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Fluorescent labeling of specific cysteine residues using CyMPL

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Abstract

The unique reactivity and relative scarcity of cysteine among amino acids makes it a convenient target for the site-specific chemical modification of proteins. Commercially available fluorophores and modifiers react with cysteine through a variety of electrophilic functional groups. However, it can be difficult to obtain specific labeling of a desired cysteine residue in a protein with multiple cysteines, in a mixture of proteins, or in a protein's native environment. CyMPL (Cysteine Metal Protection and Labeling) enables specific labeling by incorporating a cysteine of interest into a minimal binding site for group 12 metal ions (e.g. Cd^{2+} and Zn^{2+}). These sites can be inserted into any region of known secondary structure in virtually any protein and cause minimal structural perturbation. Bound metal ions protect the cysteine from reaction while background cysteines are blocked with non-fluorescent modifiers. The metal ions are subsequently removed and the deprotected cysteine is labeled specifically.

Keywords

Fluorescence; FRET; metal binding; cysteine modification

INTRODUCTION

Due to its relatively low abundance and reactive sulfhydryl moiety, cysteine is a valuable target for protein labeling, e.g. with small molecule fluorophores. Unfortunately, specific labeling of a desired protein or a desired cysteine within a protein can be difficult to achieve. Labeling a purified protein with a single cysteine is trivial. However, labeling a protein in a mixture of many proteins, a protein in a cellular environment, or a specific cysteine in a protein with multiple cysteine residues presents a great challenge. Non-specific labeling of "background" cysteines can obscure the fluorescence signal arising from a cysteine or protein of interest.

CyMPL (Cysteine Metal Protection and Labeling) is a method that provides specific cysteine labeling with minimal structural perturbation of the protein target (Puljung and Zagotta, 2011). Briefly a cysteine of interest can be protected by the binding of group 12 metal ions (Cd^{2+} or Zn^{2+}) while the background cysteines are covalently blocked with a non-fluorescent, cysteine-reactive compound like *N*-ethylmaleimide (NEM; Figure 1). After the metal ions are chelated, the deprotected cysteine is available to react with fluorophore. Metal binding affinity is selectively enriched by engineering (via mutagenesis) metal binding amino acids (histidines or other cysteines) to act as binding partners with the desired cysteine. This can be achieved in any region of regular secondary structure by placing the

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binding partners either one turn away in an α -helix or two residues away in the case of a β -strand.

STRATEGIC PLANNING

CyMPL requires the introduction of metal binding residues near a desired cysteine in a region of known secondary structure. This will selectively enrich metal binding to that cysteine, allowing for metal binding at concentrations at which single cysteines would be left unbound (Figure 2). The cysteine that is intended for labeling can be native or introduced by site-directed mutagenesis.

The affinity of a single cysteine for Cd^{2+} measured in a helical peptide was 158 μM , similar to the affinity measured for single cysteines in proteins or in glutathione (Puljung and Zagotta, 2011). In an α -helical structure, the metal binding site is created by placing binding partners one turn away on the helix from the cysteine at either position $i+3$ and $i+4$ (Figure 3). When histidines were placed at these positions relative to a cysteine in a helical peptide, the Cd^{2+} affinity increased by 12- and 10-fold, respectively (Table 1). With histidines at both positions $i+3$ and $i+4$ the affinity increased by a factor of 34. The introduction of additional cysteine residues at positions $i+1$, $i+3$, or $i+4$ increased the affinity to the low or sub μM range in both peptides and purified proteins. In a β -strand, placement of a histidine at position $i+2$, relative to the cysteine (which points in the same direction) increased the affinity to 6 μM (Figure 3, HCN S536C,K565H). These model sites should be used as guidelines in designing a new metal binding site in any protein of interest.

Proper choice of metal ions can also affect experimental outcomes. While Cd^{2+} and Zn^{2+} may both be used, Cd^{2+} , which is a softer Lewis acid, is expected to bind better to cysteine containing sites, while Zn^{2+} a harder Lewis acid binds better to histidine (Rulisek and Vondrasek, 1998). In our hands, Zn^{2+} bound less tightly to a single cysteine (K_d 1 mM, Table 1), but binding was greatly improved when a histidine was placed nearby at position $i+4$ (36-fold, Table 1).

