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Thiol-reactive dyes for fluorescence labeling of proteomic samples

Covalent derivatization of proteins with fluorescent dyes prior to separation is increasingly used in proteomic research. This paper examines the properties of several commercially available iodoacetamide and maleimide dyes and discusses the conditions and caveats for their use in labeling of proteomic samples. The iodoacetamide dyes BODIPY TMR cadaverine IA and BODIPY Fl C_1-IA were highly specific for cysteine residues and showed little or no nonspecific labeling even at very high dye:thiol ratios. These dyes also showed minimal effects on p*I*'s of standard proteins. Some iodoacetamide dyes, (5-TMRIA and eosin-5-iodoacetamide) and some maleimide dyes (ThioGlo I and Rhodamine Red C_2 maleimide) exhibited nonspecific labeling at high dye:thiol ratios. Labeling by both iodoacetamide and maleimide dyes was inhibited by tris(2-carboxyethyl)phosphine (TCEP); interactions between TCEP and dye were also observed. Thiourea, an important component of sample solubilization cocktails, inhibited labeling of proteins with iodoacetamide dyes but not with maleimide dyes. Maleimide dyes may serve as an alternative for labeling proteins where it is essential to have thiourea in the solubilization buffer. Covalent derivatization by BODIPY TMR cadaverine IA, BODIPY Fl C_1-IA or Rhodamine Red C_2 maleimide was also demonstrated to be compatible with in-gel digestion and peptide mass fingerprinting by matrix assisted laser desorption/ionization-mass spectrometry and allowed successful protein identification.

Keywords: Cysteine labeling / Fluorescence / Proteomics / Thiourea / Tris(2-carboxyethyl)phosphine
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1 Introduction

Fluorescent detection of proteins has been gaining popularity in the field of electrophoresis and proteomics. The common aim of proteomics is to quantitatively define biological processes at the protein level. This typically involves the separation, display, and comparison of complex mixtures of proteins from reference and target cells, which include protein concentrations spanning a broad range. The common adsorptive staining methods (Coomassie blue, colloidal gold, and silver stain [1]) do not provide the requisite dynamic range, being linear over only a 10- to 40-fold difference in protein concentration [2], and suffer from being tedious and requiring harsh chemicals. Fluorescence detection technologies on the other hand provide linear signal response over a much wider signal range (about three orders of magnitude) than is found for the adsorptive dye alternatives mentioned above.

Fluorescent modification of proteins can be divided into two categories: covalent and noncovalent. In covalent methods, the proteins or proteomic samples are derivatized with a dye prior to electrophoresis or other separation methods [3, 4]. The main advantages of covalent derivatization methods for protein detection are the elimination of staining and destaining of gels, and the ability to image and capture the protein separation pattern immediately after electrophoresis. In noncovalent methods, the proteins are first separated by SDS-PAGE followed by staining with dyes that bind to SDS-protein complexes, *e.g.*, the SYPRO dyes [5–10].

For covalent modification with fluorophores two main approaches have been applied to proteomic samples, (i) modification of cysteine (Cys) residues with alkylating

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Abbreviations: BODIPY Fl C_1-IA, *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-yl)methyl) iodoacetamide; **BODIPY TMR cadaverine IA**, *N*-(5-((4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-*s*-indacene-2-propionyl)amino)pentyl) iodoacetamide; **Cys**, cysteine; **Lys**, lysine; **TCEP**, tris(2-carboxyethyl)phosphine; **ThioGlo I**, 10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-methylester-3*H*-naphthol[2,1-b]pyran-*s*-carboxylic acid; **5-TMRIA**, tetramethylrhodamine-5-iodoacetamide dihydroiodide

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agents, *e.g.*, monobromobimane, and (ii) modification of lysine (Lys) residues with *N*-hydroxysuccinimidyl esters of cyanine dyes, such as Cy3 and Cy5 [11, 12]. While monobromobimane has been used to label proteomic samples, the major argument against its use has been its lower sensitivity and the fact that it excludes proteins not containing Cys residues [13]. Labeling of Lys groups on the other hand presents numerous challenges. Since Lys residues are abundant, it is desirable to label only a small percentage of them in order to keep proteins soluble. Underlabeling, however, results in lower sensitivity and makes precise quantitative comparisons difficult. In addition labeling at Lys residues may significantly alter the isoelectric points of proteins and inhibits proteolysis by trypsin at modified sites, thus affecting peptide generation for mass fingerprinting and identification.

In many ways, fluorescence-labeling of Cys residues presents a more attractive alternative than labeling of Lys residues. Cys residues are highly reactive, their frequency of occurrence in a protein is lower than most other residues, and the alkylation of Cys residues with neutral dyes can leave the isoelectric points of proteins only marginally affected. Cys residues are also not cleaved by commonly available proteases; hence their modification is likely to leave peptide maps unaltered for analysis by mass spectrometry. While it is true that the thiol-reactive dyes detect only proteins that contain Cys residues, recent analysis of the SWISS-PROT database demonstrates that 88% of all proteins in the SWISS-PROT database, 92% of yeast proteins, and 89% of human proteins have at least a single Cys residue. Of these total Cys proteins, 98% have 20 or fewer Cys residues, with 72% of proteins containing 1–10 Cys residues, while only $<2\%$ of proteins have more than 20 Cys [14]. Due to their lower abundance, Cys residues provide a greater opportunity for accurate quantification by permitting saturation of the reactive sites, resulting in more reproducible quantitative labeling and a lesser impact on protein solubility.

While there is an increasing number of commercially available thiol-reactive dyes for protein detection, the application of these dyes to proteomic studies has remained relatively limited. In this study we examine several commercially available thiol-reactive dyes for labeling of protein samples for proteomics, that can be visualized by commonly available imaging equipment. The dyes are analyzed with regard to specificity of labeling Cys residues, reaction with reductants and chaotropes, effect on the p*I* of proteins, and effect on the identification of labeled protein by in-gel digestion and peptide mass fingerprinting. The considerations and caveats for labeling Cys residues in proteomic samples are discussed.

