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Synthesis and Spectral Characterization of Sulfhydryl-Reactive Fluorescent Probes[1]

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We synthesized two sulfhydryl-reactive fluorescent probes, Br-ANT (2-amino-benzoic acid, 2-(bromoacetyl)hydrazide) and Br-MANT (*N*-[2-[(bromoacetyl)amino]ethyl]-2-(methylamino)benzamide). Br-ANT and Br-MANT contain an anthraniloyl and *N*-methyl anthraniloyl group, respectively, linked to the sulfhydryl-reactive bromoacetyl moiety. The cysteine adducts have absorption maxima at 323 and 326 nm, with molar extinction coefficients of 2100 and 2900 $M^{-1}\,cm^{-1}$, for Br-ANT and Br-MANT, respectively, making these probes excellent acceptors for tryptophan. The absorption spectra and quantum yields were constant at pH levels useful for protein studies (pH 5–8). Quantum yields of Br-ANT and Br-MANT were 0.16 and 0.42, emission maxima were 432 and 440 nm, and fluorescence lifetimes were 1.3 and 7.8 ns, respectively. The emission of Br-ANT-Cys and Br-MANT-Cys shifted to shorter wavelengths with decreasing solvent polarity. Polarization values were maximal between 330 and 375 nm. Both probes reacted selectively and stoichiometrically with the single cysteine residue of a model protein. The labeled protein exhibited relatively long lifetimes (9–10 ns), suggesting that these probes will be generally useful for rotational studies.

Fluorescence is a powerful technique to study the dynamics and structure of proteins (1, 2). Sulfhydryl-reactive extrinsic fluorescent probes (2, 3) are especially useful tools due to a highly selective covalent reaction with cysteine residues (4). There is currently interest in fluorescently labeled engineered proteins as biosensors of binding phenomena, or postranslational modifications, in cells and other biological preparations, for example. Commercially available fluorophores are not always adequate for this new interest. A telling case is the engineering of a protein to assay (in real time) inorganic phosphate in biological preparations (5). All appropriate commercially available probes were tested with six sulfhydryl mutants with a maximal fluorescence increase of 65% upon phosphate binding to the protein. However, a five- to sixfold increase in fluorescence was obtained upon phosphate binding (6) with a new coumarin derivative which differed from the commercially available analog by an aliphatic spacer arm.

One objective of our studies is to synthesize new sulfhydryl-reactive fluorescent probes to be generally useful for cysteine mutant protein studies. We noticed that the otherwise widely used anthraniloyl group had not been reported as a cysteine-reactive reagent. The small size of the anthraniloyl group (a single aromatic ring) is particularly desirable for cysteine-mutant protein labeling, avoiding potential hydrophobic interactions of larger ring systems which may perturb protein structure. Recent studies with the popular anthraniloyl nucleotide derivatives (7, 8) have shown that the anthraniloyl group has a relatively homogenous and long lifetime (9–11) useful for rotational measurements. This group is also an excellent acceptor of tryptophan (12–14) which is useful for distance measurements (15).

We report here the synthesis and spectral characterization of two new thiol selective fluorescent reagents, 2-amino-benzoic acid, 2-(bromoacetyl)hydrazide (Br-

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ANT)[3] and *N*-[2-[(bromoacetyl)amino]ethyl]-2-(methylamino)benzamide (Br-MANT), which contain the anthraniloyl and *N*-methyl anthraniloyl groups, respectively. Each probe has a different linker arm length between the fluorophore and a cysteine-reactive bromoacetyl moiety. These reagents are amply water soluble, show selectivity for protein thiols, and are easy to synthesize and further modify.

MATERIALS AND METHODS

Reagents and solvents were reagent or HPLC grade, unless otherwise noted. All solvents were purchased from J. T. Baker, except for deuterated solvents from Aldrich. All reactions were protected from room light to avoid potential photodegradation. NMR data were recorded on either a Nicolet (200 MHz, ^{1}H, ^{13}C) or Bruker AMX (300 MHz, ^{1}H) spectrophotometer. Mass spectral data were obtained on a VG 7070 mass spectrometer with FAB, EI, and CI sources at the departmental facility at Washington State University. The synthetic reactions of Br-MANT and Br-ANT are shown in Scheme 1. TLC analysis to obtain R_f values was performed on silica gel 60 F_{254} 0.25-mm thickness, glass support (EM Separations). Compounds were detected by illumination of the TLC plates with an ultraviolet lamp (Model UVGL-25 Mineralight Lamp, multiband UV-254/366 nm, UVP, Inc., San Gabriel, CA) at 254 or 366 nm.

