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A Study of Profiling the reactivity of cyclic C-nucleophiles towards electrophilic sulfur in cysteine sulfenic acid

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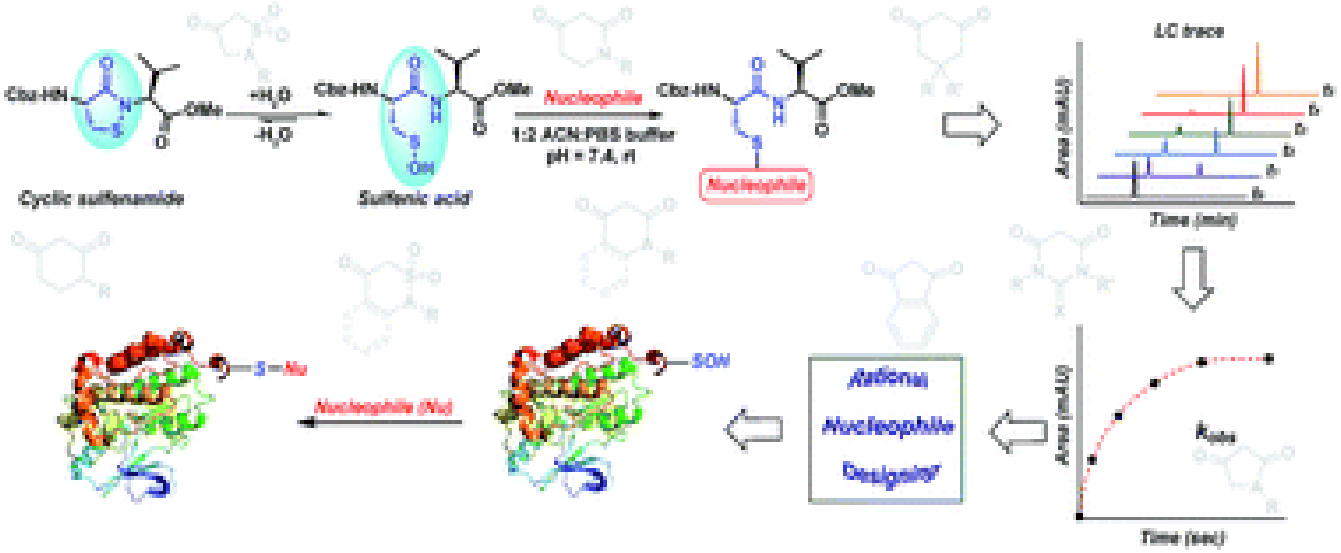
ABSTRACT

Oxidation of a protein cysteine thiol to sulfenic acid, termed S-sulfenylation, is a reversible post-translational modification that plays a crucial role in regulating protein function and is correlated with disease states. The majority of reaction-based small molecule and immunochemical probes used for detecting sulfenic acids are based on the 5,5-dimethyl-1,3-cyclohexanedione (dimedone) scaffold, which is selective, but suffers from low reactivity. In addition, mechanistic details and features that diminish or enhance nucleophile reactivity remain largely unknown. A significant hurdle to resolving the aforementioned issues has been the chemically unstable nature of small-molecule sulfenic acid models. Herein, we report a facile mass spectrometry-based assay and repurposed dipeptide-based model to screen a library of cyclic C-nucleophiles for reactivity with sulfenic acid under aqueous conditions. Observed rate constants for ~100 cyclic C-nucleophiles were obtained and, from this collection, we have identified novel compounds with more than 200-fold enhanced reactivity, as compared to dimedone. The increase in reactivity and retention of selectivity of these C-nucleophiles were validated in secondary assays, including a protein model for sulfenic acid. Together, this work represents a significant step toward developing new chemical reporters for detecting protein S-sulfenylation with superior kinetic resolution. The enhanced rates and varied composition of the C-nucleophiles should enable more comprehensive analyses of the sulfenome and serve as the foundation for reversible or irreversible nucleophilic covalent inhibitors that target oxidized cysteine residues in therapeutically important proteins.