2 Materials and methods

2.1 Materials

Yeast enolase I, human carbonic anhydrase I, bovine α-lactalbumin, horse myoglobin, and thiourea were from Sigma-Aldrich (St. Louis, MO, USA). *N*-(4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-yl)methyl) iodoacetamide (BODIPY Fl-C_1-IA), *N*-(5-((4,4-Difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-*s*-indacene-2-propionyl)amino)pentyl) iodoacetamide (BODIPY-TMR cadaverine IA), tetramethylrhodamine-5-iodoacetamide dihydroiodide (5-TMRIA) and Rhodamine Red C_2 maleimide were from Molecular Probes (Eugene, OR, USA). 10-(2,5-Dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-methylester-3*H*-naphthol[2,1-b]pyran-*s*-carboxylic acid (ThioGlo I) was from Covalent Associates (Woburn, MA, USA). Tris(2-carboxyethyl)phosphine (TCEP) and HPLC-grade TFA were from Pierce (Rockford, IL, USA). Urea was from Amersham Biotech (Piscataway, NJ, USA). Reduced Triton X-100 and CHAPS were from Calbiochem (San Diego, CA, USA). Sequencing-grade trypsin was from Promega (Madison, WI, USA) and α-cyano-4-hydroxycinnamic acid was from Agilent Technologies (Palo Alto, CA, USA). HPLC-grade acetonitrile (Burdick and Jackson) was obtained from VWR International (Plainfield, NJ, USA). Precast Tris-Gly, NuPage and IEF gels were from Invitrogen (Carlsbad, CA, USA). Fluorescent images were obtained on an AlphaImager (Alpha Innotech, San Leandro, CA, USA).

2.2 Protein labeling

In general, protein labeling with fluorescent dye was performed with ~25 μM protein reduced with variable amounts of TCEP in 8 M urea, 50 mM Tris-HCl (pH 7.5 or pH 8.0) (except for the maleimide dyes where 50 mM Tris-HCl, pH 7.2, was used) for 30 min, and then alkylated with fluorescent thiol-reactive dye for 1.5–2 h. The reaction was typically quenched by the addition of 150-fold excess of 2-mercaptoethanol for 30 min. In some cases, labeled proteins were purified from free dye using a PD-10 column (Amersham Biotech, Piscataway, NJ, USA).

2.3 TCEP reaction with dye

TCEP (0.5–5 mM) in 8 M urea, 50 mM Tris-HCl (pH 7.5) was incubated with 0.5 mM dye for 1.5 h in a 400 μL reaction volume. The reaction was stopped by acidification with 100 μL 0.1% TFA (pH 2.5). DMSO (100 μL) was added to the mixture (total 30%). Sample (50 μL) was diluted to 200 μL and a 10 μL aliquot was analyzed by RP-HPLC.

2.4 Digestion with trypsin and in-gel digestion

Approximately 300 pmol of BODIPY-labeled enolase was digested with trypsin in a 0.1 M $NaHCO_3$ buffer at a 1:10 trypsin:substrate ratio at 37°C for 18 h. For in-gel digestion dye-labeled protein (5 pmoles) was subjected to SDS-PAGE, and the bands were excised by visualization with UV transillumination. Excised bands were washed with 25 mM NH_4HCO_3, 50% acetonitrile three times, and the slices were dried under vacuum. The slices were rehydrated in 25 μL NH_4HCO_3 with 1:10 trypsin:protein and incubated at 37°C for 18 h. The digest was removed, and slices were washed three times with 50% acetonitrile, 5% TFA, and pooled. The pooled digest was concentrated to 10 μL.

2.5 SDS-PAGE and reversed-phase HPLC

SDS-PAGE was performed using precast 14 or 16% Tris-Gly gels according to the manufacturer's instructions. Reversed-phase HPLC was performed on an HP1100 HPLC system with UV/vis detection using a diode array detector and fluorescence detection. Separation was performed using a C18 reversed phase HAISIL 300 C18 5 μm (250 × 2.1 mm) column from Higgins Analytical (Mountain View, CA, USA). All separations were performed using a water-acetonitrile gradient with 0.1% TFA at a flow-rate of 0.2 mL/min. TCEP-dye adducts were analyzed with a gradient of 5% acetonitrile (0.1% TFA) at 0 min, 25% at 5 min, 60% at 25 min, and 95% at 40 min.

2.6 Mass spectrometry

MALDI-MS was performed using a Voyager DE-RP Biospectrometry workstation (PE Biosystems, Framingham, MA, USA) in the reflectron mode with delayed extraction using α-cyano-4-hydroxycinnamic acid as a matrix. Typically 0.5 μL of sample was applied to the MALDI target followed by 0.5 μL of matrix. The spot was air-dried at room temperature prior to acquiring mass spectra. MS was performed in the positive-ionization mode using an accelerating voltage of 20 kV. Peptide mass fingerprinting data was evaluated using MS-Fit program of the Protein Prospector MS analysis package (UCSF, San Francisco, CA, USA).

3 Results

A number of thiol-reactive dyes spanning different spectral ranges has become commercially available in the recent past. In our study we evaluated several neutral thiol-reactive dyes that were chosen for their high extinction coefficients, good quantum yields, pH insensitivity, and expectations of minimal effects on the p*I* of proteins. We evaluated both iodoacetamide and maleimide dyes for their specificity for Cys residues on proteins and examined the effects of reaction components such as reducing agents, chaotropes, and detergents on the labeling reaction. The structures and abbreviations of the dyes utilized in this study are shown in Fig. 1.

3.1 Specificity of dyes for cysteine-containing proteins

When considering a dye for proteomic applications with subsequent identification by MS it is essential to first establish the specificity of the dye for the residues being modified. Nonspecific derivatizations may result in erroneous quantification of proteins, cause large shifts in p*I* and mass of the protein under consideration, and lead to ambiguous identification of the proteins when databases are searched with the mass data. To evaluate the specificity of thiol-reactive dyes for Cys-containing proteins we labeled a mixture of human carbonic anhydrase I (containing a single Cys residue) and horse myoglobin (no Cys residues) with varying concentrations of dye. After labeling, the protein mix was subjected to SDS-PAGE, the fluorescent image was acquired, and the gel was stained with colloidal Coomassie blue. Figure 2 presents the result with three different dyes.

Figures 2A and B show that in the case of BODIPY TMR cadaverine IA the carbonic anhydrase band shows fluorescence at all dye concentrations used (0.5 –7.5 mM or a dye:thiol ratio of 20:1 to 300:1) (lanes 2–7). No fluorescence labeling of the lower myoglobin band is observed in the entire concentration range of dye used. The corresponding protein stain (Fig. 2A) shows equivalent amounts of both carbonic anhydrase and myoglobin in every lane. This demonstrates that BODIPY TMR cadaverine IA is highly specific for Cys proteins and does not label nonspecifically even at the highest ratio of dye:thiol used (300:1). Similar results were observed for BODIPY Fl C_1-IA (data not shown).

