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Selective disulfide reduction for labeling and enhancement of Fab antibody fragments

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Abstract

Many methods have been developed for chemical labeling and enhancement of the properties of antibodies and their common fragments, including the Fab and F(ab')₂ fragments. Somewhat selective reduction of some antibody disulfide bonds has been previously achieved, yielding antibodies and antibody fragments that can be labeled at defined sites, enhancing their utility and properties. Selective reduction of the two hinge disulfide bonds present in F(ab')₂ fragments using mild reduction has been useful. However, such reduction is often not quantitative and results in the reduction of multiple disulfide bonds, and therefore subsequent multiple labeling or conjugation sites are neither homogenous nor stoichiometric. Here, a simple and efficient selective reduction of the single disulfide bond linking the partial heavy chain and the intact light chain which compose the Fab fragment is accomplished utilizing tris(2-carboxyethyl)phosphine (TCEP) immobilized on agarose beads. The resultant reduced cysteine residues were labeled with several cysteine-selective fluorescent reagents, as well as by cysteine-directed PEGylation. These two cysteine residues can also be re-ligated by means of a bifunctional cysteine cross-linking agent, dibromobimane, thereby both restoring a covalent linkage between the heavy and light chains at this site, far removed from the antigen binding site, and also introducing a fluorescent probe. There are many other research and clinical uses for these selectively partially reduced Fab fragments, including biotinylation, toxin and drug conjugation, and incorporation of radioisotopes, and this technique enables simple generation of very useful Fab fragment derivatives with many potential applications.

Keywords

Monoclonal antibody; Selective disulfide reduction; Fab fragments; TCEP agarose; Sulfhydryl modification; Antibody conjugation

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Conflict-of-interest and financial disclosure statement

Dr. Norman is named as a co-inventor on a patent application for the use of the h2E2 humanized anti-cocaine monoclonal antibody.

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1. Introduction

The covalent linkage of probes, drugs, toxins, radioisotopes, and stabilizing agents to antibodies and their fragments has many research and clinical applications. Optimally, this labeling would be well-defined in terms of not only stoichiometry, but also location(s) of labels introduced, as well as not interfering with the function(s) of the antibody or antibody fragment. Cysteine residues are the most reactive nucleophiles in proteins, so in general, they are the best option for site-selective covalent labeling of antibodies. However, most antibodies and antibody fragments have no reduced cysteine residues (no free sulfhydryls), only disulfides (cystines). Thus, cysteine residues need to be either introduced by manipulation of the encoding DNA, or generated by reduction of existing disulfide bond(s). Therefore, somewhat selective, partial reduction of specific disulfide bonds in intact antibodies, and in $F(ab')_2$ fragments, have been previously described [1–4]. However, the procedures generating these modified antibodies for labeling seldom yield either totally selective, or quantitative, disulfide bond reduction, and usually involve partial reduction of multiple disulfide bonds. This leads to labeling heterogeneity and labeling at more than 2 cysteine residues (i.e., at multiple disulfide bond sites).

Fab fragments have better tissue penetration, and are cleared from the blood *in vivo* much faster than the larger intact mAbs, and thus may be a better way to selectively deliver radioisotopes, toxic drugs, and toxins to the antigen site than intact antibodies, by increasing access of the toxins to the intended site of delivery, while at the same time limiting the whole body exposure to these noxious compounds. Using the Fab fragment that is very efficiently generated from our h2E2 humanized anti-cocaine mAb by Endo-Lys C digestion [5], we have investigated the use of several protein disulfide reductants under several conditions to examine the feasibility to selectively reduce the single disulfide bond linking the light chain to the heavy chain fragment (composing the Fab fragment). These reductants included 2-mercaptoethylamine (2-MEA), which has been commonly used for partial reduction of the 2 hinge region disulfide bonds in intact mAbs and, more commonly, in $F(ab')_2$ antibody fragments [6]. In this work, we targeted the single inter-chain disulfide bond present in the Fab fragment for selective reduction, since it is predicted to be on the surface of the protein, and also located at the opposite end of the Fab fragment from the antigen (cocaine) binding site, making it both likely to be solvent accessible, and unlikely to be important for antigen binding.

