Posttranslational Chemical Mutagenesis: To Reveal the Role of Noncatalytic Cysteine Residues in Pathogenic Bacterial Phosphatases

Jean B. Bertoldo,*†,‡ Hernán Terenzi,§ Stefan Hüttelmaier,† and Gonçalo J. L. Bernardes*†,‡,§

†Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, U.K.
‡Institut für Molekular Medizin, Medizinische Fakultät, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Stra 3a, 06108 Halle, Germany
§Centro de Biologia Molecular Estrutural, Departamento de Bioquímica, Universidade Federal de Santa Catarina, 88040-970 Florianópolis, SC, Brazil

ABSTRACT: The field of chemical site-selective modification of proteins has progressed extensively in recent decades to enable protein functionalization for imaging, drug delivery, and functional studies. In this Perspective, we provide detailed insight into an alternative view of site-selective protein chemistry to probe the role(s) of unpaired Cys residues in the structure and function of disease relevant proteins. Phosphatases are important players in the successful infection of pathogenic bacteria, which represent a significant health burden, particularly in multi-drug-resistant strains. Therefore, a strategy for readily probing the key amino acid role(s) in structure and function may facilitate the targeting and inhibition of these virulence factors. With a dehydroalanine-based posttranslational chemical mutagenesis approach, it is possible to reveal hitherto unknown function(s) of noncatalytic Cys residues and confirm their role and interplay in pathogenic bacterial phosphatases. By selectively modifying reactive sulfhydryl side chains in different protein local environments, this posttranslational site-selective chemical mutagenesis approach reveals structural information about binding pockets and regulatory roles of the modified residues, which can be further validated by conventional site-directed mutagenesis. Ultimately, these new binding pockets can serve as templates for enhanced structure-based drug design platforms and aid the development of potent and specific inhibitors.

Cysteine (Cys) plays key roles in protein function and stability even though it is one of the least abundant amino acids. Its appearance on the genetic code is rather late,1 but its accumulation continues in modern organisms.2 Cys residues have a unique set of chemical properties, such as the ability to form disulfide bridges and to operate as redox switches3 because of the high nucleophilicity of the thiol group (R-SH).4 The thiol can be alkylated by electrophiles and oxidized by reactive oxygen (ROS) and nitrogen species (RNS),5 which presents a challenge to proteins that contain highly reactive Cys, hence their tightly regulated expression under oxidative stress.6 As a safety mechanism, many of these proteins express key Cys residues in buried motifs7 and to preserve protein activity,8 a feature that likely evolved under selective pressure. Pathogenic bacteria, however, employ enzymes with highly reactive and surface-exposed Cys.9,10 These enzymes promote the successful establishment of bacterial infection by helping bacteria circumvent host defenses.11–13 In addition, the cell host defense mechanism also involves a burst of ROS, which can lead to overoxidation; nevertheless, these enzymes manage to avoid irreversible inactivation13 by employing mechanisms that involve catalytic and noncatalytic Cys. For example, the catalytic sulfur atom on Cys can react with a nearby nitrogen atom to form a sulfenylamide.14 A noncatalytic Cys, usually termed a “backdoor” residue, can form a reversible disulfide bond with the catalytic Cys. This bond can also be formed between two noncatalytic Cys residues as seen in several phosphatases,15–18 including the low-molecular weight protein tyrosine phosphatase family (LMW-PTPs).19 Overall, these noncatalytic Cys residues can be found in pairs, clusters, in a single place or in multiple places within the protein structure. They can function as redox switches and have enhanced interplay when clustered, which provides a suitable environment to prevent irreversible inactivation.20,21 These features point to an elaborate, synergistic interplay among Cys residues in bacterial phosphatases, especially with noncatalytic residues. This fact also demands an in-depth analysis of their role in virulence effectors of pathogenic bacteria. The reactivity and localization of these noncatalytic Cys residues have a unique set of chemical properties, such as the ability to form disulfide bridges.
Cys residues can be further exploited for drug design approaches as their role may prove to be critical for the maintenance of Cys-containing proteins. In fact, noncatalytic Cys residues have already proven to be effective targets in Food and Drug Administration (FDA)-approved drugs like the kinase-targeting ibrutinib. However, the crucial point lies in the identification of which Cys residues merit further investigation; therefore, a strategy for readily revealing their roles in protein structure and activity may better inform the design of new antibacterial drugs. In this context, we seek to demonstrate how a recently developed method, which uses a chemical mutagenesis approach, can be confidently used to shed light on the role of noncatalytic Cys residues in bacterial enzymes and facilitate the discovery of suitable targets for antibacterial therapeutics. Our main goal is not to exhaustively describe and discuss the reactions developed so far for chemical site-selective protein modification that can be found in recent reviews; instead, we will focus on the potential and progress of such reactions in “posttranslational chemical mutagenesis”, a methodology that enables the modification of specific residues within the protein target, which permits the incorporation of synthetic modifications to explore amino acid side-chain diversity. This Perspective will give detailed insight into this exciting emerging area and provide robust information about an alternative use: to reveal the role(s) of unpaired Cys residues in the structure and function of disease relevant proteins to advocate, hopefully, for its addition to the current chemical biology tool kit used to probe Cys reactivity (Figure 1). The first use will be in bacterial phosphatases, and the ultimate use is to study the interplay between Cys residues in a diverse panel of multiple Cys-containing proteins relevant in disease.