Figures 2C and D show the results of labeling carbonic anhydrase and myoglobin with varying concentrations of 5-TMRIA. At lower dye concentrations (up to 40:1 dye:thiol ratio) fluorescence labeling of only the carbonic anhydrase band is observed (lanes 1–3); however, as the dye concentration increases, fluorescence labeling of both carbonic anhydrase and the lower myoglobin band is observed (Fig. 2D, lanes 4–7) indicating nonspecific labeling at these dye:thiol ratios. Of further interest is that at dye:thiol ratios of >40:1 (Fig. 2D, lanes 4–7), where labeling of myoglobin is seen, the fluorescence in the carbonic anhydrase band decreases. It is not clear why the

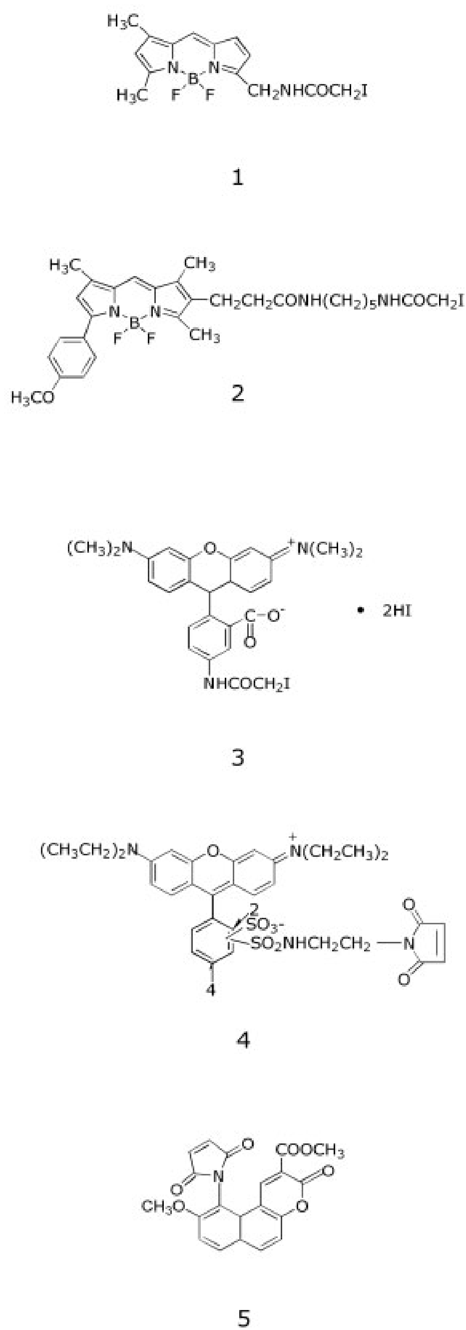

Figure 1. Structures of iodoacetamide and maleimide thiol-reactive dyes. (1) *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-yl)methyl) iodoacetamide (BODIPY® FL C_1-IA); (2) *N*-(5-((4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-*s*-indacene-2-propionyl)amino)pentyl) iodoacetamide (BODIPY® TMR cadaverine IA); (3) tetramethylrhodamine-5-iodoacetamide dihydroiodide (5-TMRIA); (4) Rhodamine Red™ C_2 maleimide; (5) 10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-methyl ester 3*H*-naphthol [2,1-b]pyran-*s*-carboxylic acid (ThioGlo I). Chemical structures of the dyes are based on structures provided by their respective manufacturers.

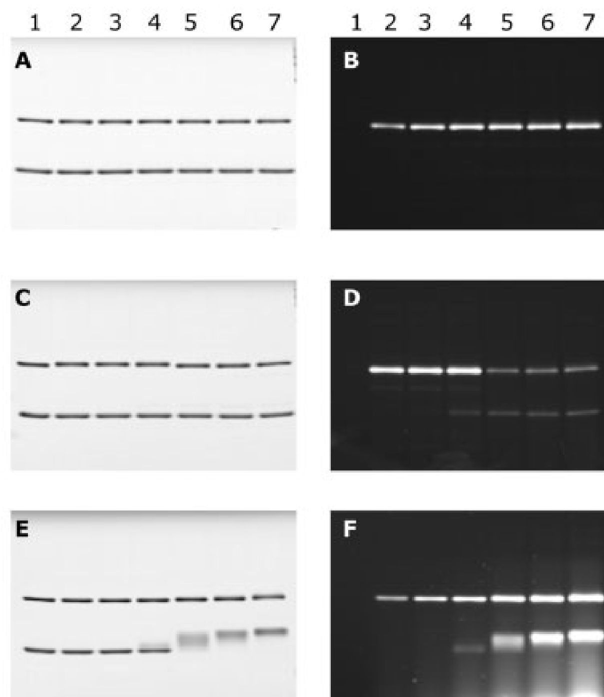

Figure 2. Specificity of thiol-reactive dyes for Cys proteins. A mixture of 25 μM enolase (upper band) and 50 μM myoglobin (lower band) was treated with a range of dye concentrations from 0.5–7.5 mM in 8 M urea, 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100 and 2 mM TCEP. The treated samples were quenched, subjected to SDS-PAGE, and the fluorescence images (B, D, F) and protein stains (A, C, E) of the gels were obtained. Panels (A) and (B) represent the data with BODIPY TMR cadaverine IA, (C) and (D) with 5-TMRIA, and (E) and (F) with ThioGlo I. In each gel, lanes 1–7 represent protein treated with 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 7.5 mM dye and correspond to dye:thiol ratios of 0, 20, 40, 80, 140, 200, and 300, respectively.

decrease in fluorescence of the carbonic anhydrase band should occur since we would expect 5-TMRIA to label carbonic anhydrase nonspecifically as well. It is possible that the decrease in the carbonic anhydrase band fluorescence may be due to fluorescence quenching with increasing amount of dye being incorporated, although other factors may be operative. It is clear from these results that labeling conditions for 5-TMRIA must be carefully optimized to prevent nonspecific labeling. We found eosin-5-iodoacetamide to be similar to 5-TMRIA in that nonspecific labeling was observed at higher dye:thiol ratios (data not shown).

Figures 2E and F show the result of labeling carbonic anhydrase and myoglobin with ThioGlo I, a maleimide dye. While there is no labeling of myoglobin at the lower dye concentrations, at higher dye concentrations ($>$ 40:1

dye:thiol) where strong labeling of carbonic anhydrase is obtained, there is also a significant labeling of the myoglobin band (lanes 4–7). The fluorescence of the carbonic anhydrase band continues to increase as expected, apparently due to labeling of non-Cys residues. No evidence of quenching is apparent for the ThioGlo I dye. In addition, the Coomassie-stained gel shows significant shifts in the apparent molecular weight of the myoglobin band as labeling is increased (Fig. 2E, lanes 4–7). This strongly suggests that ThioGlo I is nonspecific with increasing dye:thiol ratios, and caution is indicated for labeling with high dye:thiol ratios. Similar effects have also been observed with another maleimide dye, Rhodamine Red C_2 maleimide (data not shown).