We were not able to achieve partial, selective reduction of that single disulfide bond with DTT, 2-MEA or TCEP reductants in solution, but found that using a suitable amount of TCEP reductant bound to agarose beads did allow very selective and nearly quantitative reduction of this single disulfide bond. Furthermore, this selective reduction could be carried out in a variety of different buffers at a large range of pHs, using convenient experimental conditions (overnight incubation at ambient temperature), yielding similar results under most conditions tested. This is consistent with what is known of the broad range of pH conditions under which TCEP is effective for reducing protein disulfide bonds [7]. The selectively reduced Fab fragment can then be rapidly separated from the TCEP reducing beads by filtration centrifugation, and then immediately either assayed for the concentration of the generated reduced cysteine, or alkylated using a variety of reagents, with no buffer

exchange being required prior to initiation of alkylation or conjugation. The alkylation or conjugation reactions can then be terminated by adding excess cysteine, and/or by immediate application of the samples to a size exclusion column to remove excess reagents and exchange the reaction buffer to whatever buffer is desired for subsequent steps of analyses or applications for the selectively labeled Fab fragment.

The reduction and labeling stoichiometry of the resultant modified Fab fragments can be quantified by colorimetric or fluorometric assays for cysteine and incorporated labels, and also analyzed by SDS-PAGE and mass spectrometry to demonstrate the selectivity of the reduction of the targeted disulfide bond, as well as the extent, if any, of the reduction of the 4 other disulfide bonds present in the Fab fragment. This novel and efficient combination of well-established methods should be applicable to a broad range of monoclonal and polyclonal antibody Fab fragments, and will be useful as a general way to add covalent labels, drugs and toxins to Fab fragments while maintaining their antigen affinity.

2. Materials and methods

2.1. Materials

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, cat # 20490) and agarose bound Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-agarose, cat # 77712), were obtained from Thermo-Scientific Pierce. Iodoacetamide (IAM, cat. # I-6125) was from Sigma. 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F, cat # A5597) was from TCI America, while fluorescein 5-maleimide (F-MAL, cat # F-150) was purchased from Molecular Probes, and dibromobimane (DBB) was from Anaspec, Inc. (cat # AS81448). Maleimide-polyethylene glycol (5000 Da PEG chain, MPEG-MAL-5000) was from Nektar Transforming Therapeutics. The 0.22 μ m 500 μ L spin filters were from EMD Millipore (cat. # UFC30GV0S), and the 10,000 MWCO Vivaspin 500 μ L concentrators (cat. # 28-9322-25) were from GE Healthcare. To separate iodoacetamide from the Fab fragment after reduction and alkylation for mass spectral analysis, Sephadex G-25-80 size exclusion beads were from Sigma, and disposable columns were from BioRad (Econo-Pac 14 cm-high 1.5 $\times$ 12 cm polypropylene columns, cat. # 7321010EDU), equilibrated with 0.1% formic acid just before use, as previously described [8]. Tris base, NaCl, EDTA, and other buffer components, as well as sequencing grade concentrated formic acid, were from Fisher Scientific.

2.2. Methods

Fab fragment was generated and purified from the h2E2 anti-cocaine mAb as described previously [5], using Endoproteinase Lys-C for proteolysis (rather than papain). Partial selective reduction of 1.0 mg/ml (0.218 μ M) Fab fragment was performed in 100–1000 μ L samples in a variety of buffers containing 5 mM EDTA and 1/10 volume of an approximately 50% slurry of TCEP-agarose in water, either as supplied by the manufacturer, or after washing the commercial beads 3–4 times with pure water. At the time of use, the 50% TCEP-agarose bead slurry was measured to be equivalent to approximately 3.5 mM TCEP (equivalency assay performed as described in the manufacturer's product description), thus resulting in an equivalent of about 0.35 mM TCEP in the immobilized TCEP Fab reduction reaction samples. The TCEP agarose beads were also measured for their reducing

capacity after filtration of the bead solution through 0.22 μm spin filters, and resuspension of the beads with pure water. In all cases, the TCEP bead reduction reaction samples were incubated at ambient temperature (22 $^{\circ}\text{C}$) with gentle end-over-end mixing for various times.