Our protocols use maleimide-based cysteine modifiers. We found these to be quite stable when stored properly (in ethanol or DMSO at -80°C). In our hands, these reagents also had very reproducible kinetics. Finally, maleimides form a carbon-sulfur bond to cysteine, which is not readily reduced and do not catalyze disulfide bond formation through the creation of mixed disulfides (Hermanson, 1996). However, the principles of CyMPL should apply to cysteine modifiers of any functionality, e.g. benzyl halides, iodoacetamides, methanethiosulfonates, etc. Blockers and fluorophores may be chosen to maximize functionality and efficacy, limit membrane permeability or cellular toxicity, or, as is the case with methanethiosulfonates, provide reversibility. For proteins in which cysteine-cysteine sites were used for metal binding, a bifunctional fluorophore such as dibromobimane may be used if labeling with two molecules of fluorophore is not desirable (Figure 4; Kosower et al., 1979).

BASIC PROTOCOL 1

FLUORESCENTLY LABELING A SPECIFIC CYSTEINE IN A PROTEIN WITH MULTIPLE CYSTEINES OR IN A MIXTURE OF SOLUBLE PROTEINS

This protocol was originally designed to label one component of an equimolar mixture of two proteins. The general scheme is outlined in Figure 1. Briefly, a concentration of Cd^{2+} or Zn^{2+} is applied that is expected to occupy the metal binding site (cysteine-histidine or cysteine-cysteine) but not bind individual cysteines. With metal bound, the single cysteines are reacted with a non-fluorescent cysteine modifier (NEM). The metal is then chelated with

EDTA, and the deprotected cysteine is reacted with a cysteine-reactive fluorophore (Fluorescein-5-maleimide). The protocol as written is for a 100 μL reaction and uses Cd^{2+} for protection, but can be scaled and modified as necessary.

Materials—Protein mixture with engineered metal binding site for the target cysteine ($\sim 1 \mu\text{M}$ of the labeling target)

Labeling buffer (see recipe)

10 mM *N*-ethylmaleimide (in labeling buffer)

1.1 mM CdCl_2 stock solution in H_2O

10 mM fluorescein-5-maleimide (in labeling buffer)

500 mM ethylenediaminetetraacetic acid (EDTA) in water, pH 8.0

1 mM glutathione

1.5 mL microcentrifuge tubes (snap cap)

Protocol steps

1. Dilute protein mixture in labeling buffer so that the protein intended for labeling is in the low μM range.

To ensure that all of the target protein is completely bound to metal, and therefore completely protected from NEM block, it is important that the concentration of the protein intended for labeling not exceed the expected K_d value for metal binding.

2. In a 1.5 mL microcentrifuge tube, add 10 μL of 1.1 mM CdCl_2 to 100 μL of the protein mixture. The final concentration will be 100 μM .

This $[\text{Cd}^{2+}]$ should be sufficient to saturate the cysteine-cysteine or cysteinehistidine binding sites, while leaving single cysteines mostly unbound (Figure 2 and Table 1).

3. Mix gently by flicking the tube and briefly centrifuge.

4. Incubate 10 minutes to ensure that metal binding has equilibrated.

5. Add 1 μL of 10 mM NEM (final concentration 100 μM).

NEM can be prepared as a 100 mM stock in 100% ethanol and stored at -80°C indefinitely. The stock can be diluted to 10 mM in labeling buffer immediately prior to adding it to the reaction.

6. Mix gently by flicking the tube and briefly centrifuge.

7. React for 10 minutes.

8. Add 1 μL of 10 mM fluorescein-5-maleimide (final concentration 100 μM).

Fluorescein-5-maleimide can be prepared as a 100 mM stock in dimethylsulfoxide (DMSO) and stored in aliquots at -80°C indefinitely. Repeated Freezing and thawing of the stock may cause significant degradation. The stock can be diluted to 10 mM in labeling buffer immediately prior to adding it to the reaction.