3.2 Specificity of labeling Cys residues in a single protein

We next examined whether the more specific iodoacetamide exclusively labeled at Cys residues. Yeast enolase, which has a single Cys residue, was labeled with a thiol-reactive fluorescent dye, BODIPY Fl C_1-IA, at a 75:1 dye:thiol ratio. After labeling and cleanup, the labeled protein was digested with trypsin, and peptides were separated by RP-HPLC with UV and fluorescence detection. Comparison of the HPLC chromatogram with UV and fluorescence detection (Fig. 3, panel 1) showed that, of the several peaks seen in the chromatogram with 214 nm detection, only a single peak at 41.6 min was observed with fluorescence detection (Ex 485 nm, Em 502 nm, panel 3) or by visible detection (502 nm, panel 2). This suggests that a single peak/peptide was labeled with the fluorescent dye as expected. The fluorescent peak was collected and analyzed by MALDI-MS (Fig. 3, panel 4). The MALDI-MS analysis shows two peaks, one at m/z 1605.8 and another at *m/z* 1822.8. The peak at *m/z* 1605.1 corresponded to the mass of the peptide IGLDCASSEFFK from yeast enolase modified by a single BODIPY Fl residue. The other mass at *m/z* 1822.1, which co-eluted in this HPLC peak corresponds to a non-Cys peptide SGETEDTFIADLVVGLR from yeast enolase with no dye label attached. These data show that BODIPY-Fl C_1-IA was highly specific for and modified only the Cys residue on the protein, even at high dye:thiol ratios.

3.3 Effect of TCEP and other reducing agents on protein labeling

An important requirement for the effective labeling of Cys residues is reduction of the disulfide bonds. This is typically achieved by the use of reagents such as dithiothreitol, β-mercaptoethanol, TCEP, or tributylphosphine (TBP). TCEP has the advantage of being water-soluble, nonvolatile, and reduces disulfide bonds more rapidly at a broader pH range [15]. Hence, in our studies we chose to use TCEP as a reducing agent. While it is widely accepted that DTT and β-mercaptoethanol show reactivity with iodoacetamides and maleimides, it is generally thought that phosphines such as TCEP are nonreactive with thiol-reactive compounds and need not be removed prior to the use of alkylating reagents. Since reducing agents are essential to obtain labeling of proteins and proteomic samples we evaluated whether TCEP interferes with labeling of proteins by thiol-reactive dyes. For this study

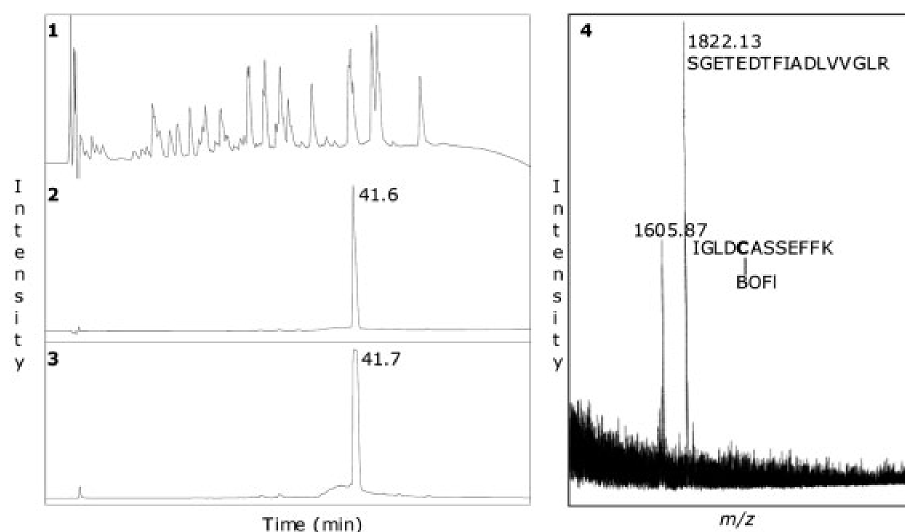

Figure 3. Specificity of BODIPY Fl C_1-IA for Cys residues. Enolase was labeled with BODIPY Fl C_1-IA at a dye:thiol ratio of 75:1 in a buffer of 8 M urea, 50 mM Tris-HCl (pH 7.5), 0.1% Triton and 1 mM TCEP. The treated protein was purified by gel filtration and the purified protein digested with trypsin. Approximately 250 pmol was analyzed by RP-HPLC. Panel (1) represents the HPLC chromatogram of the digest with UV detection at 214 nm, panel (2) with detection at 502 nm, and panel (3) with fluorescence detection (Ex 495 nm, Em 510 nm). Panel (4) represents the MALDI-mass spectrum of the collected fluorescent fraction seen at 41.7 min in the HPLC chromatogram.

we utilized yeast enolase, which has one Cys residue and no disulfide bonds, so that we could examine the effects on protein alkylation independent of the effects on protein reduction.

Yeast enolase was treated with BODIPY TMR cadaverine IA (Figs. 4A, B), BODIPY Fl C_1-IA (Figs. 4C, D) or Rhodamine Red C_2 maleimide (Figs. 4E, F) in the presence of 8 M urea and 0.1% Triton with concentrations of TCEP ranging from 0.5–10 mM. The gels were imaged using UV transillumination (Figs. 4B, D, F) and then stained with colloidal Coomassie blue (Figs. 4 A, C, E). While the protein stain shows equivalent amounts of protein in each lane (Figs. 5A, C, E), the fluorescence in the enolase band diminishes as the concentration of TCEP increases (Figs. 5B, D, F) for all three dyes. In the case of BODIPY TMR cadaverine IA, no influence on fluorescence is observed up to 1 mM TCEP (Fig. 4B, lanes 1, 2, 3), while at 2.5 mM and 10 mM TCEP there is a 58% (lane 4) and a 95% (lane 6) decrease in fluorescence, respectively. With BODIPY Fl C_1-IA, a similar inhibition of fluorescence-labeling of the enolase band is seen (Figs. 4C, D). A similar inhibition of fluorescence-labeling is seen when a maleimide dye, Rhodamine Red C_2 maleimide, is used for thiol alkylation. Figure 4E shows that, for an equivalent amount of protein, there is a constant decrease in band fluorescence with increasing amount of TCEP (Fig. 4F, lanes 1–6). Fluorescence quantification indicates an 80% decrease in intensity at 10 mM TCEP. These results demonstrate that increasing concentrations of TCEP inhibit labeling of proteins with both iodoacetamide and maleimide dyes. We next examined whether the decrease in labeling is due to a reaction between TCEP and thiol-reactive dyes.