After rapid removal of the TCEP agarose by centrifugation through 0.22 μm spin filters, the partially reduced samples were either assayed for cysteine content using Ellman's reagent (DTNB) for 15 min at ambient temperature (extinction coefficient of the resultant 2-nitro-5-thiobenzoate product is $14,150\text{ M}^{-1}\text{ cm}^{-1}$ at 412 nm), or immediately alkylated or conjugated by addition of a concentrated solution of alkylation reagent. After 30 min (for most reactions) at room temperature, the alkylation reaction was either terminated by addition of excess cysteine, or the Fab fragment separated from excess reagents and buffer exchanged into phosphate buffered saline (PBS) by size exclusion chromatography at room temperature. For samples analyzed by mass spectral methods, the partially reduced Fab fragment devoid of TCEP agarose was treated by addition of freshly dissolved iodoacetamide (IAM) to a final concentration of 20 mM, and alkylated for 30 min at 22 $^{\circ}\text{C}$ in the dark. Immediately following the alkylation, all excess reagents were removed and the buffer was exchanged at 22 $^{\circ}\text{C}$ for 0.1% formic acid by application of the sample to a 4–5 ml G-25 column, poured in water, and then equilibrated with 0.1% formic acid just before use. The absorbance of each 0.5 ml fraction (280 nm) was determined and the fractions containing Fab fragments were pooled and concentrated using 10,000Da molecular weight cut off (MWCO) spin filters. After concentrating to a volume of approximately 30–50 μL , a 5 μL aliquot was diluted 20-fold for determination of protein concentration (using an extinction coefficient of $73,965\text{ M}^{-1}\text{ cm}^{-1}$ for the Fab fragment, equivalent to Fab concentration of $1.0\text{ mg/ml} = 1.612$ absorbance at 280 nm). SDS-PAGE analysis of about 0.5–1.0 μL of each preparation was performed, with or without additional reduction with DTT, to check for the desired reduction of the inter-chain disulfide, which results in the appearance of 2 bands near 25 kDa, in the absence of DTT reduction prior to electrophoresis.

The IAM alkylated Fab samples were analyzed by electrospray ionization time-of-flight mass spectrometry (ESI-Tof MS), as described previously [8]. Briefly, samples were diluted by transferring 1 μL of the concentrated sample in 0.1% formic acid into 19 μL of 50% methanol/0.1% formic acid. The mass spectral (MS) system was set up for loop injection with a constant flow of 500 nL/min in 50% methanol/0.1% formic acid using a 2 μL loop, thus producing about a 4 min mass spectral profile for each sample. Spectral data were processed using the protein reconstruct algorithm in the PeakView ver 2.1 software from Sciex to generate the reconstructed mass profiles.

In other experiments, the TCEP gel partially reduced Fab fragments prepared by approximately 20 h of ambient temperature TCEP-agarose reduction were also alkylated with 1 mM F-Mal, 0.5 mM ABD-F, 0.1 mM dibromobimane, or 0.1 mM MPEG-MAL-5000 using the methods described above, terminating the reactions by loading the samples onto Sephacryl S-100 size exclusion columns equilibrated with PBS buffer.

3. Results

After investigating various disulfide reducing reagents under several conditions in attempts to get selective reduction of the single inter-chain disulfide present in the Fab fragment of the anticocaine h2E2 monoclonal antibody, the only successful strategy tested involved using TCEP bound to agarose beads. This makes sense since this disulfide bond, unlike the 4 intra-chain disulfide bonds present in the Fab fragment, is predicted to be solvent exposed in crystal structures, and the bead-bound reductant would not be likely to have access to any Fab cystine residues that are not on the exposed surface of the molecule. Fig. 1 shows the time course of selective reduction of the Fab fragment by the TCEP agarose beads at ambient temperature (22 °C) in 100 mM Tris-HCl, 5 mM EDTA, pH = 8.0. As seen in the filled-in squares, the results are reproducible, and approximated by a power function fit (dashed line). Also shown (filled triangles) are multiple experiments in which the selectively reduced samples were subsequently alkylated with various sulfhydryl selective reagents under the same buffer conditions. Not shown is an experiment using pH = 6.8 Tris-HCl, 5 mM EDTA buffer, which yielded very similar results (1.99 cys/Fab after 23 h reduction at ambient temperature). Other experiments (also not shown) demonstrated that overnight incubation under the same buffer conditions at 4 °C resulted in much less than the 2.0 cys/Fab expected for a single selectively and stoichiometrically reduced disulfide. Fig.1 also contains data obtained using the TCEP agarose (in water) as supplied by the manufacturer, i.e., not washed with water prior to use (open circles). As can be seen, there appears to be rapid reduction of more than 2.0 cys/Fab. However, this is most likely due to direct reaction of free, solution phase, TCEP (released from the TCEP agarose upon storage at 4 °C) with the DTNB reagent used to quantify free sulfhydryls, since about 8% of the reducing activity of this material passed through a 0.22 µm filter, and the total reducing activity of the supplied 50% gel bead slurry was equal to the filter-retained beads plus the soluble filtrate activities (not shown). Thus, it is important to wash the TCEP agarose with water multiple times to eliminate any free, soluble TCEP prior to use.