PROBING CYS REACTIVITY IN PTPS

Proteome-wide Analysis. As a result of the susceptibility of the thiol side chain to ROS, and its involvement in the direct regulation of PTPs, the first methods for probing Cys reactivity were developed to detect oxidized PTPs. The most common, indirect techniques rely on the ability of active PTPs to react stoichiometrically and irreversibly with alkylation agents, i.e., iodoacetic acid (IAA) or N-ethylmaleimide, and the resistance of oxidized PTPs to alkylation. Generally, cells are lysed in the presence of a labeled alkylation agent (e.g., radioactive), and oxidation is monitored with a decrease in the level of detection of that probe. Oxidized PTPs are converted to modified forms that can be detected by mass spectrometry or other analytical techniques. The ability to detect oxidized PTPs has been used in proteomics studies to identify targets for therapeutic intervention. However, these methods are limited in their ability to identify the specific residues that are being modified, and they do not provide information about the effects of the modifications on protein structure and function.

Figure 1. Overview of a posttranslational mutagenesis approach for probing the amino acid sequence, structure, and activity of multiple Cys-containing proteins. A chemical probe, in this case α,α′-dibromo-adipyl(bis)amide, designed to react with free Cys residues and convert these into Dha is incubated with the respective native Cys protein. PTM protein Dha-protein is assessed for enzymatic activity or binding activity. Once the effect on the activity or structure is assigned, the preferentially modified residue(s) is identified by mass spectrometry. A single plasmid construct that contains an Ala or Ser substitution of the preferentially modified residue(s) is cloned and expressed. The recombinant mutant protein is assessed, and the role of the modified residues in the structure (by circular dichroism for secondary structural content and, for example, by molecular dynamics simulations for local structural changes) and activity is confirmed. For the molecular dynamics simulations, structural ensembles derived from 0.5 μs molecular dynamics simulations on chemical mutant Dha53 of PtpA. Both the global three-dimensional shape and the local structure of the protein are retained upon chemical mutation. Cys11 and Cys16 of the active site are also shown.
into the active state (by using reducing agents), captured, and
detected by using a PTP-reactive probe (e.g., biotin-tagged IAA).35
Although effective at detecting oxidized PTPs, these
approaches failed to depict other reactive noncatalytic Cys
because the outcome depends mostly on enzyme activity [e.g.,
in-gel phosphatase assay, IAP-biotin, and activity-based probes
(ABPs)36]. In addition, the alkylating agent added to the
reaction mixture invariably modifies all reduced Cys residues
except the oxidized residue, which prevents an understanding
of the intrinsic Cys reactivity and their interplay. Furthermore,
the antibodies used to detect oxidized PTPs in proteomic
analysis are raised against the conserved PTP signature motif
VHCSO3HSAG, which covers only the catalytic cleft.37 Although
useful, this approach probes only oxidized catalytic Cys. This
caveat is also present in the analysis of recombinantly
expressed PTPs, which makes the study of the interplay
between Cys residues difficult. However, over the past few
years, more direct and improved approaches have been
developed (recently reviewed38), based on Cys-reactive
probes, which enabled the analysis of highly reactive Cys
residues regardless of their catalytic nature.10 These new
approaches were based on the pioneering work by Cravatt and
co-workers.39 The authors developed an activity-based protein
profiling probe called isoTOP-ABPP (isotopic tandem
orthogonal proteolysis activity-based protein profiling) that,
combined with liquid chromatography–tandem mass spec-
trometry analysis, allowed the identification of highly reactive
noncatalytic Cys residues in different proteomes. Their
findings also revealed that some of the identified noncatalytic
Cys residues possessed a previously unknown function and
were significantly more conserved in eukaryotic organisms.
Their findings provided strong evidence that noncatalytic Cys
residues play essential roles in the proteome and merit special
attention. Reasonably, proteome-wide reactivity profiling aids
in the identification of highly reactive noncatalytic Cys
residues, both in proteomic analysis and in purified protein
systems. However, the functional assignment of the identified
residues still requires detailed kinetic and mutagenic exper-
iments. In this scenario, we believe that our methodology may
be useful in targeted chemical mutagenesis for studying specific
protein targets. If one multiple Cys-containing protein is
selected, then no structural or activity information is available.
Usually, to probe new functionalities, a previous putative role is
assigned to the amino acids to be studied, based generally on
conserved structural motifs identified through bioinformatics.
Thus, consecutive rounds of cloning, expression, and
purification are required for each targeted mutation, which
may result in a time-consuming, trial-and-error investigation.
To facilitate inquiry into the functionality of new amino acids,
a direct, chemistry-driven mutagenesis approach can be used to
obtain otherwise unknown information about the function and
interplay of specific amino acid(s). For example, by targeting
the most reactive Cys within a protein with multiple Cys
residues, suitable chemical probes used in a posttranslational
chemical mutagenesis can provide new insights into the
reactivity and role(s) of the preferentially modified residue(s).
Site-directed mutagenesis is employed because the residue(s)
is identified by mass spectrometry. Upon in vitro confirmation,
the role of the chemically modified residues can be further
evaluated in transiently transfected cells or stably by expressing
cell lines that contain the Cys mutant form of the protein
target. In this way, a more biologically relevant protein activity
or regulation can be uncovered.

