

2.1 Introduction to Thiol Modification and Detection

Common Applications for Thiol-Reactive Probes

Labeling Biopolymers

In contrast to the amine-reactive reagents described in Chapter 1, thiol-reactive dyes are principally used to prepare fluorescent peptides, proteins and oligonucleotides for probing biological structure, function and interactions. Because the thiol functional group is not very common in most proteins and can be labeled with high selectivity, thiol-reactive reagents often provide a means of selectively modifying a protein at a defined site. Thiol-reactive probes can be used to:

- Analyze the topography of proteins in biological membranes using polar thiol-reactive fluorescent reagents
- Determine distances within the protein or between the protein and a ligand using excited-state energy transfer (see Fluorescence Resonance Energy Transfer (FRET) in Section 1.3)
- Follow changes in protein conformation using environment-sensitive probes
- Site-selectively label proteins in order to study protein–protein and protein–nucleic acid interactions using fluorescence anisotropy

Thiol-reactive dyes can also be reacted with thiolated primers for DNA sequencing,^{1,2} with thiouridine-modified tRNA for studying its association with protein synthesis machinery^{3,4} and with thiol-containing proteins to facilitate their electrophoretic detection.⁵

Derivatizing Low Molecular Weight Molecules

Several of the thiol-reactive probes described in this chapter are also useful for derivatizing low molecular weight thiols for various analytical assays that employ chromatographic and electrophoretic separation. An extensive review by Shimada and Mitamura describes the use of several of our thiol-reactive reagents for derivatizing thiol-containing compounds.⁶

Quantitating Thiols

Thiols play a principal role in maintaining the appropriate oxidation–reduction state of proteins, cells and organisms. However, the susceptibility of thiols to oxidation can lead to the formation of disulfides and higher oxidation products, often with loss of biological activity. Measuring the oxidation state of thiols within live cells is complicated by the extraordinarily high concentration of reduced glutathione in cells, which makes them difficult to assay with reagents that stoichiometrically react with the thiol (Section 15.6). However, good reagents and methods have been described for the quantitative assay of thiols (and disulfides) in solution, a few of which are described below.

Reactivity of Thiol Groups

In proteins, thiol groups (also called mercaptans or sulfhydryls) are present in cysteine residues. Thiols can also be generated by selectively reducing cystine disulfides with reagents such as dithiothreitol⁷ (DTT, D-1532) or β -mercaptoethanol, each of which must then be removed by dialysis or gel filtration before reaction with the thiol-reactive probe.⁸ Unfortunately, removal of DTT or β -mercaptoethanol is sometimes accompanied by air oxidation of the thiols back to the disulfides. Reformation of the disulfide bond can be avoided by using the reducing agent tris-(2-carboxyethyl)phosphine^{9,10} (TCEP, T-2556), which usually does not need to be removed prior to thiol modification because it does not contain thiols (Figure 2.1). However, TCEP has been reported to react with haloacetamides or maleimides under certain conditions.^{9,11} TCEP is stable at a higher pH than is DTT and for a longer period of time in buffers without metal chelators such as EGTA; DTT is more stable in solutions that contain metal chelators.⁹ TCEP is also more stable in the presence of Ni^{2+} levels that commonly contaminate proteins eluted from Ni^{2+} affinity

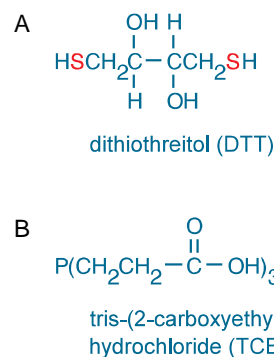


Figure 2.1 Structural comparison of the reducing agents DTT (A) and TCEP (B). Unlike DTT (dithiothreitol, D-1532), TCEP (tris-(2-carboxyethyl)phosphine, hydrochloride; T-2556) does not contain a free thiol, and therefore does not require removal before reaction with a thiol-reactive probe.

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columns and that rapidly oxidize DTT.⁹ Spin labels in TCEP are two to four times more stable than those in DTT, an advantage for electron paramagnetic resonance (EPR) spectroscopy.⁹ TCEP is also used to stabilize solutions of ascorbic acid.¹²

TCEP is generally impermeable to cell membranes and to the hydrophobic protein core, permitting its use for the selective reduction of disulfides that have aqueous exposure. The pH-insensitive and less polar phosphine derivative tris-(2-cyanoethyl)phosphine (T-6052) may yield greater reactivity with buried disulfides. Several reagents have also been developed for introducing thiols into proteins, nucleic acids and lipids, permitting the use of any of the fluorescent or chromophoric thiol-reactive reagents described in this chapter for their covalent modification. Because the selective introduction of thiols is particularly important for crosslinking two biomolecules, these reagents are discussed in Chapter 5.