The stoichiometry data in Fig. 1 suggest, but do not show directly, that the single disulfide connecting the heavy chain fragment with the light chain in the Fab was selectively reduced. To address this question directly, the selectively reduced Fab was analyzed by non-reducing SDS-PAGE (with *N*-ethylmaleimide in the sample buffer to prevent any disulfide exchange or re-oxidation during heating of the samples before electrophoresis). Samples from one of the two time course experiments using washed TCEP agarose (see Fig. 1, filled squares) were analyzed in this fashion, and the results are shown in Fig. 2. As is evident by the disappearance of the approximately 45 kDa intact Fab band, the targeted disulfide bond is almost completely reduced by 24 h, when the cys/Fab stoichiometry was about 2.4 to 1, suggesting selective reduction of the intended disulfide bond, with only slight reduction of any of the 4 intra-chain disulfides.

To confirm and further investigate the selectivity of reduction of the single Fab disulfide bond, the selectively reduced Fab was alkylated with IAM, following our established procedures for quantitative alkylation and preparation of the sample for mass spectral analyses [8]. Two independent samples were analyzed on different days, sample 1 was reduced for 24 h, and had 2.7 cys/Fab, while sample 2 was reduced for 17 h and had 1.9

cys/Fab fragment prior to alkylation. In Fig. 3, the mass spectral results for both samples are overlaid for ease of comparison and to show reproducibility (and the non-reduced SDS-PAGE results for these 2 samples prior to mass spectral analyses are shown in Fig. 3 inset A). Assuming only the single target disulfide is reduced (and subsequently quantitatively alkylated by IAM), the theoretical masses were calculated as follows:

Light chain (LC) = 22,748.6 (for the 215 amino acids) – 17.03 (for the *N*-terminal Gln to pyroGlu [8]) – 4.04 Da (4×1.01 (for 2 intact internal disulfides)) + 57.05 (from one IAM) = 22,784.6 Da (compared to the observed mass of the main peak seen in Fig. 3 of 22,785.9 Da, they differ by + 1.3 Da).

Heavy Chain fragment (HC) = 23,143.96 (for the 218 amino acids) – 4.04 Da (4×1.01 (for 2 intact internal disulfides)) + 57.05 (IAM) = 23,197.0 (compared to the observed mass of the main peak seen in Fig. 3 of 23,197.8 Da, they differ by + 0.8 Da).

If one intra-molecular LC or HC disulfide was non-selectively reduced (and subsequently alkylated with IAM), then an additional species of +116.12 Da would be observed (+116.12 Da = 2×1.01 Da for cys reduction + 2×57.05 Da for IAM alkylation). There is a small light chain peak observed in both samples at 22903.6 Da, which is close (+1.6 Da) to the theoretical size of 22902.0 Da (22785.9 + 116.12). In addition, there is also a small HC peak observed in both samples at 23316.4 Da, which is somewhat close (+2.5 Da) to the predicted mass of 23313.9 (23197.8 + 116.12) which would be expected if one HC intra-chain disulfide had been partially reduced. Both of these potential nonselective reduction/alkylation peaks are indicated by asterisks in Fig. 3. In addition, a signal consistent with the higher level of the intact Fab in sample 2 is detected at 22935.4 (# symbol). This signal represents a common artifact when deconvoluting charge-state spectra into narrow mass ranges in which the even charges states for a higher mass protein are represented by a peak at one half the actual mass (in this case the intact Fab is 45871.4 Da—see inset B). This mass is consistent with one heavy and one light chain each with 2 internal disulfides and the Gln to pyroGlu for the LC for a calculated mass of 45867.5.