9. Add 5 μL of 500 mM EDTA to chelate Cd^{2+} (final concentration 2.5 mM).
10. Mix gently by flicking the tube and briefly centrifuge.
11. React for 10 minutes.
12. (Optional) Kill reaction by adding 100 μL of 1 mM glutathione (final concentration 500 μM).

Labeling can be verified by running 10 μL of each reaction on an SDS-PAGE gel. In-gel fluorescence can be imaged using a standard UV gel box and ethidium bromide filter.

BASIC PROTOCOL 2

SPECIFIC LABELING OF AN EXTRACELLULAR PROTEIN DOMAIN IN *XENOPUS* OOCYTES

This basic protocol was used to label a pair of cysteine residues on the extracellular side of a transmembrane protein expressed in *Xenopus laevis* oocytes (Puljung and Zagotta, 2011). Due to the presence of other transmembrane proteins, extracellular matrix proteins, and the vitelline membrane, oocytes exhibit a high background labeling with cysteine reactive dyes. The overall reaction scheme is the same as in Protocol 1 (Figure 1). Oocytes were blocked with NEM in the presence of a concentration of Cd^{2+} expected to protect cysteines in a metal binding site, but not individual cysteine residues. The oocytes were then removed to a solution containing EDTA and a cysteine-reactive fluorophore and labeled. Fluorescent labeling was stronger in oocytes expressing the protein of interest than in controls. Labeling in a cellular environment allows for the removal of excess reactants by changing solutions, which cannot be achieved when labeling soluble proteins. Furthermore, if the solutions are perfused over the cells or are in large volume relative to the cells being labeled, it can be assumed that the free concentration of reactants and metal ions are approximately equal to the solution concentration. This protocol can be readily adapted for use in other cell types. Oocytes were prepared as described previously (Gordon and Zagotta, 1995).

Materials—*Xenopus laevis* oocytes (defolliculated and injected with RNA encoding the protein intended for labeling or sham injected)

35 mm polystyrene culture dishes

OR2 solution (see recipe)

10 mg/mL bovine serum albumin (BSA) in water

10 mM NEM in OR2

1 mM Alexa Fluor 546 in OR2

500 mM EDTA in water, pH 8.0

10 mM CdCl_2 in water

Orbital shaker

Glass Pasteur pipette, broken and fire polished

Protocol steps

1. Place 3 mL of OR2 in four 35 mm culture dishes.

2. Add 30 μL of 10 mg/mL BSA to each dish (final concentration 100 $\mu\text{g/mL}$).

The BSA is to prevent the oocytes from sticking to the bottom of the culture dish. Agar was also used to prevent sticking, but the reactants diffused into the agar, making it difficult to control the concentration of reactants to which the oocytes were exposed.

3. Add 30 μL of 10 mM CdCl_2 to two of the dishes (final concentration 100 μM).

4. Add the desired number of oocytes to each dish.

Oocytes expressing the protein of interest can be divided between Cd^{2+} -containing and Cd^{2+} -free solutions so that labeling may be assessed with and without metal protection. The same can be done for control (sham-injected) oocytes. Oocytes are transferred using a Pasteur pipette that has been broken back and fire polished to form a larger diameter hole. This will prevent damage from shear forces.

5. Incubate with shaking for 10 minutes.

The defolliculated oocytes still have the vitelline membrane, in addition to the plasma membrane. Furthermore the oocyte surface is highly invaginated. The incubation step is meant to ensure that the Cd^{2+} has time to overcome these diffusional barriers and equilibrate at its binding site.

6. Add 30 μL of 10 mM NEM to each dish (final concentration 100 μM).

NEM can be prepared as a 100 mM stock in 100% ethanol and stored at -80°C indefinitely. The stock can be diluted to 10 mM in OR2 immediately prior to adding it to the reaction.

7. Incubate with shaking for 30 minutes.

8. Transfer oocytes to four new 35 mm dishes containing 3 mL of OR2 + 100 $\mu\text{g/mL}$ BSA.

Oocytes can be rinsed by pipetting into one or more separate, clean dishes of OR2 prior to placing them into the dish for the labeling reaction.