**Posttranslational Chemical Mutagenesis.** Chemical
biologists have been designing chemical probes for Cys
modification since 1965, when Wilchek and colleagues
demonstrated the conversion of serine (Ser) into Cys in
small peptides.40 Wilchek’s work demonstrated how the
protein amide backbone and side chains could be exploited
for selective chemical mutagenesis. Soon thereafter, new
milestones in chemical mutations on proteins were achieved
(Table 1). Some of the first examples of chemical mutagenesis
used conditions that were considered to be harsh for proteins.
For example, in the pioneering work by Clark and Lowe, the
nucleophilic Cys of papain was chemically converted into Ser
by means of alkylation with phenacyl bromide and subsequent
photolysis and reduction with NaBH4.41 Basic or acidic
conditions, high temperatures, organic solvents, and other
non-ideal conditions have been used continuously until
recently. In addition, these reactions suffered from poor
selectivity, which results in a mixture of modified products at
different sites within the protein sequence. To avoid these
drawbacks, improved reagents and site-selective chemical
reactions that proceed efficiently under mild conditions were
developed.23–27 These new reactions proved to be reliable
alternatives to maintain, or even extend, protein activity
without disrupting how it folds, for instance, fluorescently
labeled proteins for imaging in which a precise position for
modification is required.26,28 Antibody–drug conjugates used
for cancer diagnostics, and delivery payloads of antitumor
drugs.24,42 In addition, these reactions enabled the chemoselective
labeling of ribosomally incorporated noncanonical
amino acids that bear unnatural side chains such as alkynes,
alkenes, tetrazines, ketones, and azides on endogenously
expressed proteins.35,44 These unusual side chains permitted
bioorthogonal protein labeling and functionalization, which
facilitates a detailed understanding of biological processes and
biomolecules within their native habitat, cells and live
organisms.45,46

Another use of site-selective modification is the specific
installation of suitable mimics of posttranslational
modifications (PTMs) in recombinantly expressed proteins at defined