The thiol-reactive functional groups are primarily alkylating reagents, including iodoacetamides, maleimides, benzylic halides and bromomethylketones. Arylating reagents such as NBD halides react with thiols or amines by a similar substitution of the aromatic halide. Reaction of any of these functional groups with thiols usually proceeds rapidly at or below room temperature in the physiological pH range (pH 6.5–8.0) to yield chemically stable thioethers.

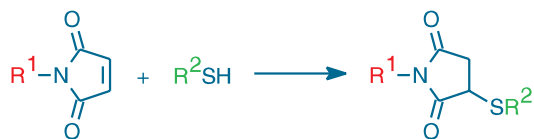
Thiols also react with many of the amine-reactive reagents described in Chapter 1, including isothiocyanates and succinimidyl esters. However, the products appear to be insufficiently stable to be useful for routine modification of thiols in proteins. Although the thiol–isothiocyanate product (a dithiocarbamate) can react with an adjacent amine to yield a thiourea, the dithiocarbamate is more likely to react with water, consuming the reactive reagent without forming a covalent adduct.



Alkyl halide or
Haloacetamide (X = I, Br, Cl)

Thioether

Figure 2.2 Reaction of a thiol with an alkyl halide.



Maleimide

Thioether

Figure 2.3 Reaction of a thiol with a maleimide.



Symmetric disulfide

Mixed disulfide

Figure 2.4 Reaction of a thiol with a symmetric disulfide (e.g., didansyl-L-cystine, D-146).

Iodoacetamides

Iodoacetamides readily react with all thiols, including those found in peptides, proteins and thiolated polynucleotides, to form thioethers (Figure 2.2); they are somewhat more reactive than bromoacetamides. However, when a protein's cysteine residues are blocked or absent, iodoacetamides can sometimes react with methionine residues.^{13,14} They may also react with histidine¹⁵ or tyrosine, but generally only if free thiols are absent. Although iodoacetamides can react with the free base form of amines, most aliphatic amines, except the α -amino group at a protein's N-terminus, are protonated and thus relatively unreactive below pH 8. In addition, iodoacetamides react with thiolated oligonucleotide primers, as well as with thiophosphates and thiouridine residues present in certain nucleic acids,^{1,3,4,16} but usually not with the common nucleotides.

Iodoacetamides are intrinsically unstable in light, especially in solution; reactions should therefore be carried out under subdued light. Adding cysteine, glutathione or mercaptosuccinic acid to the reaction mixture will quench the reaction of thiol-reactive probes, forming highly water-soluble adducts that are easily removed by dialysis or gel filtration. Although the thioether bond formed when an iodoacetamide reacts with a protein thiol is very stable, during amino acid hydrolysis the bioconjugate loses its fluorophore to yield *S*-carboxymethylcysteine.

Maleimides

Maleimides are excellent reagents for thiol-selective modification, quantitation and analysis. The reaction involves addition of the thiol across the double bond of the maleimide to yield a thioether (Figure 2.3). The applications of these fluorescent and chromophoric analogs of *N*-ethylmaleimide (NEM) strongly overlap those of iodoacetamides, although maleimides apparently do not react with methionine, histidine or tyrosine. Reaction of maleimides with amines usually requires a higher pH than reaction of maleimides with thiols. Hydrolysis of maleimides to a mixture of isomeric nonreactive maleamic acids can compete significantly with thiol modification, particularly above pH 8. Furthermore, maleimide adducts can hydrolyze, which may cause a significant change in the fluorescence properties of the conjugate,¹⁷ or they can ring-open by nucleophilic reaction with an adjacent amine to yield crosslinked products.¹⁸ This latter reaction can potentially be enhanced by raising the pH above 9 after conjugation. Several maleimides — including 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM), as well as the pyrene and stilbene derivatives — are not appreciably fluorescent until after conjugation with thiols, and may therefore be useful for thiol quantitation.