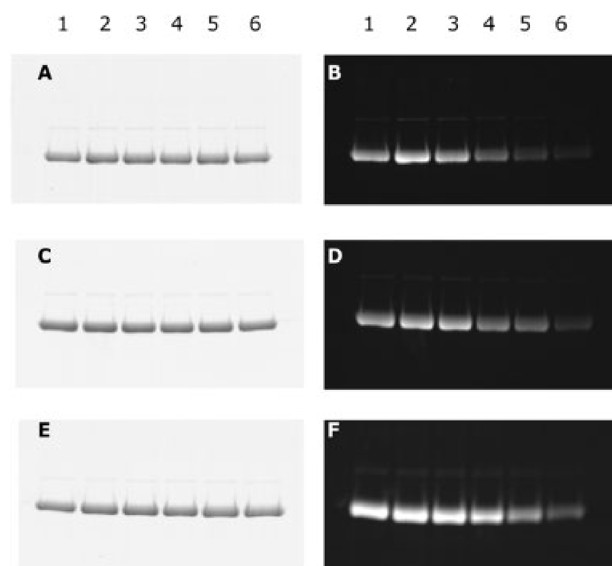

Figure 4. Alkylation of proteins with thiol-reactive dyes in the presence of TCEP. 25 μM enolase was alkylated with BODIPY TMR cadaverine IA, BODIPY® FL C_1-IA or Rhodamine Red C_2 maleimide with varying concentrations of TCEP in 8 M urea, 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100 for 2 h. Samples were subjected to SDS-PAGE, and images of the protein stain (A, C, E) and fluorescence (B, D, F) are presented. (A) and (B) represent the images with BODIPY TMR cadaverine IA, (C) and (D) with BODIPY Fl C_1-IA, and (E) and (F) with Rhodamine Red™ C_2 maleimide. Lanes 1–6 represent protein alkylation in the presence of 0, 0.5, 1.0, 2.5, 5, and 10 mM TCEP.

Iodoacetamide (BODIPY Fl C_1-IA and BODIPY TMR cadaverine IA) and maleimide (Rhodamine Red C_2 maleimide) dyes (0.5 mM) were treated with different concentrations of TCEP (0.5–5 mM) and the resultant mixtures were analyzed by RP-HPLC. The HPLC chromatograms in Fig. 5 show the results of the reaction of TCEP (0–2.5 mM) with the three different dyes. Figure 5A, panel 1 shows that in the absence of TCEP, a dominant dye peak at 34.47 min is seen for BODIPY TMR cadaverine IA. On treatment with a equimolar concentrations of TCEP and dye (0.5 mM), about 20% of the dye peak at 34.47 min is consumed, and a new peak, presumably corresponding to the dye-TCEP adduct, appears at 29.53 min (Fig. 5A, panel 2). On treatment with 2.5 mM TCEP (5 × over dye concentration), the dye peak at 34.47 min is completely consumed and only the TMR-TCEP adduct peak at

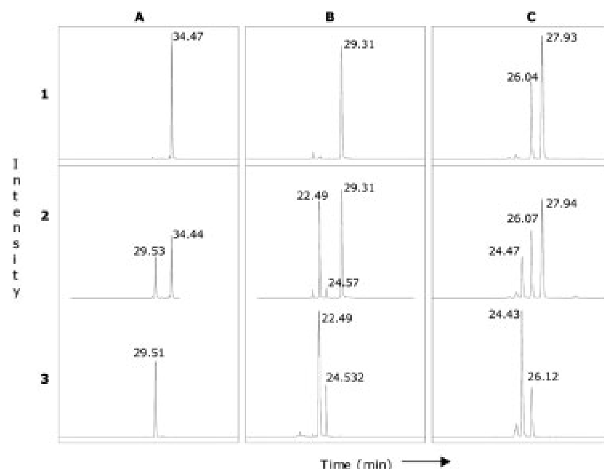

Figure 5. Interaction of thiol-reactive dyes with TCEP. 0.5 mM fluorescent dye was reacted with different concentrations of TCEP (0–5 mM) in 8 M urea, 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100 for 2 h. The mixture was then quenched with 0.1% TFA and an aliquot analyzed by RP-HPLC. (A), (B), and C depict the HPLC chromatograms with fluorescence detection of the reaction of BODIPY TMR cadaverine IA (Ex 546, Em570), BODIPY Fl C_1-IA (Ex 495, Em 510) and Rhodamine Red C_2 maleimide (Ex 560, Em 580 nm) with 0, 0.5, and 2.5 mM TCEP (panels 1–3, respectively).

29.51 min remains (Fig. 5A, panel 3). A similar result was observed for BODIPY Fl C_1-IA (Fig. 5B). The dye peak at 29.31 min (Fig. 5B, panel 1) is completely consumed on reaction with TCEP and two new adduct peaks appear, a dominant product at 22.49 min and another at 24.53 min (Fig. 5B, panel 3). These results demonstrate direct interactions between TCEP and iodoacetamide dyes and indicate depletion of the reactive dye species in the presence of TCEP.

Figure 5C shows the reaction of TCEP with a maleimide dye, Rhodamine Red C_2 maleimide. In panel 1, two peaks are seen in the chromatogram with no TCEP; these correspond to the main dye peak at 27.93 min and a second peak at 26.04 min (a possible hydrolysis product). On treatment with 0.5 mM TCEP the dye peak decreases, and a new peak at 24.43 min appears (panel 2). On treatment with 2.5 mM TCEP, the dye peak at 27.93 min is completely consumed and replaced by the new peak at 24.43 min (panel 3). The peak at 26.04 min is relatively nonreactive with TCEP compared to the main dye peak at 27.93 min. These results are consistent with the reaction of Rhodamine Red C_2 maleimide with TCEP and a decrease of the reactive dye species.

These results suggest that the reaction of TCEP with the iodoacetamide and maleimide dyes is competitive with Cys residues. This is consistent with our observation that the best labeling usually occurs at dye concentrations equal to or greater than TCEP concentration. It is important for the labeling of proteomic samples with thiol-reactive dyes that the concentration of reducing agent is such that maximum reduction is obtained and that the dye concentration is sufficient to overcome the inhibitory effects of the reducing agent. In our hands a typical ratio of ~9:1 for TCEP over thiol and ~1.125:1 of dye over TCEP (or 10:1 of dye over thiol) to be effective in reducing and labeling proteins that have multiple disulfide bonds, such as α-lactalbumin (data not shown).

3.4 Completion of labeling reaction

For differential proteomics, it is important that the labeling reaction goes to saturation or that it consistently goes to the same degree of completion, so that multiple spots for each protein are not observed and quantification is accurate. Saturation labeling may be verified either by saturation of band fluorescence or by mobility shifts of fully labeled protein in SDS-PAGE. Figure 6 shows the result of one such labeling experiment using BODIPY Fl C_1-IA. As each dye molecule adds a mass of 291 Da to the protein molecular mass, we expect that the partially labeled forms of the protein might be resolvable by SDS-PAGE and the fully labeled protein distinguished. Samples of

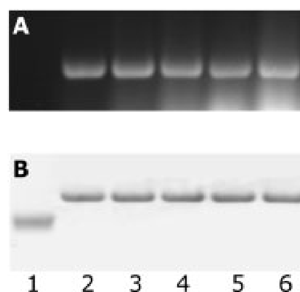

Figure 6. Degree of completion. 25 μM α-lactalbumin was reduced with 1 mM TCEP in 8 M urea, 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100 for 30 min and then alkylated for 90 min with varying ratios of dye (BODIPY Fl C_1-IA):thiol (lane 1, 0:1; lane 2, 3:1; lane 3, 6:1; lane 4, 12:1; lane 5, 18:1; and lane 6, 25:1). An aliquot of each sample was subjected to SDS-PAGE, and (A) the fluorescence and (B) protein stain images of the gel were obtained.