Br-ANT. One millimole of dried anthranilic acid hydrazide (AAH, Pfaltz and Bauer Inc., Waterbury CT; R_f = 0.38 in EtOAc) was dissolved in 4 ml DMF and slowly added to 1 mmol BrNHS in 2 ml of DMF with constant stirring. After reaction at room temperature under argon for 2–3 h, the sulfhydryl-reactive product was detected by TLC (R_f = 0.72 in EtOAc, see also Reaction of Br-MANT and Br-ANT with L-cysteine) and purified by flash chromatography on a 2.5 × 30-cm column using 95 g of 230–400 mesh, 60-Å silica gel (Merck) as the stationary phase, and EtOAc as the eluent. The overall yield for this reaction and purification was 75–80%. The purity of the product as estimated from TLC and NMR analysis was greater than 98%.

^{13}C-NMR (solvent CD_3CN): ppm 117–155 (6-s, phenyl); ppm 167–170 (2-s, carbonyls); ppm 28 (1-s, methylene). ^{1}H-NMR (solvent CD_3CN): ppm 7.4 (d, phenyl, $\int$ = 1.0); ppm 7.3 (t, phenyl, $\int$ = 0.95); ppm 6.8 (d, phenyl, $\int$ = 1.1); ppm 6.65 (t, phenyl, $\int$ = 1.0); ppm 3.95 (s, methylene, $\int$ = 2.1). Mass Spectrum: Parent peak at m/e = 271 with m + 2 peak corresponding to the naturally occurring bromine isotope. Main fragment peaks were also observed at m/e = 120, 80, 93, and 162, respectively (listed in order of decreasing intensity).

Intermediate 1 (N-methylisatoic anhydride–ethylenediamine adduct). *N*-Methylisatoic anhydride (NMIA; Molecular Probes) was dried before use by lyophilization from ACN then lyophilized for 12 h at 1 μm Hg. Twenty milliliters of freshly distilled (from calcium hydride) ethylenediamine (Sigma Chemicals, St. Louis, MO) was added to NMIA (723 mg, 3.69 mmol) and heated to 35°C under argon with a reflux condenser. Excess ethylenediamine was removed after 62 h of reaction, first by rotary evaporation at 25°C for 2–3 h to an oil and then by lyophilization (at 1 μm Hg) for 12 h from 20 ml of dioxane (Aldrich) to ensure that all excess ethylenediamine was removed. The oily solid was dissolved in 50 ml of MeOH and purified by preparative TLC (Kiesel gel 60 F_{254} 2.0-mm thickness, glass support; EM Separations) in 95% MeOH:5% ammonium hydroxide. Three fluorescent spots corresponding to the starting material, Intermediate 1, and an uncharacterized side product at R_f = 0.93, 0.46, and 0.35, respectively, were observed. The product was eluted with 50% MeOH:50% CH_2Cl_2. The overall yield for this reaction and purification was ~40%.

^{13}C-NMR (solvent MeOD): ppm 172.49 (s, carbonyl); ppm 151.45–111.89 (6-s, phenyls); ppm 43.14 (s, methylene); ppm 42.09 (s, methylene); ppm 29.86 (s, *N*-methyl). ^{1}H-NMR (solvent MeOD): ppm 7.34–6.49 (m, phenyls, $\int$ = 3.835); ppm 3.33 (t, methylene, $\int$ = 1.955); ppm 2.753 (t, methylene); ppm 2.724 (s, *N*-methyl); $\int$ = 5.126 for latter methylene and *N*-methyl. Mass spectrum: Parent peak at m/e = 193, main fragment peaks were also observed at m/e = 134, 164, 77, 105, 91, and 150, respectively (listed in order of decreasing intensity).

Br-MANT (from Intermediate 1). BrNHS (78.0 mg) (Sigma Chemicals) was added with constant stirring to a solution of 60.2 mg Intermediate 1 dissolved in 2.5 ml DMF. After 3 h at 37°C the reaction solution was dried to a yellow oily solid by repeatedly dissolving in MeOH and rotary evaporating to dryness to remove all DMF. This was applied to two preequilibrated preparative TLC plates and developed three times in EtOAc. The product (R_f = 0.59 in EtOAc) was eluted from the silica with EtOAc/MeOH and was found to be sulfhydryl reactive (see Reaction of Br-MANT and Br-ANT with L-cysteine). The overall yield for this reaction and purification was 70–80%. The purity of the product as estimated from TLC and NMR analysis was greater than 98%.

