated for 10 min with 300 pM mature HGF/SF (HGF), or with 10 nM / 100 nM **K1/S**, **cK1-1f/S** and **cK1-2f/S**. Cell lysates were then analyzed by specific total MET and ERK or phospho-MET, phospho-Akt and phospho-ERK Western blot; f) Cell scattering assay. Human Capan 1 isolated cell islets were incubated for 18 h in culture media with 300 pM mature HGF/SF (HGF), or 100, 10, 1 nM and 100 and 10 pM **K1B**, **cK1-1f** and **cK1-2f**.

References

1. Dawson P. E., Muir T. W., Clark-Lewis I. & Kent S. B. H. Synthesis of proteins by native chemical ligation. *Science* **266**, 776-779 (1994).
2. Agouridas V., El Mahdi O., Diemer V., Cargoet M., Monbaliu J.-C. M. & Melnyk O. Native chemical ligation and extended methods. Mechanisms, catalysis, scope and limitations. *Chem. Rev.* **12**, 7328-7443 (2019).
3. Kulkarni S. S., Sayers J., Premdjee B. & Payne R. J. Rapid and efficient protein synthesis through expansion of the native chemical ligation concept. *Nat. Rev. Chem.* **2**, 0122 (2018).
4. Conibear A. C., Watson E. E., Payne R. J. & Becker C. F. W. Native chemical ligation in protein synthesis and semi-synthesis. *Chem. Soc. Rev.* **47**, 9046-9068 (2018).
5. Agouridas V., El Mahdi O., Cargoët M. & Melnyk O. A statistical view of protein chemical synthesis using NCL and extended methodologies. *Bioorg. Med. Chem.* **25**, 4938-4945 (2017).
6. Raibaut L., Ollivier N. & Melnyk O. Sequential native peptide ligation strategies for total chemical protein synthesis. *Chem. Soc. Rev.* **41**, 7001-7015 (2012).
7. Jbara M., Laps S., Morgan M., Kamnesky G., Mann G., Wolberger C. *et al.* Palladium prompted on-demand cysteine chemistry for the synthesis of challenging and uniquely modified proteins. *Nat. Commun.* **9**, 3154 (2018).
8. Maity S. K., Mann G., Jbara M., Laps S., Kamnesky G. & Brik A. Palladium-assisted removal of a solubilizing tag from a Cys side chain to facilitate peptide and protein synthesis. *Org. Lett.* **18**, 3026-3029 (2016).
9. Maity S. K., Jbara M., Laps S. & Brik A. Efficient palladium-assisted one-pot deprotection of (acetamidomethyl)cysteine following native chemical ligation and/or desulfurization to expedite chemical protein synthesis. *Angew. Chem. Int. Ed.* **55**, 8108-8112 (2016).
10. Jbara M., Maity S. K., Seenaiah M. & Brik A. Palladium mediated rapid deprotection of N-terminal cysteine under native chemical ligation conditions for the efficient preparation of synthetically challenging proteins. *J. Am. Chem. Soc.* **138**, 5069-5075 (2016).
11. Reddy P. S., Dery S. & Metanis N. Chemical synthesis of proteins with non-strategically placed cysteines using selenazolidine and selective deselenization. *Angew. Chem. Int. Ed.* **55**, 992-995 (2015).
12. Poole L. B. The basics of thiols and cysteines in redox biology and chemistry. *Free Radic. Biol. Med.* **80**, 148-157 (2015).
13. Klomsiri C., Karplus P. A. & Poole L. B. Cysteine-based redox switches in enzymes. *Antioxid. Redox Signal.* **14**, 1065-1077 (2011).