α-lactalbumin (containing 8 cysteine residues) were subjected to labeling with varying BODIPY Fl C_1-IA dye concentrations, and the product was analyzed by SDS-PAGE. The fluorescent image and protein stain are shown in Figs. 6A and B, respectively. The figure shows a banding pattern consistent with the unlabeled (lane 1) and a slower migrating, completely labeled α-lactalbumin (lanes 4, 5, and 6) as dye concentration is increased. In general it was observed that in a 90 min reaction time, complete labeling was achieved at a dye:thiol ratio of 10:1 and a TCEP:thiol ratio of 9:1.

3.5 Effect of denaturants and chaotropes on protein labeling

Detergents and chaotropes are essential components of solubilization cocktails that ensure maximum recovery and complete denaturation of proteins. Hence compatibility of thiol-reactive dyes with commonly used chaotropes such as urea and thiourea is important in the labeling of proteomic samples. Figure 7 shows the influence of detergents and chaotropes on thiol-labeling of proteins. Yeast enolase and bovine α-lactalbumin were labeled with BODIPY TMR cadaverine IA in the presence of chaotropes and detergents and then analyzed by SDS-PAGE. Figure 7A shows the protein stain of the gel while Fig. 7B shows the fluorescence image of the same gel. The results show that efficient labeling of both enolase and α-lactalbumin takes place in the presence of 8 M urea with and without CHAPS (lanes 2 and 4). In the case of α-lactalbumin one can also see the shift of the band to a slower migrating, completely labeled product (lanes 2 and 4 *vs.* lane 1 and 3).

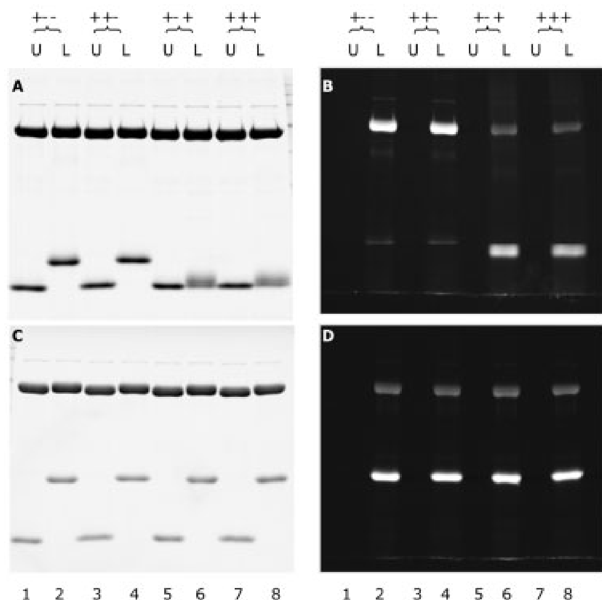

Figure 7. Effect of detergents and chaotropes on protein alkylation with thiol-reactive dyes. A mixture of 25 μM each of enolase (upper band) and α-lactalbumin (lower band) was reduced in a buffer containing 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100 and 1 mM TCEP along with 8 M urea (lanes 1, 2), 8 M urea, 2% CHAPS (lanes 3, 4), 2 M thiourea, 7 M urea (lanes 5, 6), and 2 M thiourea, 7 M urea, and 2% CHAPS (lanes 7. 8). Of these the samples in lanes 2, 4, 6, 8 were alkylated with BODIPY TMR cadaverine IA (panels A, B) or Rhodamine Red C_2 maleimide (panels C, D) for 1.5 h; the others (1, 3, 5, 7) served as unlabeled controls. The samples were subjected to SDS-PAGE, and the protein stain (panels A, C) and fluorescence (panels B, D) images of the gel were obtained. U and L refer to unlabeled and labeled samples, respectively.

Labeling in the presence of 2 M thiourea/7 M urea, however, results in decreased fluorescence intensity of the enolase band (Fig. 7B, lanes 6 and 8) compared to labeling in the absence of thiourea (lanes 2 and 4). The band intensity of enolase labeled in the presence of thiourea is only 25% of the intensity of the band labeled in its absence. Similarly for α-lactalbumin we see reduced and incomplete labeling in the presence of thiourea (lanes 6 and 8) *vs.* labeling in urea or urea/CHAPS mixtures. This is also clearly seen in the Coomassie-stained gel (Fig. 7A) where the lower α-lactalbumin band labeled in the presence of urea or urea/CHAPS moves to a slower migrating, completely labeled product (lanes 2 and 4). In the samples labeled in the presence of thiourea, one or more indistinct bands of intermediate apparent MW are observed, suggesting partial labeling of α-lactalbumin under these conditions (lanes 6 and 8). Studies with BODIPY Fl C_1-IA also demonstrate similar results (data not shown). Our results thus corroborate the observation by Galvani *et al.* [20] that inhibition of alkylation with iodoacetamide occurs in the presence of thiourea. We did not observe any significant effects on thiol labeling from detergents (Triton X-100 or sulfobetaines) in the labeling mixture (data not shown).

We extended this study to examine whether labeling by maleimide dyes was also inhibited in the presence of thiourea. Again enolase and bovine α-lactalbumin were labeled in the presence of detergents and chaotropes with a maleimide dye (Rhodamine Red C_2 maleimide or BODIPY FL-N-(2-aminoethyl)maleimide) and then analyzed by SDS-PAGE. The results obtained with Rhodamine Red C_2 maleimide are shown in Figs. 7C and D. To our surprise, under all conditions tested (urea, urea/CHAPS, urea/thiourea, and urea/thiourea/CHAPS), efficient fluorescence labeling of both enolase and α-lactalbumin was obtained (Fig. 7D, lanes 2, 4, 6, 8). This is also supported by the protein stain of the gel, where we see the shift of the lower α-lactalbumin band in the control to a slower migrating more completely labeled product under all four conditions (Fig. 7C, lanes 2, 4, 6, and 8). Similar results were obtained for BODIPY FL-N-(2-aminoethyl)maleimide, showing that labeling by this maleimide dye was not affected by thiourea (data not shown). Thus the data suggest that, unlike iodoacetamide dyes, labeling by maleimide dyes appears to be unaffected by thiourea. Hence, for samples where the use of thiourea in the solubilization cocktail is essential, labeling with maleimide dyes may be a better option.