To demonstrate a few of the many potential applications for the selectively reduced Fab fragments, various alkylating and conjugation reagents were reacted with the selectively reduced Fab fragments. The sulfhydryl selective fluorescent reagents, fluorescein 5-maleimide (F-Mal) and 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F), as well as the PEGylation reagent maleimide-polyethylene glycol 5000 (MPEG-MAL-5000), and the bifunctional cross-linking sulfhydryl reactive reagent dibromobimane (DBB), which both reforms a covalent bond between the reduced cysteine residues, as well as introduces a single fluorophore where the disulfide bond had been present, were used. No attempts were made to optimize these alkylation/conjugation reactions, they were only evaluated as examples of possible downstream reactions that make the selectively reduced Fab fragment suitable for a variety of applications. In addition, it is noted that some of the reactions included in Fig. 4 were performed at varying pHs and under very different buffer conditions (e.g., the ABD-F labeling was done in a borate buffer (50 mM borate/5 mM EDTA, pH = 8.0), while the F-Mal and Mal-PEG reductions and alkylations were done using MOPS buffer (50 mM MOPS/5 mM EDTA, pH = 7.4)). All these TCEP agarose reductions yielded

similar cys/Fab reduction stoichiometries (about 2 cys/Fab after 17–24 h reduction at ambient temperature – see the filled-in triangles in Fig. 1).

4. Discussion

There are many research and clinical uses for antibodies and their fragments. Covalent modification of antibodies and antibody fragments have been used for many purposes, including the introduction of fluorescent or radioactive labels, as well as for conjugation to small molecule drugs for localized drug delivery, and attachment of polymers to improve the stability and plasma half-life of the antibody fragments. Recently, smaller fragments of antibodies have been used and proposed for the targeting of toxins to disseminated cancers, due to their more efficient penetration into tissues [9]. Fab and F(ab')₂ fragments of antibodies have been historically generated by cleavage of IgG with papain and pepsin, respectively. However, IdeS protease (Immunoglobulin G-degrading enzyme of *Streptococcus pyogenes* [10]), and Endoproteinase-Lys-C [11] have recently been shown to be attractive alternative proteases to generate F(ab')₂ and Fab fragments, respectively, leading to better fragment yields and less non-specific cleavage.

In this study, the anti-cocaine monoclonal antibody h2E2 was cleaved by Endo-Lys-C to generate a Fab fragment, as described previously [5]. Various protein reduction agents were evaluated for their ability to selectively reduce the targeted disulfide bond, but only TCEP bound to agarose beads proved to be effective in this regard. TCEP itself in solution was quite effective in reducing the other (non-targeted) disulfides in the Fab fragment (not shown).

The overnight ambient temperature incubations with TCEP agarose resulted in near stoichiometric reduction of 2.0 cys/Fab fragment under a variety of buffer conditions, as shown by the quantification of the cysteine generated (Fig. 1). The inter-chain, targeted disulfide was reduced selectively as a function of time (Fig. 2). Confirmation of the selectivity of the inter-chain disulfide reduction, and additional information concerning the possibility of a very small amount of non-specific reduction of intra-chain disulfides is evident from the mass spectral results (Fig. 3). The utility of the two selectively generated reduced cysteine residues, located at the opposite end of the Fab fragment from the antigen (cocaine) binding site is demonstrated by the labeling with fluorescent sulfhydryl specific reagents, as well as PEGylation, and reformation of a covalent linkage incorporating a single fluorophore (i.e., using the dibromobimane cross-linker), as shown in Fig. 4. Although no attempt was made to optimize these, or other, downstream sulfhydryl-selective conjugation reactions, the results presented clearly demonstrate the utility for laboratory and clinical applications of the selectively reduced Fab fragment, which is readily generated by reduction of a single disulfide bond using TCEP agarose.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

mAb	monoclonal antibody
h2E2	humanized monoclonal antibody against cocaine
Endo Lys-C	Endoproteinase Lys-C
IAM	iodoacetamide
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
DTT	dithiothreitol
DTNB	5,5'-dithio-bis-[2-nitrobenzoic acid] (Ellman's reagent)
ABD-F	aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole
F-MAL	fluorescein 5-maleimide
DBB	dibromobimane
MPEG-MAL-5000	maleimide–polyethylene glycol (5000 Da PEG chain)
TBS	trisbuffered saline
PBS	phosphate buffered saline
ESI-Tof MS	electrospray ionization time-of-flight mass spectrometry