[3] Abbreviations used: Br-ANT, 2-aminobenzoic acid, 2-(bromoacetyl)hydrazide; Br-MANT, *N*-[2-[(bromoacetyl)amino]ethyl]-2-(methylamino)benzamide; NMIA, *N*-methylisatoic anhydride; BrNHS, bromoacetic acid *N*-hydroxy succinimide ester; DMF, dimethylformamide; EtOAc, ethyl acetate; CD_3CN, deuterated acetonitrile; MeOD, deuterated methanol; AAH, anthranilic acid hydrazide; Br-MANT-Cys, product of L-cysteine and Br-MANT reaction; Br-ANT-Cys, product of L-cysteine and Br-ANT reaction; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); DTT, dithiothreitol.

^{13}C-NMR (solvent CD_3CN): ppm 167–171 (2s, carbonyls); ppm 115–153 (6s, phenyls); ppm 30–40 (4s, methylenes). ^{1}H-NMR (solvent CD_3CN): ppm 7.3–7.6 (m, phenyls, $\int$ = 2.2); ppm 6.7–6.8 (m, phenyls, $\int$ = 2.0); ppm 3.85 (s, methylene adjacent to halogen, $\int$ = 1.6); ppm 3.3–3.5 (m, methylenes, $\int$ = 4.1); ppm 2.9 (s, *N*-methyl, $\int$ = 2.5). Mass Spectrum: Parent peak at m/e = 313 with an m + 2 peak corresponding to the naturally occurring bromine isotope. Main fragment peaks were observed at m/e = 149, 134, 161, 189, and 234, respectively (listed in order of decreasing intensity).

Reaction of Br-MANT and Br-ANT with L*-cysteine.* Fifty microliters of 200 mM L-cysteine (in 100 mM Tris–Cl, pH 7.5) was added to 50 μl of the halogen-containing compound (~1 mM) and allowed to react for 15 min at room temperature. The products of these reactions, Br-MANT-Cys and Br-ANT-Cys, do not migrate in the solvent system (EtOAc) used in the TLC analysis. This procedure was used to demonstrate complete reactivity of Br-MANT and Br-ANT with sulfhydryl-containing compounds and can be used as a simple test for intact haloacetyl substituents.

Molar extinction coefficients of Br-MANT and Br-ANT. The extinction coefficients for Br-MANT and Br-ANT were determined both gravimetrically and by stoichiometric reaction with L-cysteine. To determine the extinction coefficients by the latter method, Br-MANT and Br-ANT were dissolved in either water or EtOH and the absorption spectra were recorded. A known excess of L-cysteine (in 100 mM Tris–Cl, pH 7.5) was added to each compound. After the reaction was completed (see above), the amount of unreacted L-cysteine was determined using DTNB (16). The concentrations of the fluorescent compounds and the extinction coefficients were then calculated. The coefficients determined by either method were within 5% and the average of all determinations is reported.

Steady-state fluorescence measurements. Steady-state fluorescence measurements were made at 25°C on an SLM 4800 fluorometer (SLM Instruments, Division of Milton Roy, Rochester, NY) which was interfaced with a computer which utilizes DOS (Microsoft Corp.)-based software supplied by SLM. Internal fluctuations of the xenon lamp and variation in the efficiency of the excitation monochrometer were corrected by using a rhodamine B quantum counter in the reference position. All samples were filtered through a 0.45-μm Millipore filter to minimize light scattering and were excited at 326 nm (except where noted).