3.6 Effect of thiol labeling on protein isoelectric points

In order to examine the suitability of thiol-reactive dyes for labeling proteomic samples we next examined the effects of labeling on the p*I* of proteins. Table 1 summarizes the results of various labeling experiments. In these experiments, the proteins were labeled with the dyes indicated under optimized conditions, purified by gel filtration, and the isoelectric points of the labeled proteins were measured by comparing them to the migration of standard proteins in IEF gels. The table shows that for several of the proteins labeled (some of which had multiple Cys residues), alkylation with BODIPY Fl C_1-IA or BODIPY TMR cadaverine IA did not significantly alter the p*I* of the protein. In some cases, *e.g.*, soybean trypsin inhibitor, a small shift in the p*I* of the labeled protein (5.09) *vs.* unlabeled protein (4.61) is observed. In general, we found that where p*I* shifts were observed, the shifts were similar or identical for both BODIPY dyes. A more detailed study with several different standard proteins or complex mix-

Table 1. Isoelectric points of standard proteins labeled with thiol-reactive dyes

Protein	p*I* of unmodified protein	p*I* of BODIPY Fl C_1-IA adduct	p*I* of BODIPY TMR cadaverine IA adduct
Ovalbumin (chicken egg)	4.55	4.58	4.58
Trypsin inhibitor (soybean)	4.61	5.09	5.09
β-Lactoglobulin A (bovine)	5.20	5.04–5.34	5.12–5.32
Carbonic anhydrase I (human)	6.44	6.28	6.36
Carbonic anhydrase II (human)	7.4	7.4	7.4

Proteins were labeled with thiol-reactive dyes as described in Section 2.2 and then purified by gel-filtration. Isoelectric points were obtained from IEF gels by comparison to standards.

tures with the thiol-reactive dye of choice should be performed to determine the effect of labeling on protein p*I* for the particular dye.

In addition to potential shifts in p*I* of proteins, alkylation of Cys residues with dyes can also give rise to apparent mass shifts of the proteins when analyzed by gels. This shift is more observable in smaller proteins with multiple Cys residues when run in high percentage gels, *e.g.*, for BODIPY Fl C_1-IA-labeled α-lactalbumin (Figs. 6A and B). The smaller dyes are better in this regard since they give rise to smaller apparent mass shifts when compared to larger, bulkier dyes. However, as long as complete or at least consistent labeling is obtained, the shifts in apparent mass should not matter if the samples to be compared are treated identically.

3.7 Compatibility with MALDI-MS

In order to determine compatibility with MALDI-MS, in-gel tryptic digestion was performed on enolase covalently labeled with BODIPY Fl C_1-IA, BODIPY TMR cadaverine IA, or Rhodamine Red C_2 maleimide. Proteins were run on SDS-PAGE and excised without fixing or staining by visualizing under UV. After in-gel digestion, peptides were analyzed by MALDI-MS. High quality mass fingerprints were obtained for all three labeled preparations. All peptide masses were identified as belonging to yeast enolase I (Fig. 8). The figure shows that for all three dyes the peptide profiles are virtually identical except for differences in relative intensity. Submission of the masses obtained to the SWISS-PROT database using the MS-Fit program showed yeast enolase I to be the primary protein identified with about 43% sequence coverage obtained. No artifactual modifications or nonspecific labeling of any of the peptides with any of the dyes was observed. Our results demonstrate that the thiol-reactive dyes we used showed good compatibility with MS methods, no nonspecific labeling, and did not interfere with the release of non-Cys containing peptides.

4 Discussion

The use of neutral fluorescent thiol-reactive dyes for covalent labeling of Cys residues in proteins presents a powerful approach for proteomic analysis, allowing highly sensitive detection of proteins, accurate quantification, largely unaltered p*I*, and no post-separation staining and destaining, thereby leading to good recovery by in-gel digestion for MS and protein identification. While a large number of thiol-specific dyes is commercially available, each dye needs to be investigated thoroughly with regard to its suitability for labeling proteomic samples.

For many thiol-alkylating reagents specificity is taken for granted, and a number of examples exist in the literature where activity-based studies using dyes are performed with the apparent assumption that modification is specific. For the purpose of labeling proteomic samples, specificity is even more critical since, at a minimum, nonspecific labeling can adversely affect quantitative comparisons of proteins as well as identification of proteins by MS.

Our studies show that while most thiol-reactive dyes are specific for Cys residues at a 10–40-fold excess over thiol groups, higher ratios can in some cases result in nonspecific labeling. Of the iodoacetamide dyes, BODIPY-Fl-C_1-IA and BODIPY-TMR cadaverine were highly specific for Cys residues at all ratios used and showed the greatest suitability for labeling proteomic samples under our criteria. However, other iodoacetamide dyes such as 5-TMRIA and eosin-5-iodoacetamide as well as maleimide dyes ThioGlo I and Rhodamine Red C_2 maleimide showed nonspecific labeling at high dye concentrations (>40-fold excess over thiol) emphasizing the importance of careful consideration of the dye: thiol ratios used in the labeling mixtures. Nonspecific labeling by some of the iodoacetamides in the absence of free thiols has been attributed to the labeling of methionine, tyrosine or histidine residues on proteins [21]. Maleimides on the other hand are more reactive towards amine residues, and at higher pH's this reaction is favored [22, 23]. However, hydrolysis of maleimides to a mixture of isomeric maleamic acids can compete significantly with thiol modification particularly above