I. INTRODUCTION

Reactive oxygen species (ROS) are continuously generated, transformed and consumed in living organisms as a consequence of aerobic life. Due to their role in both physiology and pathology, ROS are considered scientific equivalents of "antiheroes".[1] Once generated, ROS mediates diverse arrays of reversible and irreversible modifications on biomolecules such as proteins, lipids DNA and RNA.[2,3] Due to their strong nucleophilic character and low redox potential in proteins (E^o, –0.27 to –0.125 V) side chain thiol(ate) of cysteines (Cys-SH) are one of the more common targets of ROS.[4] Indeed, thiolate oxidation by hydrogen peroxide (H_2O_2) represents a widely studied area of redox-based post-translational protein modification. Nucleophilic attack of a protein thiolate on electrophilic H_2O_2 releases water and results in the formation of cysteine sulfenic acid (Cys-SOH) also known as S-sulfenylation. Depending upon the protein microenvironment where the thiolate is located, the rate of oxidation by H_2O_2 can vary substantially (1–10^8 M^{-1} s^{-1}). This stark difference in oxidation rates is highlighted by the reaction rates of two major targets of H_2O_2 signaling in cells, peroxiredoxin 2 (Prx2; 10^8 M^{-1} s^{-1}) and protein tyrosine phosphatase type 1B (PTP1B; 9 M^{-1} s^{-1}).[4,5] Reversible Cys-SOH formation plays a regulatory role among transcription factors, kinases (EGFR, JAK2, Akt2, IKK-β, RegB, PGKase, L-PYK), phosphatases (PTP1B, YopH, PTEN, Cdc25a, SHP-1 and SHP-2), ion channels, peroxidases and cysteine proteases, human serum albumin (HSA) and many other proteins.[6–20] Moreover, aberrant S-sulfenylation correlates with tumor progression and can lead to noncanonical scurvy in mice.[10,21] The aforesaid examples and many other reports demonstrate that protein S-sulfenylation constitutes a global signal mechanism, not unlike phosphorylation.

The cellular lifetime of Cys-SOH depends on numerous factors, including the level of ROS and/or duration of ROS signaling as well as the local protein environment. Essentially, the absence of proximal thiols capable of generating an intramolecular disulfide is considered to be a primary stabilizing factor; limited solvent access and proximal hydrogen bond acceptors also contribute toward Cys-SOH stabilization. Cys-SOH is the first oxidation product that results from the reaction between a cysteine thiolate and H_2O_2 (Fig. 1A, Reaction 1). High ROS, chronic oxidative stress, and/or the lack of adjacent thiols may cause –SOH to undergo further oxidization to sulfinic (–SO_2H) or sulfonic acid (–SO_3H) (Fig. 1A, Reactions 2 and 3). In contrast to biologically reversible Cys-SOH, these higher oxoforms are essentially irreversible (the only exception to this statement has been found to date is with Prx-SO_2H, which can be reduced to Prx-SH by the ATP-dependent enzyme, sulfiredoxin[22]). An important biological reaction of Cys-SOH is disulfide bond formation. Mechanistically, the electrophilic sulfur atom of Cys-SOH reacts with the thiolate nucleophile to give the disulfide with concomitant loss of water (Fig. 1A, Reaction 4). Due to the abundance of biological thiols (mM levels) including protein and low-molecular weight molecule thiols, such as glutathione (GSH), this reaction can be facile and constitutes a major pathway for disulfide formation. The nascent disulfide may undergo thiol–disulfide exchange to give the initial thiol (Fig. 1A, Reactions 4 and 5). Cys-SOH may also undergo intramolecular reaction with adjacent amide nitrogen, which results in the formation of isothiazolidinone, also known as cyclic sulfenamide (Fig. 1A, Reaction 6).[23,24] The cyclic sulfenamide species may be reduced back to thiol via disulfide formation (Fig. 1A, Reactions 7 and 5). On the basis of the reversible/irreversible reactions that Cys-SOH can undergo, this post-translational modification serves as an important hub within the redox milieu. Accordingly, an important goal to dissect regulatory redox pathways has been to develop robust,

sensitive and rapid detection techniques to identify sites, conditions and the cellular lifetime of protein S-sulfenyl modifications