Other Thiol-Reactive Reagents

A variety of other thiol-reactive probes are available. These include alkyl halides and arylating agents (NBD chloride, ABD fluoride). We also offer two fluorescent disulfides, BODIPY FL L-cystine (B-20340, Section 2.2, Figure 2.6) and *N,N'*-didansyl-L-cystine (D-146, Section 2.3). Symmetric disulfides undergo a thiol–disulfide interchange reaction to yield a new asymmetric disulfide (Figure 2.4), a reaction that is freely reversible and thiol-specific. The covalent adducts from these thiol-reactive probes

are, in general, more resistant to hydrolysis than those from iodoacetamides or maleimides, which are somewhat sensitive to hydrolysis at the amide linkage connecting the fluorophore to its reactive group. See Thiol-Reactive Probes Discussed in Other Chapters of the Handbook for more information about thiol-reactive probes with specialty applications.

Reagents for Quantitating Thiols

Thiol and Sulfide Quantitation Kit

Ultrasensitive colorimetric quantitation of both protein and nonprotein thiols is now possible using our Thiol and Sulfide Quantitation Kit (T-6060). In this assay, which is based on a method reported by Singh,^{19,20} thiols or sulfides reduce a disulfide-inhibited derivative of papain, stoichiometrically releasing the active enzyme (Figure 5.5). Activity of the enzyme is then measured using the chromogenic papain substrate L-BAPNA. Although thiols and inorganic sulfides can also be quantitated using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent, D-8451), the enzymatic amplification step in our quantitation kit yields a sensitivity for detection of thiols or sulfides of approximately 0.2 nanomoles, or about 100-fold better than that obtained with DTNB. Thiols in proteins and potentially other high molecular weight molecules can be detected indirectly by incorporating the disulfide cystamine into the reaction mixture. Cystamine undergoes an exchange reaction with protein thiols, yielding 2-mercaptoethylamine (cysteamine), which then releases active papain. The Thiol and Sulfide Quantitation Kit contains:

- Papain-SSCH₃, the disulfide-inhibited papain derivative
- L-BAPNA, a chromogenic substrate for papain
- DTNB (Ellman's reagent), for calibrating the assay
- Cystamine
- L-Cysteine, a thiol standard
- A reaction buffer
- Protocols for measuring thiols, inorganic sulfides and maleimides

Sufficient reagents are provided for approximately 50 assays using standard 1 mL cuvettes or 250 assays using a microplate format. This kit can also be used to detect phosphines, sulfites and cyanide (Section 22.2) with detection limits of about 0.5, 1 and 5 nanomoles, respectively.

Ellman's Reagent (DTNB) for Quantitating Thiols

Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB; D-8451) is an important reagent for quantitating thiols in proteins, cells and plasma by absorption measurements.²¹ It readily forms a mixed disulfide with thiols, liberating the chromophore 5-mercapto-2-nitrobenzoic acid (absorption maximum 410 nm, $\epsilon \sim 13,600 \text{ cm}^{-1}\text{M}^{-1}$).²² Only protein thiols that are accessible to this water-soluble reagent are modified.^{23,24} Inaccessible thiols can usually be quantitated by carrying out the titration in the presence of 6 M guanidinium chloride. DTNB conjugates of glutathione and other thiols can be separated by HPLC and quantitated based on their absorbance.²⁵

Fluorometric Reagents for Quantitating Thiols

Several of the "fluorescent" maleimides described in Section 2.3 actually have very low fluorescence until they react with thiols to form fluorescent adducts, permitting their use for both the quantitation of thiols and their semiquantitative localization in cellular organelles. Monobromobimane (M-1378, M-20381; Section 2.3) is also essentially nonfluorescent until it reacts with thiols and can be used for their quantitation.

Most of the fluorescent thiol-reactive reagents in this chapter can be used as derivatization reagents for analysis of thiols by techniques such as HPLC that utilize a separation step. 5-(Bromo-methyl)fluorescein (B-1355, Section 2.2) is the reagent with the greatest intrinsic sensitivity for this application. See Section 15.6 for a further discussion of methods to quantitate reduced glutathione in cells.

TECHNICAL NOTE

Thiol-Reactive Probes Discussed in Other Chapters of the Handbook

Molecular Probes offers a number of other thiol-reactive probes with special applications. For example, our DetectaGene β -galactosidase substrates and our CellTracker and MitoTracker probes are mildly thiol-reactive chloromethyl derivatives that show superior cellular retention when compared with their nonchloromethyl counterparts. These and other thiol-reactive probes are discussed in the chapter appropriate for their application.

Chapter 4 Biotin Derivatives and Haptens — Including thiol-reactive biotinylation and haptenylation reagents.