9. Add 30 μL of 500 mM EDTA (final concentration 5 mM).

10. Incubate with shaking for 5 minutes.

11. Add 30 μL of 1 mM Alexa Fluor 546.

Alexa Fluor 546 can be prepared as a 100 mM stock in DMSO and stored at -80°C indefinitely. The stock can be diluted to 1 mM in OR2 immediately prior to adding it to the reaction.

12. Incubate with shaking for 30 minutes.

13. Rinse the oocytes thoroughly by repeatedly transferring them to successive dishes of clean OR2 + 100 $\mu\text{g/mL}$ BSA.

REAGENTS AND SOLUTIONS

Labeling solution (1 L)—130 mM NaCl (7.60 g)

30 mM HEPES (7.15 g)

Bring to pH 7.2 with NaOH

OR2 solution (1 L)—82.5 mM NaCl (4.82 g)

2.5 mM KCl (0.19 g)

1 mM MgCl₂·6H₂O (0.20 g)

5 mM HEPES (1.19 g)

Bring to pH 7.6 with NaOH

COMMENTARY

Background Information—Fluorescence spectroscopy allows for the real-time investigation of protein structures and conformational changes. One major advantage of fluorescence over other structural techniques is that it can be applied both in purified systems and in a protein's native environment (Lakowicz, 2006; Taraska and Zagotta, 2010). In order to faithfully measure atomic-scale structural changes in proteins, it is best to use small fluorescent probes that only minimally perturb the structure of the molecule intended for study (Taraska et al., 2009a; Taraska et al., 2009b). This is best achieved by labeling proteins with small-molecule fluorophores that selectively react with the amino acid cysteine. Labeling of a specific cysteine in a protein is trivial, provided the protein can be purified and has only one accessible cysteine residue. However, in many experiments, the protein intended for study may have multiple reactive cysteines, may be present in a mixture of other proteins with cysteines, or may be in a native context in which many other cellular proteins will contribute to the fluorescence background.

Several approaches to specific cysteine labeling have been described previously. The simplest of these methods involves the identification of solvent-accessible cysteines in a protein and the determination of their modification rates (Ratner et al., 2002). The differential reaction kinetics can then be exploited to control cysteine labeling. In order to be effective, this method requires a purified protein of known structure and also requires that the different cysteines have substantially different reaction rates. A more general approach would allow the experimenter to directly manipulate the reaction kinetics of a desired cysteine in order to provide labeling specificity. Proteins that exist in multiple conformations may have regions that change their solvent-accessibility in a state-dependent fashion. If a cysteine is inserted into such a region, and the conformational state can be reproducibly controlled, e.g. with ligand binding, then the desired cysteine can be maintained in an inaccessible state while the background cysteines are blocked. Following block, the protein can be restored to a state in which the cysteine of interest is accessible and can be labeled. This approach has been used to label residues on ligand-gated ion channels (Islas and Zagotta, 2006; Zheng and Zagotta, 2000). Unfortunately, this method only works on proteins that undergo a conformational change that can be directly manipulated by the experimenter. Furthermore, it requires some knowledge about the location and state-dependence of the target cysteine and does not provide for labeling of cysteines in regions of the protein that do not exhibit any conformation-dependent changes in accessibility. Cysteines can also be prevented from reacting by reversible incorporation into disulfide bonds (Smith et al., 2005). This method is highly specific in purified proteins, but in a cellular environment, many proteins have disulfide bonds or cysteines that can be readily incorporated into disulfide bonds under the conditions used to oxidize the cysteines intended for labeling. When the target cysteines are reduced for labeling, these background cysteines will also be available to react with an applied fluorophore. A similar method involves the protection of pairs of cysteines in close proximity with phenylarsine oxide while background cysteines are blocked (Kuiper et al., 2009). This method is limited in that it can only be applied to pairs of cysteines, both of which would label with fluorophore, and requires the

use of the harsh reducing agent dithiothreitol to remove the protecting group. Dithiothreitol may expose cysteines in native disulfide bonds so that they can react with fluorophores. Finally, cysteines can be incorporated into zinc finger domains, which reversibly bind group 12 metal ions like Cd^{2+} and Zn^{2+} that protect the cysteine from reacting (Smith et al., 2005). This is a highly specific method, but requires the insertion of a structured domain of at least 18 amino acids into the target protein.