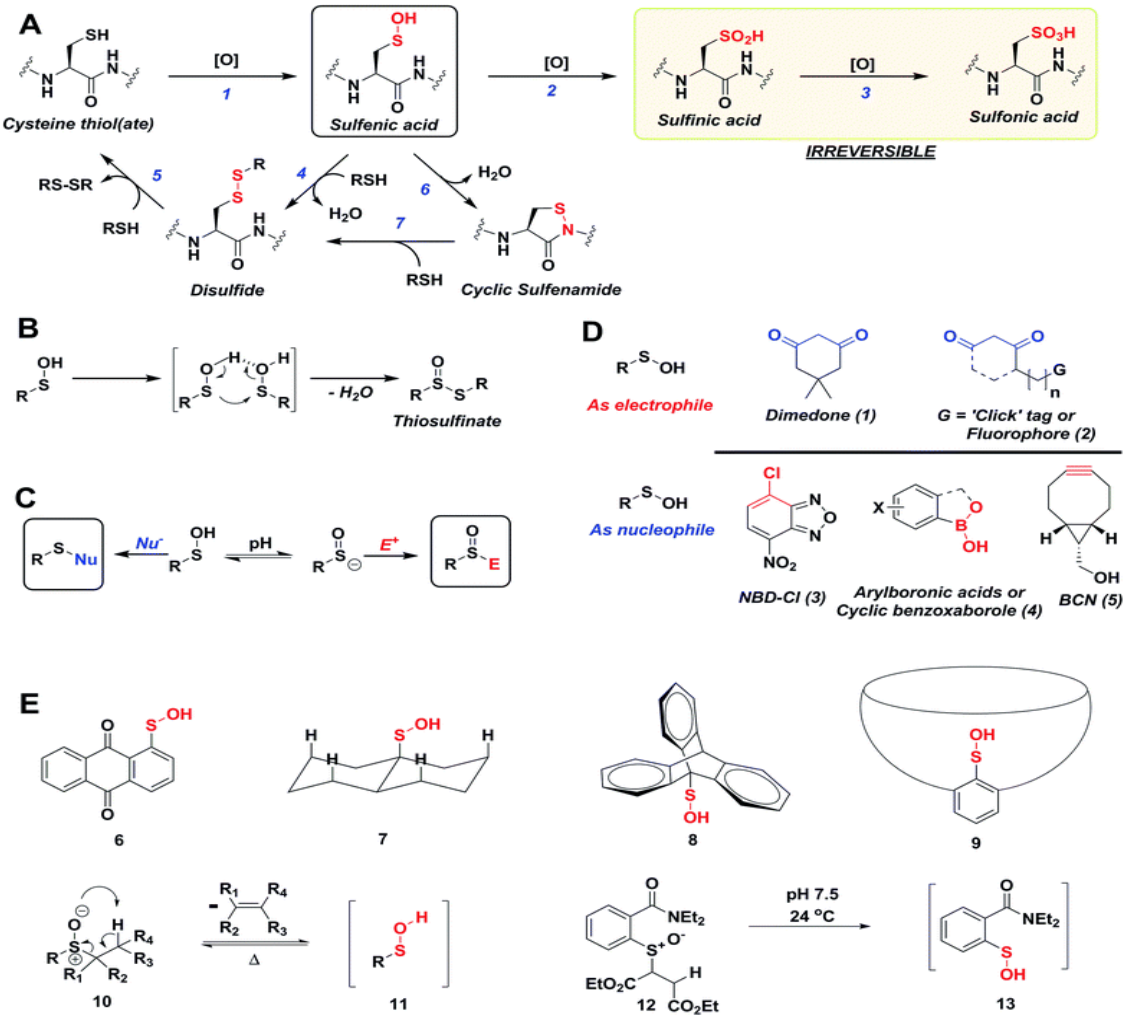


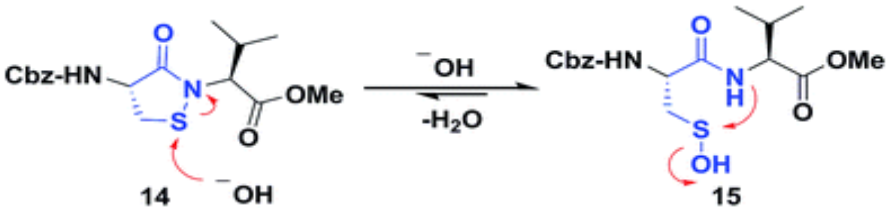
Fig. 1 (A) Biological cysteine oxoforms. (B) Sulfenic acid acts as a nucleophile and an electrophile. (C) Nucleophilic probes (Nu^-) result in the formation of a thioether-type linkage and electrophilic probes (E^+) result in the formation of a sulfoxide. (D) General structures of nucleophilic and electrophilic sulfenic acid probes. (E) Examples of currently known stable and transient small-molecule sulfenic acids.