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positions to study and modulate enzyme activity. In a seminal work, Davis and colleagues engineered a Cys on the surface of kinase p38α. The same group has previously described the use of α,α′-dibromo-adipyl(bis)amide to selectively convert Cys into dehydroalanine (Dha). Subsequently and through thiol-Michael addition with sodium thiophosphate, a phospho-Cys mimic is installed. The similarity of phospho-Cys to naturally occurring phospho-Tyr was sufficient to switch the kinase to its active form. Several synthetic mimics of PTMs were facilitated by the ability to generate Dha in a site-selective way on proteins. A regioisomer of histidine (His), iso-His (Hisiso), installed through βγ,C,Naza-Michael mutagenesis was used to probe the contributing roles of size and hydrogen bonding in the activity of Mycobacterium tuberculosis (Mtbc) pantothenate synthetase (PanC). His mimic H44Hisiso which lacks the ability to donate H-bonds, provided the mechanistic detail for the inhibition of PanC. The chemical mutant preferentially adopted a rotamer that is oriented toward the solvent, which made it unable to interact with the β-phosphate from ATP. PanC ATP-dependent condensation activity is important for the formation of vitamin B5 (pantothenate), on which Mtbc relies to grow and for the proper function of its virulence factors. Because of its important activity, PanC is also a target for anti-tuberculosis therapy. Interestingly, the same mimic Hisiso installed in the protease subtilisin from Bacillus lentus (S156Hisiso), revealed that H-bond donors are not necessary to maintain enzymatic activity. Acetylated and methylated Lys mimics were also produced by site-selective chemical mutagenesis and successfully used to study writer/erase activity of histone deacetylases (HDACs) in chromatin modification. To date, the acetyl-Lys mimic installed through Dha in this study is the most convenient way to investigate HDAC activity on full-length histones. Dha is currently used as a chemical precursor of mono-, di-, and trimethylated Lys, acetylated Lys, phosphorylated Ser, and glycosylated Ser and has proven to be a reliable chemical handle for mimicking PTMs and modulating enzymatic activity.

Interestingly, what the aforementioned approaches do not address are the reasons why nature has evolved to enhance the reactivity of particular side chains within a protein sequence and what are the structural and functional consequences. Why, in a complex mixture of residues spread along the protein sequence, do some residues have enhanced reactivity that allows them to be preferentially chemically modified? Do they have a structural or functional role? Do physicochemical properties dictate their reactivity? Does reactivity mean that these residues perform a special role? The case is not simple for Cys, but it may provide insights into why a preferentially modified Cys in a multiple-Cys-containing protein might play an important role in redox sensing, binding to an antigen, or catalytic activity. It is recognized that structural context dictates reactivity, and highly polarizable sulphydryl side chains are often presented in buried motifs, which affects their accessibility and protonation state. Secondary structures, such as α-helices, can exhibit a dipole moment with a positive charge at the N-terminal end, which decreases the pKa and makes the Cys residues located within these constraints more reactive. Such features enhance Cys nucleophilicity by reducing its pKa from an “unperturbed” value of 8.5 to values as low as 3.5. In addition, some proteins have specialized catalytic pocket designs that also strongly enhance the reactivity of Cys toward specific substrates, such as peroxiredoxins. Often, if there are two free Cys units, the reactivity of one is dependent of the other. Understanding Cys reactivity and interplay in this context might prove to be a challenge.

To address this challenge, we developed an approach that combined Dha-based chemical mutagenesis with site-directed mutagenesis. We hypothesized that Cys-to-Dha converter compound α,α′-dibromo-adipyl(bis)amide could react with highly reactive Cys in multiple Cys-containing bacterial phosphatases in accordance with Cys reactivity, which may highlight a possible key role. We then tested the Cys high reactivity/key function correlation by producing mutants of the preferentially modified residues and analyzing the structure and enzymatic activity of both chemical and site-directed mutants (Figure 1). Our findings indicated that a Dha-based posttranslational chemical mutagenesis can be used as means to interrogate Cys function. We present here two case studies on which to base this assertion.

**Case Study 1: Noncatalytic Cys16 and Cys53 from M. tuberculosis Protein Tyrosine Phosphatase A (PtpA).** Our analysis of PtpA provides the first evidence to support the use of posttranslational chemical mutagenesis in revealing the critical role of noncatalytic Cys residues in bacterial phosphatases. PtpA possesses three Cys residues, Cys11, Cys16, and Cys53, and its phosphatase activity relies on the nucleophilicity and reactivity of catalytic Cys11. As a result of PtpA dephosphorylation activity, Mtbc is able to avoid the macrophage proteolysis machinery. PtpA disrupts key components of the macrophage endocytic pathway, which leads to the inhibition of phagosome maturation and its late fusion with the lysosome, which ultimately allows Mtbc to survive inside the host macrophages. As a result of its key activity, PtpA has become an important druggable target in anti-tuberculosis chemotherapy. To probe Cys reactivity and function in PtpA, we applied our posttranslational chemical mutagenesis approach (Figure 1). In this new approach, in vitro reactions of recombinant PtpA and an increasing number of equivalents of α,α′-dibromo-adipyl(bis)-amide were performed under mild conditions [50 mM NaH2PO4 (pH 8.0)]. The reaction resulted in a single protein product from which the mutant C53A was isolated as a target of S-nitrosylation by nitric oxide. The selective single modification of Cys53 was achieved readily and without further optimization. Upon tandem mass spectrometry analysis of the single modified protein digestes, Cys53 (Dha53) was identified as being preferentially modified. Structural (circular dichroism and molecular dynamics) and functional (phosphatase activity) assays revealed that the conformation and phosphatase activity of the Dha53 mutant were not altered relative to those of nonmodified PtpA. Subsequently, to corroborate the results from chemical mutagenesis, a site-directed C53A mutant was produced and its activity profile was analyzed relative to that of the Dha53 mutant. As expected, the C53A mutant presented the same phosphatase activity as the wild type and Dha53 mutant. Previously, Cys53 had been identified as a target of S-nitrosylation by nitric oxide; however, in this case the phosphatase activity was partially suppressed, which could be an indication that Cys53 is a regulatory site, modulated by nitric oxide.