Chapter 5 Crosslinking and Photoreactive Reagents — Including several reagents that contain maleimide groups for crosslinking biomolecules or synthesizing thiol-reactive probes, as well as reagents for introducing thiols into biopolymers.

Chapter 10 Enzyme Substrates — Including fluorogenic substrates for glycosidases, proteases and microsomal dealkylase that contain mildly thiol-reactive chloromethyl or pentafluorobenzoyl groups.

Chapter 12 Probes for Organelles — Including thiol-reactive MitoTracker mitochondrion-selective probes.

Chapter 14 Fluorescent Tracers of Cell Morphology and Fluid Flow — Including thiol-reactive CellTracker probes.

Chapter 16 Probes for Endocytosis, Receptors and Ion Channels — Including a cell-permeant, thiol-reactive derivative of dichlorodihydrofluorescein.

Chapter 21 pH Indicators — Including a thiol-reactive, cell-permeant derivative of the SNARF-1 pH indicator.

References

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Data Table — 2.1 Introduction to Thiol Detection and Modification

Cat #	MW	Storage	Soluble	Abs	Em
D-1532	154.24	D	H ₂ O	<300	none
D-8451	396.35	D	pH >6	324	none
T-2556	286.65	D	pH >5	<300	none
T-6052	193.19	D	MeCN	<300	none

For definitions of the contents of this data table, see "How to Use This Book" on page viii.

Product List — 2.1 Introduction to Thiol Detection and Modification

Cat #	Product Name	Unit Size
D-8451	5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent)	10 g
D-1532	dithiothreitol (DTT)	1 g
T-6060	Thiol and Sulfide Quantitation Kit *50–250 assays*	1 kit
T-2556	tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP)	1 g
T-6052	tris-(2-cyanoethyl)phosphine	1 g

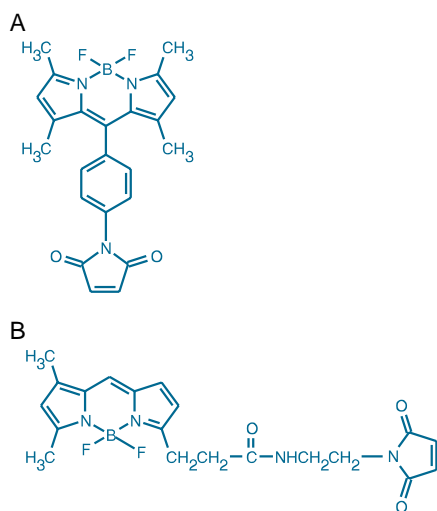


Figure 2.5 Comparison of the fluorophore orientation relative to the reactive moiety of two spectrally similar thiol-reactive BODIPY dyes, A) BODIPY 499/508 maleimide (D-20350) and B) BODIPY FL N-(2-aminoethyl)maleimide (B-10250).

2.2 Thiol-Reactive Probes Excited with Visible Light

The probes described in this section are those that have visible absorption maxima beyond 400 nm. Our thiol-reactive probes that have peak absorption below 400 nm are described in Section 2.3. Among all the thiol-reactive probes, the BODIPY, Alexa Fluor, fluorescein, Oregon Green, tetramethylrhodamine and Texas Red derivatives have the strongest absorptivity and highest fluorescence quantum yields. This combination of attributes makes these compounds the preferred reagents for preparing protein and low molecular weight ligand conjugates to study the structural properties, diffusion and interactions of proteins and ligands using techniques such as fluorescence recovery after photobleaching (FRAP), fluorescence polarization (FP), fluorescence correlation spectroscopy (FCS) or fluorescence resonance energy transfer (FRET, see Section 1.3). In this section and in Section 2.3, thiol-reactive reagents with similar spectra, rather than the same reactive group, are generally discussed together. The exception to this organization is the description of our reactive BODIPY and Alexa Fluor fluorophores, which are available in several choices of excitation and emission wavelengths; they are therefore each considered as groups. Table 2.1 summarizes the iodoacetamide and maleimide probes listed in this section.

BODIPY Derivatives

Like their amine-reactive BODIPY counterparts (Section 1.4), BODIPY iodoacetamides, BODIPY maleimide and BODIPY methyl bromides yield thiol adducts with several important properties:

- High extinction coefficients ($\epsilon > 60,000 \text{ cm}^{-1}\text{M}^{-1}$)
- High fluorescence quantum yields, often approaching 1.0, even in water