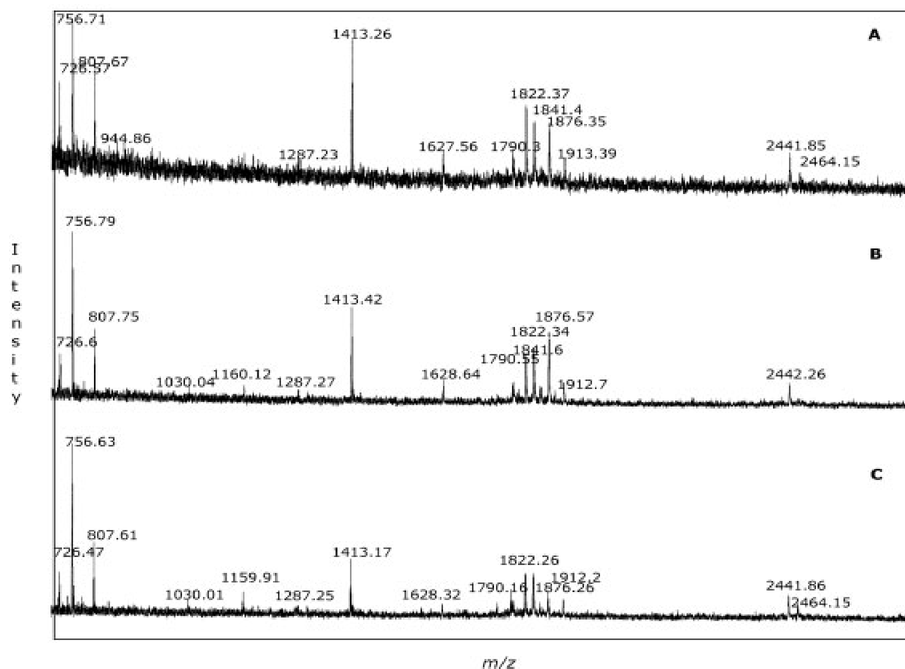

Figure 8. Compatibility of covalent labeling by thiol-reactive dyes with protein identification by MALDI-MS. Yeast enolase I was labeled with thiol reactive dyes and the protein subjected to 14% SDS-PAGE. The figure shows the MALDI-mass spectrum of peptide profiles generated by in-gel digestion of protein labeled with (A) BODIPY Fl C_1-IA, (B) BODIPY TMR cadaverine IA, and (C) Rhodamine Red C_2 maleimide.

pH 8 [24, 25]. Thus careful attention must be given to the use of maleimide dyes in protein labeling, both with respect to the dye:thiol ratio and the pH of the labeling reaction, in order to specifically label Cys residues.

In considering conditions necessary to obtain optimum protein labeling we demonstrate that the concentration of reducing agents such as TCEP and DTT is very important. While the interaction of DTT with iodoacetamides and maleimides is widely accepted, the data concerning TCEP are not so clear. In the study of Getz *et al.* [16] using TMR-IA and TMR-maleimide, inhibition of labeling of a myosin fragment by TCEP or DTT was demonstrated. Since, in our hands, 5-TMRIA appears to label relatively nonspecifically (Figs. 2C and D), it is not clear in the studies of Getz *et al.* whether the effect of TCEP on labeling is due solely to its effects on alkylation of thiols. Shafer *et al.* [17] demonstrated the depletion of TCEP with differing amounts of iodoacetamide and maleimide. While their data are suggestive of interactions between TCEP and iodoacetamide and *N*-ethylmaleimide, these findings do not necessarily imply reduced alkylation of proteins by these reagents in the presence of TCEP.

Our results show that protein alkylation by both iodoacetamide and maleimide dyes is inhibited with increasing concentrations of TCEP and that the reactive dye molecule forms a new species on interaction with TCEP. Thus, reducer concentrations must be carefully chosen to ensure optimum reduction of proteins and maximum alkylation. This may be accomplished in many cases by removing reducing agents prior to alkylation or by using enough dye to overcome the competition from the reducer. In our hands, approximately equimolar concentrations of TCEP and dye, in approximately 10–20-fold excess over protein thiols have worked well with most dyes. Nevertheless, these conditions are general and should be optimized for each individual dye and reducing condition.

Detergents (*e.g.*, CHAPS, Triton, and sulfobetaines – SB-12, SB-14 and SB-3) and chaotropes (urea, thiourea) are essential components of solubilization cocktails for proteomic samples, required for maximal recovery and complete denaturation of proteins. While all of the above detergents were compatible with the labeling process, our results demonstrate that protein labeling by all of the iodoacetamide dyes tested was strongly inhibited by the presence of thiourea. This corroborates the results observed by Galvani *et al.* [20] that direct interaction of thiourea and iodoacetamide occurs, resulting in diminished protein labeling.

Our studies demonstrate that for maleimide dyes Rhodamine Red C_2 maleimide and BODIPY Fl maleimide, no inhibition of protein labeling in the presence of thiourea was observed. Hence, for samples where the presence of thiourea in the solubilization cocktail is essential, maleimide dyes may be the labeling method of choice to obtain efficient Cys labeling. This finding may also be relevant to the nonfluorescent alkylation of proteomic samples before electrophoresis (including the first-dimensional IEF/IPG separation) where *N*-ethylmaleimide could potentially be used in place of iodoacetamide to obtain derivatization of free sulfhydryl groups.

Labeling of proteins with at least two iodoacetamide dyes, BODIPY-Fl and BODIPY-TMR, was evaluated with regard to the effect on protein p*I*. Our results demonstrate that the p*I*'s of several proteins (p*I*'s 4.5–8.0) were mostly unchanged except in a few cases where minimal shifts were observed. These observations further point to the advantage of modifying Cys residues, which are a minority in amino acid composition, but are highly reactive. Even for dyes that produce a significant shift in p*I* of the protein on modification, fluorescence labeling is still useful as long as the shifts are reproducible, since protein identification is based on peptide mass fingerprinting. However, in labeling with thiol or other reactive dyes it is important that conditions be optimized such that saturation/complete labeling is achieved to ensure complete or consistently labeled product. Incomplete or inconsistent labeling risks ambiguous quantification, and the potential for the creation of artifactual trains of underalkylated proteins. Our future studies will focus on labeling complex lysates with thiol-reactive dyes and further evaluation of their effects on protein isoelectric points.

We also evaluated the recovery of peptides from covalently labeled proteins after in-gel digestion of separated proteins. Covalent prelabeling methods are extremely useful in this regard since there are no time-consuming fixing and staining steps after protein separation by SDS-PAGE. This also avoids harsh chemicals and detergents (*e.g.*, formaldehyde, glutaraldehyde, Tween 20) that frequently interfere with peptide identification *via* MS. Samples can be directly visualized using standard UV transilluminators and the desired bands imaged and excised for in-gel digestion. It has been reported that monobromobimane-labeled proteins gave poor peptide mass profiles and that the dye is not compatible with MS-based identification methods [2]. In contrast with the thiol-reactive dyes we evaluated, no artifactual modifications were seen, and thiol-labeling was found to be compatible with MS-based identification methods. A more detailed study on peptide mass fingerprints from a greater number of proteins with multiple Cys residues needs to be undertaken along with determination of the efficiency of release from dye labeled proteins.

In conclusion, fluorescence detection technologies will play an increasingly important role in the visualization and identification of proteins for proteomic analyses. Covalent derivatization with neutral thiol-reactive dyes exhibits good specificity for Cys residues under the right conditions, has minimal effects on the p*I*'s of proteins, and can provide precise quantification due to saturation labeling. Furthermore, thiol-labeling can be specific, sensitive, and compatible with commonly available imaging equipment and in-gel digestion for identification by peptide mass fingerprinting. While fluorescence preseparation labeling has its great advantages, its implementation in proteomic analyses requires considerable attention to the potential for nonspecific labeling, and demands systematic optimization.

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