Intrigued by these findings, we decided to submit the protein variants (wild type, Dha53 mutant, and C53A mutant) to incubation with H2O2 and nitric oxide. Surprisingly, the chemical Dha53 and C53A mutants were extremely prone to...
H$_2$O$_2$ inactivation relative to the wild type and failed to be S-nitrosylated by NO. These findings have shown that the Dha-based posttranslational chemical mutagenesis facilitated the discovery of noncatalytic Cys53 as an oxidation-sensitive residue involved in a redox regulation mechanism; thus, it functions as a scavenger of ROS and RNS and reduces the level of overoxidation of catalytic Cys11. There is still the need to corroborate the role of Cys53 in the cellular activity and regulation of PtpA, and our results made its investigation in Mtb infection even more promising. The crystal structure of PtpA further showed that Cys53 was surface-exposed and the solvent accessibility was the first answer for its preferential modification by the $\alpha,\alpha'$-dibromo-adipyl(bis)amide compound (Figure 2). A prolonged protein incubation time with $\alpha,\alpha'$-dibromo-adipyl(bis)amide revealed an incomplete stoichiometry for their specificiations at backdoor Cys residues, such as Cys53, in PtpA with small molecules may avoid off-targets in drug development because it is not conserved among PTPs. In fact, drug design strategies, like tethering and covalent fragment-based ligand discovery, can aid in the development of such small molecules. 

**Figure 2.** Cys53 in *M. tuberculosis* PtpA and Cys259 in *Yersinia enterocolitica* YopH identified as the preferentially modified residues in our posttranslational chemical mutagenesis approach. Noncatalytic Cys53 is mostly exposed to the solvent in PtpA, whereas in YopH, noncatalytic Cys259 is mostly buried. Their localization may account for their specific role, as PtpA Cys53 acts as a scavenger of reactive oxygen and nitrogen species and YopH Cys259 may act as a “backdoor” Cys as a result of its proximity to catalytic Cys403. Cartoons were generated with the CCP4 Molecular Graphics Program (University of York) with Protein Data Bank entries 1U2P (PtpA) and 4YAA (YopH).

$dibromo-adipyl(bis)$amide revealed an incomplete stoichiometric correlation in the chemical reactions, because it was not possible to modify all of the PtpA’s Cys residues completely under mild conditions.

In addition, failed attempts to chemically modify site-directed double mutant C11/53A, which contains only Cys16, and the combined FTIR findings of disturbed O–H stretches in Dha11 and C11S mutants led us to identify noncatalytic Cys16 as a “backdoor” residue for our newly discovered Cys–Cys water-bridged motif (Figure 3). The elegant interplay between these noncatalytic residues (Cys16 and Cys53) shows how PtpA may prevent critical inhibition of its phosphatase activity under oxidative stress. More fascinating is the fact that all this new information in regard to new structural motifs and regulation in PtpA may be used to design innovative anti-tuberculosis (anti-PTP) drugs. As a start, the design of Cys-directed electrophilic drug-like molecules that react preferentially with Cys53, which may disrupt its role as scavenger, by making the protein more susceptible to oxidative damage or by inducing structural changes may lead to an inactive PtpA conformation. Targeting highly reactive noncatalytic Cys residues, such as Cys53, in PtpA with small molecules may avoid off-targets in drug development because it is not conserved among PTPs. In fact, drug design strategies, like tethering and covalent fragment-based ligand discovery, can aid in the development of such small molecules. These medicinal chemistry approaches have indeed led to the generation of FDA-approved drugs such as kinase inhibitors irbritinib and EGFR inhibitor afatinib, both of which target highly reactive noncatalytic Cys residues.