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Fig. 1. Time course of reduction of Fab fragment by TCEP agarose

The amount of free -SH (cysteine residues) generated by treatment with TCEP agarose after incubation with gentle end-over-end mixing in 100 mM Tris-HCl/5 mM EDTA, pH = 8.0 at 22 °C is shown for several samples. Filled squares are from duplicate washed TCEP agarose gel time course experiment samples which were also used to generate the samples analyzed in Fig. 2. Open circle symbols are from samples treated with unwashed TCEP agarose (used as supplied from the manufacturer), while filled triangles are from samples subsequently used for alkylations (using washed TCEP agarose in a variety of buffers after 22 °C incubations from 17 to 24 h) with various reagents. The dotted line represents the 2.0 cysteine/Fab fragment stoichiometry expected if only the targeted disulfide is (quantitatively) reduced. The dashed line represents a power function curve fitted to the two replicate data sets (filled squares) of time course experiments.

Fig. 2. SDS-PAGE analysis of time course of Fab reduction by washed TCEP agarose
Approximately 5 µg aliquots of the samples were analyzed by 10% SDS-PAGE, after dilution with sample buffer containing *N*-ethylmaleimide (no DTT) and boiling for 3 min prior to electrophoresis. The gel was subsequently stained with Coomassie blue. The incubation time at 22 °C for each sample is indicated at the top of each lane, while the stoichiometry of reduction (number of cysteine formed per Fab fragment) is indicated at the bottom of each lane. The migration position of the intact, non-reduced Fab fragment is indicated.

Fig. 3. Mass spectral analysis of iodoacetamide (IAM) alkylated, selectively TCEP-reduced Fab fragments

Sample 1 (S1, blue) was reduced for 24 h and measured to have 2.7 cys/Fab and sample 2 (S2, pink) was reduced for 17 h and measured to have 1.9 cys/Fab fragment before IAM alkylation. Each sample was evaluated by loop injection ESI-ToF-MS as described in the methods section. An overlay of the deconvoluted mass spectrum for each sample is presented over the mass range of 22.6–23.6 kDa. Inset A is a corresponding Coomassie-stained gel of each sample. Inset B shows the deconvoluted mass spectrum in the range of 45–46 kDa with a mass peak consistent with the presence of increased intact Fab seen in sample 2 (Inset A). Asterisks represented masses likely arising from partial reduction and alkylation of internal disulfide bonds in both the heavy and light chains (see text for details). # represents a deconvolution algorithm artifact that is confirmed by inset B, and corresponds to the intact Fab fragment seen in gel sample S2 in the gel shown in Inset A, lane 3.

Fig. 4. Examples of alkylations and conjugations of selectively reduced Fab fragment

Note that none of the alkylation and conjugation reaction conditions were optimized for stoichiometric derivatization in this study. Each lane contains 4 μ g of Fab fragment. The samples were treated with TCEP agarose and then alkylated with various reagents. They were not reduced with DTT prior to electrophoresis, but instead boiled for 3 min in SDS-PAGE non-reducing sample buffer containing *N*-ethylmaleimide (to prevent cysteine oxidation and possible disulfide formation during heating). The gel was photographed first for fluorescence (with 366 nm *trans*-illumination), and then stained with Coomassie blue and destained, and photographed again. Samples were; (1) molecular weight standards, (2) untreated control Fab, (3) 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole Fab (ABD-Fab), (4) dibromobimane Fab (DBB-Fab) (5) fluorescein maleimide Fab (F-Mal Fab), and (6) maleimide-polyethylene glycol 5000 Da Fab (MPEG-MAL-5000-Fab).