Cysteine Metal Protection and Labeling (CyMPL) uses the same principle of cysteine protection through reversible metal binding, but does not require the insertion of a large, structured domain into the protein intended for labeling (Puljung and Zagotta, 2011). CyMPL uses minimal metal binding sites composed of a cysteine-histidine pair or cysteine-cysteine pair. These sites can be inserted into any region of known secondary structure (α -helix or β -strand) and are not expected to greatly perturb the local protein environment.

Critical Parameters and Troubleshooting—One critical concern when using CyMPL is the proper design of the metal binding site. Table 1 lists the apparent K_d for different cysteine-histidine and cysteine-cysteine combinations. Ideally, one would use a binding site with the greatest stabilization of metal binding relative to single cysteines. When using Cd^{2+} , cysteine-cysteine pairs offer the greatest stabilization, but have the disadvantage of containing two reactive residues following metal removal. Cysteine-histidine pairs stabilize Cd^{2+} binding to a lesser degree, but have the obvious advantage of leaving only one reactive residue after metal dissociation. If a single histidine does not offer enough stabilization, one can engineer a binding site with two histidines. Alternatively, Zn^{2+} can be used. While we have not characterized Zn^{2+} binding as extensively as Cd^{2+} binding, in the one case we tried, it bound much more strongly to a cysteine-histidine site than to cysteine alone (Table 1; Puljung and Zagotta, 2011). In fact, the stabilization offered by the cysteine-histidine site over cysteine alone was much greater with Zn^{2+} binding than with Cd^{2+} . This is an expected result, as Zn^{2+} , a harder Lewis acid, should bind more strongly to histidine than to cysteine (Rulisek and Vondrasek, 1998).

The success of CyMPL depends on controlling the kinetics of the reaction of a cysteine of interest, i.e. making modification of the desired cysteine by non-fluorescent covalent blockers slower than that of background cysteines. However, since metal binding is reversible, and covalent cysteine modification is essentially irreversible, even protected cysteines will modify if the reaction is allowed to come to equilibrium. Therefore, it is necessary to manipulate the concentration of reactants so that the blocking reaction does not proceed long enough to modify even the target cysteines. If this occurs, the specific signal will be very small. Care should also be taken to ensure that the concentration of covalent blocker is in excess of the background cysteines that need to be blocked.

Another possible issue is that long-term exposure to cysteine modifiers can damage the cells being studied. Therefore, it is important that the blocking reaction does not proceed any longer than necessary. Characterization of the rate of modification of background cysteines can be useful for determining reaction conditions. Furthermore, if a particular blocker is found to be too toxic, there are several covalent cysteine modifying reagents with varying functionalities and membrane permeabilities that may be used.

Background fluorescence may arise from factors other than reaction of a fluorophore with cysteines. For example, in a cellular labeling experiment, certain lipophilic fluorophores may accumulate in cell membranes. While CyMPL does not address this issue, the proper choice of fluorophore and reaction time will ameliorate this problem. Charged fluorophores can be used that do not strongly partition into the membrane. Certain fluorophores, like bimane C_3 -maleimide, only strongly fluoresce when they have reacted with cysteines and

will reduce any background from non-specific membrane association (Puljung and Zagotta, 2011).

Anticipated Results—In our efforts to label a single component in a mixture of two proteins, we observed 40-fold greater labeling of a protein with a cysteine-histidine site compared to a protein with a single cysteine and 20 fold greater labeling of a cysteine-cysteine containing protein over a single cysteine containing protein (Puljung and Zagotta, 2011). In both experiments, the two proteins were present in equimolar amounts. In our extracellular labeling experiments in *Xenopus* oocytes, we had a greater than three-fold increase in the signal/background ratio (Puljung and Zagotta, 2011). Our chosen fluorophore, Alexa Fluor 546, bound non-specifically to the oocyte membranes. It is likely that with a different fluorophore or reaction conditions, the signal/background could be further optimized.

Time Considerations—As written, Basic Protocol 1 should require 30 to 45 minutes. Basic Protocol 2 requires 1.25 to 1.5 hours. Times may be adjusted as needed.