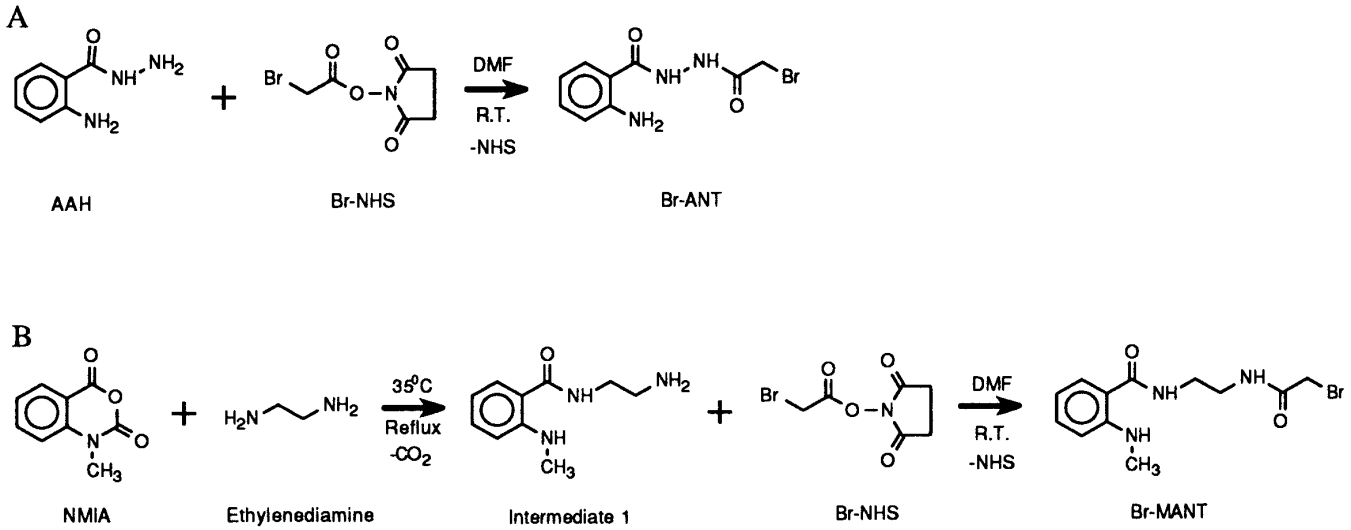
Time-resolved fluorescence measurements. Frequency-domain fluorescence lifetime measurements were performed on an ISS Model K2 multifrequency phase fluorometer equipped with digital acquisition electronics and fast fourier transform data-processing capability. The 351-nm line of a Spectra-Physics Model 2045 argon ion laser was used for excitation, and emission was monitored through both 3-144 and 3-74 Corning colored glass cut-off filters. Sample lifetimes were determined by the phase shift and demodulation of the fluorescence relative to that of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP; assuming a lifetime of 1.35 ns) (17, 18). The excitation beam was polarized parallel to the vertical laboratory axis and emission was collected through a Glan Thompson polarizer oriented at 55° to that same axis to avoid an influence of sample polarization on the observed lifetime (19). Data were analyzed using the Globals Unlimited software obtained from the University of Illinois.

Protein labeling. Smooth muscle myosin regulatory light chain (RLC20) was isolated from frozen chicken gizzards as described previously (20). The RLC20 concentration was determined using $\epsilon_{277}^{1\%}$ = 3.35 (20, 21). The labeled RLC20 concentrations were determined by the bicinchoninic acid method (22) relative to an unlabeled RLC20 standard. A solution of RLC20 (1.5 mg/ml) in 50 mM ammonium bicarbonate, pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA was purged with argon for 30 min on ice. DTT was then added to 5 mM and the sample was purged another 5 min on ice before the reaction was sealed and allowed to proceed for 12 h on ice. After sufficient dialysis versus the above buffer to remove nearly all the excess DTT as determined with DTNB (16), a fourfold excess of Br-ANT or Br-MANT (dissolved in MeOH) over the remaining sulfhydryl concentration was added and the reaction was stirred for 3–12 h on ice. Complete labeling was confirmed using DTNB. Excess unreacted probe was removed from 800-μl protein samples by centrifugal gel filtration with Sephadex G-25 (Sigma) in 5-ml columns (23).

RESULTS

Synthesis and properties of Br-MANT and Br-ANT. The syntheses of Br-ANT and Br-MANT (see Scheme 1) involved coupling the sulfhydryl-reactive bromoacetyl moiety to either AAH (for Br-ANT) or NMIA (for Br-MANT). Both reactants are commercially available. Br-ANT was synthesized by reacting the N-amino group of the hydrazide, AAH, with the NHS ester of bromoacetic acid (BrNHS) at room temperature (yield 80–90%). Br-MANT synthesis required two steps for the addition of the bromoacetyl group. NMIA was reacted neat in ethylenediamine at 35°C to form Intermediate 1 with the subsequent loss of carbon dioxide gas (24). The primary amine of purified Intermediate 1 was then reacted with BrNHS at room temperature as described for the Br-ANT synthesis. Purification of the final product gave an overall average yield of 25–30%.