14. Greene T. W. in *Protection for the Thiol Group* (eds Wuts P. G. M.) 837-894 (Wiley, 2014).
15. Lees W. J. & Whitesides G. M. Equilibrium constants for thiol-disulfide interchange reactions: a coherent, corrected set. *J. Org. Chem.* **58**, 642-647 (1993).
16. Reich H. J. & Cohen M. L. Organoselenium chemistry. Dealkylation of amines with benzeneselenol. *J. Org. Chem.* **44**, 3148-3151 (1979).
17. Krief A. & Hevesi L. *Organoselenium Chemistry I. Functional Group Transformations* (Springer-Verlag, 1988).
18. Ouyang K., Hao W., Zhang W.-X. & Xi Z. Transition-Metal-Catalyzed Cleavage of C–N Single Bonds. *Chem. Rev.* **115**, 12045-12090 (2015).
19. Loibl S. F., Harpaz Z. & Seitz O. A type of auxiliary for native chemical peptide ligation beyond cysteine and glycine junctions. *Angew. Chem. Int. Ed.* **54**, 15055-15059 (2015).
20. Yin H., Lu D., Wang S. & Wang P. Development of Powerful Auxiliary-Mediated Ligation To Facilitate Rapid Protein Assembly. *Org. Lett.* **21**, 5138-5142 (2019).
21. Dawson P. E., Churchill M. J., Ghadiri M. R. & Kent S. B. H. Modulation of reactivity in native chemical ligation through the use of thiol additives. *J. Am. Chem. Soc.* **119**, 4325-4329 (1997).
22. Johnson E. C. & Kent S. B. H. Insights into the mechanism and catalysis of the native chemical ligation reaction. *J. Am. Chem. Soc.* **128**, 6640-6646 (2006).
23. Melnyk O. & Agouridas V. From protein total synthesis to peptide transamidation and metathesis: playing with the reversibility of *N,S*-acyl or *N,Se*-acyl migration reactions. *Curr. Opin. Chem. Biol.* **22**, 137-145 (2014).
24. Ruff Y., Garavini V. & Giuseppone N. Reversible native chemical ligation: a facile access to dynamic covalent peptides. *J. Am. Chem. Soc.* **136**, 6333-6339 (2014).
25. Reich H. J. & Hondal R. J. Why Nature Chose Selenium. *ACS Chem. Biol.* **11**, 821-841 (2016).
26. Rohde H., Schmalisch J., Harpaz Z., Diezmann F. & Seitz O. Ascorbate as an alternative to thiol additives in native chemical ligation. *ChemBioChem* **12**, 1396-1400 (2011).
27. Dery S., Reddy P. S., Dery L., Mousa R., Dardashti R. N. & Metanis N. Insights into the deselenization of selenocysteine into alanine and serine. *Chem. Sci.* **6**, 6207-6212 (2015).
28. Metanis N., Keinan E. & Dawson P. E. Traceless ligation of cysteine peptides using selective deselenization. *Angew. Chem. Int. Ed.* **49**, 7049-7053 (2010).
29. Wan Q. & Danishefsky S. J. Free-radical-based, specific desulfurization of cysteine: a powerful advance in the synthesis of polypeptides and glycopolypeptides. *Angew. Chem. Int. Ed.* **46**, 9248-9252 (2007).
30. Yokoyama A., Sakurai H. & Tanaka H. Syntheses of related compounds of selenocysteamine and their complex formation with metal ions. *Chem. Pharm. Bull.* **19**, 1089-1094 (1971).
31. Jencks W. P. & Regenstein J. in *Ionization constants of acids and bases* (eds Lundblad R. L. & MacDonald F. M.) 595–635 (CRC, 2010).
32. Tanini D. & Capperucci A. Ring opening reactions of heterocycles with selenium and tellurium nucleophiles. *New J. Chem.* **43**, 11451-11468 (2019).
33. Ollivier N., Blanpain A., Boll E., Raibaut L., Drobecq H. & Melnyk O. Selenopeptide transamidation and metathesis. *Org. Lett.* **16**, 4032-4035 (2014).
34. Camarero J. A., Fushman D., Sato S., Giriat I., Cowburn D., Raleigh D. P. *et al.* Rescuing a destabilized protein fold through backbone cyclization. *J. Mol. Biol.* **308**, 1045-1062 (2001).
35. Iwai H. & Pluckthun A. Circular beta-lactamase: stability enhancement by cyclizing the backbone. *FEBS Lett.* **459**, 166-172 (1999).
36. Camarero J. A. & Muir T. W. Biosynthesis of a head-to-tail cyclized protein with improved biological activity. *J. Am. Chem. Soc.* **121**, 5597-5598 (1999).
37. Craik D. J. Chemistry. Seamless proteins tie up their loose ends. *Science* **311**, 1563-1564 (2006).
38. Craik D. J., Fairlie D. P., Liras S. & Price D. The future of peptide-based drugs. *Chem. Biol. Drug Des.* **81**, 136-147 (2013).
39. Krishna M. M. & Englander S. W. The N-terminal to C-terminal motif in protein folding and function. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 1053-1058 (2005).
40. Mulvenna J. P., Wang C. & Craik D. J. CyBase: a database of cyclic protein sequence and structure. *Nucleic Acids Res.* **34**, D192-D194 (2006).
41. White C. J. & Yudin A. K. Contemporary strategies for peptide macrocyclization. *Nat. Chem.* **3**, 509-524 (2011).
42. Camarero J. A. & Muir T. W. Chemoselective backbone cyclization of unprotected peptides. *Chem. Commun.*, 1369-1370 (1997).

43. Ollivier N., Dheur J., Mhidia R., Blanpain A. & Melnyk O. *Bis*(2-sulfanylethyl)amino native peptide ligation. *Org. Lett.* **12**, 5238-5241 (2010).
44. Boll E., Drobecq H., Ollivier N., Blanpain A., Raibaut L., Desmet R. *et al.* One-pot chemical synthesis of small ubiquitin-like modifier (SUMO) protein-peptide conjugates using *bis*(2-sulfanylethyl)amido peptide latent thioester surrogates *Nat. Protoc.* **10**, 269-292 (2015).
45. Ollivier N., Vicogne J., Vallin A., Drobecq H., Desmet R., El-Mahdi O. *et al.* A one-pot three-segment ligation strategy for protein chemical synthesis. *Angew. Chem. Int. Ed.* **51**, 209-213 (2012).
46. Simonneau C., Berenice L., Mougel A., Adriaenssens E., Paquet C., Raibaut L. *et al.* Semi-synthesis of a HGF/SF kringle one (K1) domain scaffold generates a potent in vivo MET receptor agonist. *Chem. Sci.* **6**, 2110-2121 (2015).
47. Ultsch M., Lokker N. A., Godowski P. J. & de Vos A. M. Crystal structure of the NK1 fragment of human hepatocyte growth factor at 2.0 A resolution. *Structure* **6**, 1383-1393 (1998).
48. Mekki M. S., Mougel A., Vinchent A., Paquet C., Copin M. C., Leroy C. *et al.* Hypoxia leads to decreased autophosphorylation of the MET receptor but promotes its resistance to tyrosine kinase inhibitors. *Oncotarget* **9**, 27039-27058 (2018).