**Case Study 2: Noncatalytic Cys259 from Yersinia enterocolitica Protein Tyrosine Phosphatase YopH.** YopH tyrosine phosphatase from *Yersinia* uses its catalytic Cys403 residue to hydrolyze a phosphotyrosine substrate, which usually involves the formation of a Cys–phosphate intermediate. YopH contains an N-terminal signal, a chaperone binding region, and a PTase catalytic C-terminal domain, which is conserved among PTPs. YopH Cys-phosphatase activity prevents invasin-triggered internalization of bacteria by disrupting the focal adhesion complex and suppresses the oxidative burst in macrophages and neutrophils. A combined inactivation of PRAM-1/SKAP-HOM and SLP76/Vav/PLCγ2 signal transduction axes and the modulation of lymphocytic activation ultimately mediates the survival of *Yersinia*. Moreover, YopH contains four additional Cys residues in addition to catalytic Cys403. Intriguingly, when YopH was submitted to the same posttranslational chemical reactions as PtpA, it yielded once again preferential modifications at noncatalytic Cys residues, except this time the preferentially modified Cys259 was located in a buried motif (Figure 2). A closer look at the crystal structure revealed that Cys259 is in the proximity with Cys403, so these residues might interact with each other. Cys259 in YopH may also act as “backdoor” Cys and play a role similar to that of Cys16 in PtpA. The presence of the Cys–Cys water-bridged motif in YopH remains to be confirmed. However, this new structural motif, which involves noncatalytic Cys units, is also present in SptpA from *Staphylococcus aureus* and is likely conserved within the LMW-PTP family (Figure 3).

Our results clearly show that the $\alpha,\alpha'$-dibromo-adipyl(bis)$amide compound was able to distinguish residues based intrinsically on Cys reactivity and that such reactivity is not necessarily determined by solvent accessibility, structural, or folding constraints. It is, however, affected by a combination of all these features within the local environment and is highly...
Bacterial phosphatases with multiple noncatalytic Cys residues are typically involved in polysaccharide production and as secreted virulence effectors, which downregulate the host cell signaling pathways. SptP, a tyrosine phosphatase from *Salmonella typhimurium*, is another enzyme that relies on its Cys481 catalytic residue to circumvent host immune defenses. It shares 30% sequence homology with YopH, the presence of multiple noncatalytic Cys units, with unknown amino acid functions in a biologically relevant context. As we have shown with the phosphatases, developing a drug-oriented strategy that uses information provided by posttranslational mutagenesis may prove to be effective. The reactivity of the designed chemical probes and the residues of which they preferentially target can yet reveal unusual structural motifs and regulatory effects, which can then be targeted in an alternative and more direct drug design platform. The structural information may ultimately drive the synthesis of potent allosteric inhibitors. Eventually, by

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**Figure 3.** Cys16 in *M. tuberculosis* PtpA identified as the “backdoor” Cys involved in the Cys11→Cys16 water-bridged motif by our posttranslational chemical mutagenesis approach. The motif is also found in *S. aureus* SptP. It is likely that noncatalytic Cys13 may act as a “backdoor” Cys for SptP in a mechanism similar to that identified for Cys16 in *M. tuberculosis* PtpA. Cartoons were generated with the CCP4 Molecular Graphics Program (University of York) with Protein Data Bank entries 1U2P (PtpA) and 3ROF (SptpA). Green numbers denote the Cys8→Cys13 water-bridged motif in *S. aureus* SptP. Blue numbers denote the Cys11→Cys16 water-bridged motif in *M. tuberculosis* PtpA.
revealing important noncatalytic residues, we can extend this platform to a vast set of proteins, not just enzymes, and structural proteins. This panel can include transcription factors, DNA and RNA binding proteins, receptors, and chaperones. We have successfully tested the feasibility of the platform in a chaperone (manuscript submitted for review) and in an RNA binding protein (unpublished). Finally, as this robust chemical biology strategy develops over the next few years, it will afford a fast way to uncover the functionality of other key residues, such as lysines, will aid in the answer of challenging biological questions, and may ultimately mediate the development of a better and more specific drug design platform.

AUTHOR INFORMATION

Corresponding Authors
*E-mail: gbernardes@medicina.ulisboa.pt or gb453@cam.ac.uk.
*E-mail: jean.bertoldo@medizin.uni-halle.de.

ORCID
Jean B. Bertoldo: 0000-0002-6897-6822
Gonçalo J. L. Bernardes: 0000-0001-6594-8917

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REFERENCES