Results

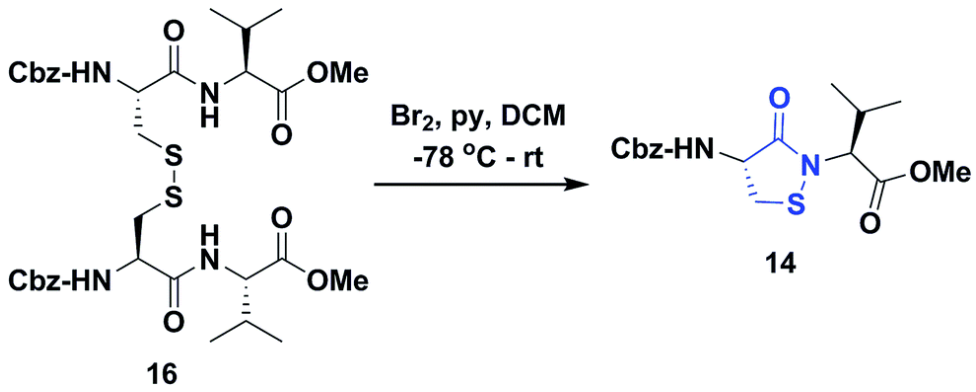
1) Synthesis and validation of a dipeptide-based sulfenic acid model

Several literature-reported persistent and transient sulfenic acid models were surveyed, but the example that caught our attention was a dipeptide-based model for its isostere, cyclic sulfenamide (Scheme 1, **14**). Dipeptide **14** was originally reported by Shiau et al. at Sunesis pharmaceuticals and employed as a model of cysteine oxidation to cyclic sulfenamide in PTP1B.[40] Owing to the combination of ring strain and electronic factors, we reasoned that the sulfur of cyclic sulfenamide might also be moderately electrophilic (Scheme 1, **15**). Furthermore, we were curious about the stability of the sulfenamide under aqueous conditions and

wondered whether the cyclic structure could be a synthon of sorts, existing in equilibrium with the corresponding sulfenic acid (Scheme 1). The reported synthesis is low in yield but a straight-forward sequence with well-established synthetic precedent for the key oxidative cyclization step.[41] Even so, following the reported procedure, we obtained the target cyclic sulfenamide (**14**) in poorer and variable yield. Closer analysis of reaction products revealed the presence of precursor disulfide (Cbz-Cys-Val-OMe)$_2$ (**16**) and a new compound, identified as cyclic sulfinamide (**17**) (Scheme S1A†). To address the issue of yield and variability, we varied the ratio of bromine to pyridine and avoided the aqueous workup. With these modifications in place, the cyclization step was successfully standardized at gram scale to give the dipeptide based cyclic sulfenamide product in >85% yield after silica gel based column purification .



Scheme 1 Dipeptide based cyclic sulfenamide model is hypothesized to exist in equilibrium with corresponding sulfenic acid under aqueous conditions.



Scheme 2 Synthesis of dipeptide based cyclic sulfenamide **14**.

With the dipeptide cyclic sulfenamide (**14**) in hand, we next evaluated its stability under aqueous conditions. In these experiments, we observed that dipeptide cyclic sulfenamide (**14**) reacted over time to form cyclic sulfinamide (**17**) and (Cbz-Cys-Val-OMe)$_2$ (**16**) (Scheme S1A†). The mechanism shown in Scheme S1B† accounts for the formation of **16** and **17** and is consistent with our proposal that cyclic sulfenamide (**14**) exist in equilibrium with sulfenic acid (**15**) under aqueous conditions. In the absence of other reactive groups, cyclic sulfenamide **14** can be reformed from **15** through attack by nitrogen. In addition, **15** can condense with itself (or cyclic sulfenamide **14**) to give thiosulfinate (**18**) as an intermediate, the eventual rearrangement of which was observed over the time (Scheme S2B†). In subsequent steps, the amide nitrogen nucleophile attacks the electrophilic sulfinyl sulfur, producing cyclic sulfinamide (**17**) and dipeptide thiolate (**19**). Thiolate **19** subsequently reacts with sulfenic acid **15** (or with cyclic sulfenamide **14**) resulting in the formation of dipeptide disulfide **16**. Importantly, the dipeptide cyclic sulfenamide **14** was stable in acetonitrile over the same period of time (and longer) demonstrating that H_2O is required for decomposition (Scheme S3†). Further

chemical evidence for the formation of sulfenic acid (**15**) was obtained through the addition of methyl iodide and NBD-Cl to the reaction, giving corresponding methyl and aryl sulfone respectively (Schemes S4 and S5†).