Acknowledgments

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Figure 1. Schematic diagram depicting the specific cysteine protection method, CyMPL

The group 12 metal ion (M^{2+}) binding affinity of a desired cysteine is increased by placing an additional metal-binding amino acid nearby. M^{2+} is used to protect the desired cysteine (i) while background cysteines (in circles) are reacted with a non-fluorescent modifying reagent like NEM (ii). Upon M^{2+} removal with a chelator like EDTA (iii), the specific cysteine is available to react with fluorophore (iv). Adapted from Puljung and Zagotta, 2011.

Figure 2. Cd^{2+} affinity of helical peptides with introduced minimal metal binding sites
 Concentration-response curve showing the rate (normalized to 0 Cd^{2+}) of the reaction of Cys Only peptide and Cys/Cys i+3 peptide with bimane C_3 -maleimide as a function of $[CdCl_2]_{total}$. The dashed box marks a concentration range at which single cysteines are not expected to be bound to Cd^{2+} , but a site consisting of two cysteines should be fully bound. Affinity was assessed by examining the concentration-dependent reduction of the rate of reaction of the peptides with bimane C_3 -maleimide. $n = 5-6$ for each concentration. Adapted from Puljung and Zagotta, 2011.

Figure 3. Metal binding sites

Cartoons depicting three possible cysteine-histidine binding sites for group 12 metal ions. Cys/His i+3 and Cys/His i+4 are helical peptides with histidines placed three and four positions away from a cysteine, respectively. HCN S563C,K565H depicts one β -strand from the C-terminal fragment of the mouse HCN2 ion channel with a cysteine and histidine placed two residues apart.

Figure 4. Dibromobimane reacted with two cysteines

Cartoon depicting the bifunctional fluorophore dibromobimane reacted with two cysteines spaced three residues apart on a model α -helix.

Table 1
Metal affinities and binding energies

Values for K_d and ΔG of metal ion binding are given for peptides and proteins in aqueous buffer. MBP refers to *E. coli* maltose binding protein. Cysteines were placed in a helical portion of MBP. HCN refers to the soluble, cysteine-free C-terminal region of the mouse HCN2 ion channel. The mutations listed were placed in a β -strand of HCN. All numbers are expressed as mean $\pm$ SEM. n refers to the number of experiments. Affinity was assessed by examining the concentration-dependent reduction of the rate of reaction of the above peptides or proteins with bimane C₃-maleimide. Adapted from Puljung and Zagotta, 2011.

		K_d (mM)	ΔG (kcal mol ⁻¹)	n
Helical Peptides + Cd ²⁺	Cys Only	158 $\pm$ 8	-5.10 $\pm$ 0.032	5
	Cys/His i+1	27.8 $\pm$ 3.2	-6.13 $\pm$ 0.066	5
	Cys/His i+3	13.5 $\pm$ 0.8	-6.54 $\pm$ 0.038	5
	Cys/His i+4	16.2 $\pm$ 1.8	-6.44 $\pm$ 0.065	5
	Cys/His i+3,4	4.69 $\pm$ 0.58	-7.17 $\pm$ 0.087	5
	Cys/Cys i+1	1.79 $\pm$ 0.67	-8.03 $\pm$ 0.361	5
	Cys/Cys i+3	1.77 $\pm$ 0.52	-7.89 $\pm$ 0.219	6
	Cys/Cys i+4	0.94 $\pm$ 0.24	-8.18 $\pm$ 0.174	5
Proteins + Cd ²⁺	MBP E22C,K25C	3.68 $\pm$ 1.29	-7.47 $\pm$ 0.205	6
	MBP K25C,K26C	3.06 $\pm$ 1.08	-7.53 $\pm$ 0.195	5
Helical Peptides + Zn ²⁺	Cys Only	1,090 $\pm$ 290	-4.07 $\pm$ 0.171	5
	Cys/His i+4	28.1 $\pm$ 3.3	-6.12 $\pm$ 0.071	5
β -strand + Cd ²⁺	HCN S563C	157 $\pm$ 42	-5.17 $\pm$ 0.143	5
	HCN S563C,K565H	6.04 $\pm$ 2.32	-7.27 $\pm$ 0.267	6