Since formation of sulfinamide **17** and disulfide **16** has the potential to interfere with downstream kinetic analysis, we determined the second-order rate constant for this reaction (Scheme S2D†). In this analysis, a value of 1.2 M^{-1} s^{-1} was obtained and deemed acceptable given the anticipated rate constants for our assay (see below). Since the rate-limiting step in this rearrangement is formation of **18** and the sulfenate anion is required for facile self-condensation of sulfenic acid, we were presented with the opportunity to determine the pK_a of sulfenic acid **15**. Pseudo first-order rate constants (k_{obs}) were obtained for the rearrangement from pH 3–9 (Scheme S6B†). The plot of k_{obs}versus pH gave a pK_a value of 7.1 for sulfenic acid **15** (Scheme S6C†). This value agrees well with small-molecule sulfenic acid pK_as, which generally range between 4 and 8 depending upon their stability.[4] The measured pK_a value of 7.1 is significant as it indicates that under our aqueous experimental conditions, sulfenic acid **15** and the corresponding sulfenate anion are present in roughly equal amounts. The existence of both species is required for facile formation of thiosulfinate (**18**), which is clearly observed in our assay. Collectively, the aforementioned data provide strong support for the formation of sulfenic acid **15** under aqueous conditions.

2) Ring size and C-nucleophile reactivity

In subsequent studies, we examined the effect of C-nucleophile ring size on reaction rate constants with sulfenic acid. To this end, we selected four commercially available nucleophiles: 1,3-cyclopentanedione (**21a**), 1,3-cyclohexanedione (**22a**), 1,3-cycloheptanedione (**23**) and 2,4-pentanedione (**24**) (Chart 1). The resulting pseudo first-order rate constants show an increase in reactivity with increasing ring size. Due to resonance stabilization of the enolate, the pK_a of the α-carbon nucleophile in 1,3-dicarbonyls is relatively low (<14) (Scheme 4) and, consequently, these compounds will have varied anionic character at physiological pH. For example, the enol tautomer of **21a** (pK_a ~ 4.3) is the dominant form under aqueous conditions at pH 7.4 and its low pK_a leads to a highly stabilized enolate. Consequently, **21a** has a lower tendency to react with sulfenic acid **15** (k_{obs} = 0.02 min^{-1}) compared to **22a** (k_{obs} = 0.4 min^{-1}).

Discussion

Although numerous studies profiling electrophiles as reactivity probes for thiols have been reported,[27,63–66] to our knowledge, this study represents the first of its kind to comprehensively profile nucleophiles as reactivity probes for the related sulfur oxoform, sulfenic acid. Herein, we have conceived, synthesized and screened several classes of cyclic C-nucleophiles for their reactivity with a novel model dipeptide sulfenic acid using a newly developed, facile LC-MS assay. The observed rate constants obtained from the fits to the ensuing data enables the stratification of C-nucleophiles based on their reaction kinetics. Our approach is user-friendly and utilizes a simply prepared dipeptide that can be stored in stable form until it is needed for conversion to sulfenic acid under aqueous conditions. Thus, this work addresses a fundamental, previously unmet need for a workflow that expedites the identification of compounds, which react with cysteine sulfenic acid over a broad range of time scales (10 to 2×10^5 M^{-1} min^{-1}).

Conclusion

We have reported a facile mass spectrometry-based assay and repurposed dipeptide-based model to screen a library of cyclic C-nucleophiles for reactivity with sulfenic acid under aqueous conditions. Observed rate constants for ~100 cyclic C-nucleophiles were obtained and, from this collection, we have identified novel compounds with more than 200-fold enhanced reactivity, as compared to dimedone (**1**). The increase in reactivity and retention of selectivity of these C-nucleophiles were validated in secondary assays, including a protein model for sulfenic acid. Together, this work represents a significant step toward developing new chemical reporters for detecting protein S-sulfenylation with superior kinetic resolution. The enhanced rates and varied composition of the C-nucleophiles should enable more comprehensive analyses of the sulfenome and serve as the foundation for reversible or irreversible nucleophilic covalent inhibitors that target oxidized cysteine residues in therapeutically important proteins.

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