To test the purified Br-ANT and Br-MANT for a reactive bromoacetyl functionality, L-cysteine was added and the reaction was monitored by TLC (25). It ap-



SCHEME 1. Overview of synthesis of fluorescent derivatives Br-ANT (A) and Br-MANT (B).

peared that both Br-ANT and Br-MANT were quantitatively converted to the cysteine adduct under these conditions, as evidenced by the change in R_f values (see Materials and Methods). Br-ANT and Br-MANT remained L-cysteine reactive after 3 h irradiation with ultraviolet light at ambient temperature (Mineralight lamp, 254 nm at a distance of 3 cm; data not shown). We recommend storage of Br-ANT and Br-MANT in the solid form or in MeOH or DMF solutions at −20 to −80°C. Basic aqueous conditions (>pH 10) should be avoided as the bromine substituent is susceptible to hydrolysis. Exposure to heat between 40 and 50°C for 10 h or more decomposed the compounds as evidenced by development of new fluorescent spots on TLC which migrated below either Br-ANT or Br-MANT (data not shown).

Molar extinction coefficients. The molar extinction coefficients for Br-ANT and Br-MANT in ammonium bicarbonate buffer at pH 7.9 were 2100 M^{-1} cm^{-1} (λ_{max} at 335 nm) and 2060 M^{-1} cm^{-1} (λ_{max} at 343 nm), respectively. The extinction coefficients in MeOH were 2650 M^{-1} cm^{-1} (λ_{max} at 335 nm) and 2800 M^{-1} cm^{-1} (λ_{max} at 340 nm) for Br-ANT and Br-MANT, respectively. The molar extinction coefficients for Br-ANT-Cys and Br-MANT-Cys in water (pH 7) were 2100 M^{-1} cm^{-1} (λ_{max} at 323 nm) and 2900 M^{-1} cm^{-1} (λ_{max} at 326 nm) (Fig. 1).

The extinction coefficients of both Br-ANT-Cys and Br-MANT-Cys were dependent upon pH (Fig. 1) with the protonated forms lacking the lowest energy absorption band. Analysis of pH titration curves (Fig. 1, insets) indicated that the apparent pK_a values were approximately 2.5 and 3.1, for Br-ANT and Br-MANT, respectively. These values are similar to those obtained with anthraniloyl nucleotide analogs (7, 8) and are consistent with the lack of modification of the aryl amine upon addition of BrNHS (see Materials and Methods). The emission spectrum exhibited a similar pH dependence in which the fluorescence decreased with decreasing pH (data not shown). These data suggest, as has been found previously for anthraniloyl derivatives, that the deprotonated form is responsible for the long-wave absorption band and thus the fluorescence (7, 8).

Corrected emission spectra and relative quantum yields of Br-ANT-Cys and Br-MANT-Cys. Figure 2 shows the effect of increasing EtOH concentrations upon the fluorescence emission spectra of Br-ANT-Cys (top) and Br-MANT-Cys (bottom). The emission maximum of Br-ANT-Cys and Br-MANT-Cys shifted continuously to shorter wavelengths as the solvent polarity decreased, as was observed with other methyl anthranilate derivatives (13). For Br-ANT-Cys, the relative quantum yield increased to a maximum of 0.86 in 100% EtOH. In contrast, the quantum yield of Br-MANT-Cys was highest at 40% EtOH. Similar results were obtained when the EtOH titration was performed in ammonium bicarbonate buffer at pH 7.9 (data not shown). Table 1 summarizes the emission maxima and relative quantum yields for Br-ANT-Cys and Br-MANT-Cys in various solvents.

Fluorescence lifetimes of Br-ANT-Cys and Br-MANT-Cys. Table 2 shows the fluorescence lifetimes of both Br-ANT-Cys and Br-MANT-Cys in various solvents corresponding to those described in Table 1. The data were fit to a two-exponential decay model. Analysis of the data using a single- or a unimodal laurentian distribution of decay rates resulted in significantly higher χ^2 values (data not shown). A triple-exponential decay model resulted in lifetime values that were less than or equal to zero. In most cases examined, greater than 95% of the emission intensity is attributable to a

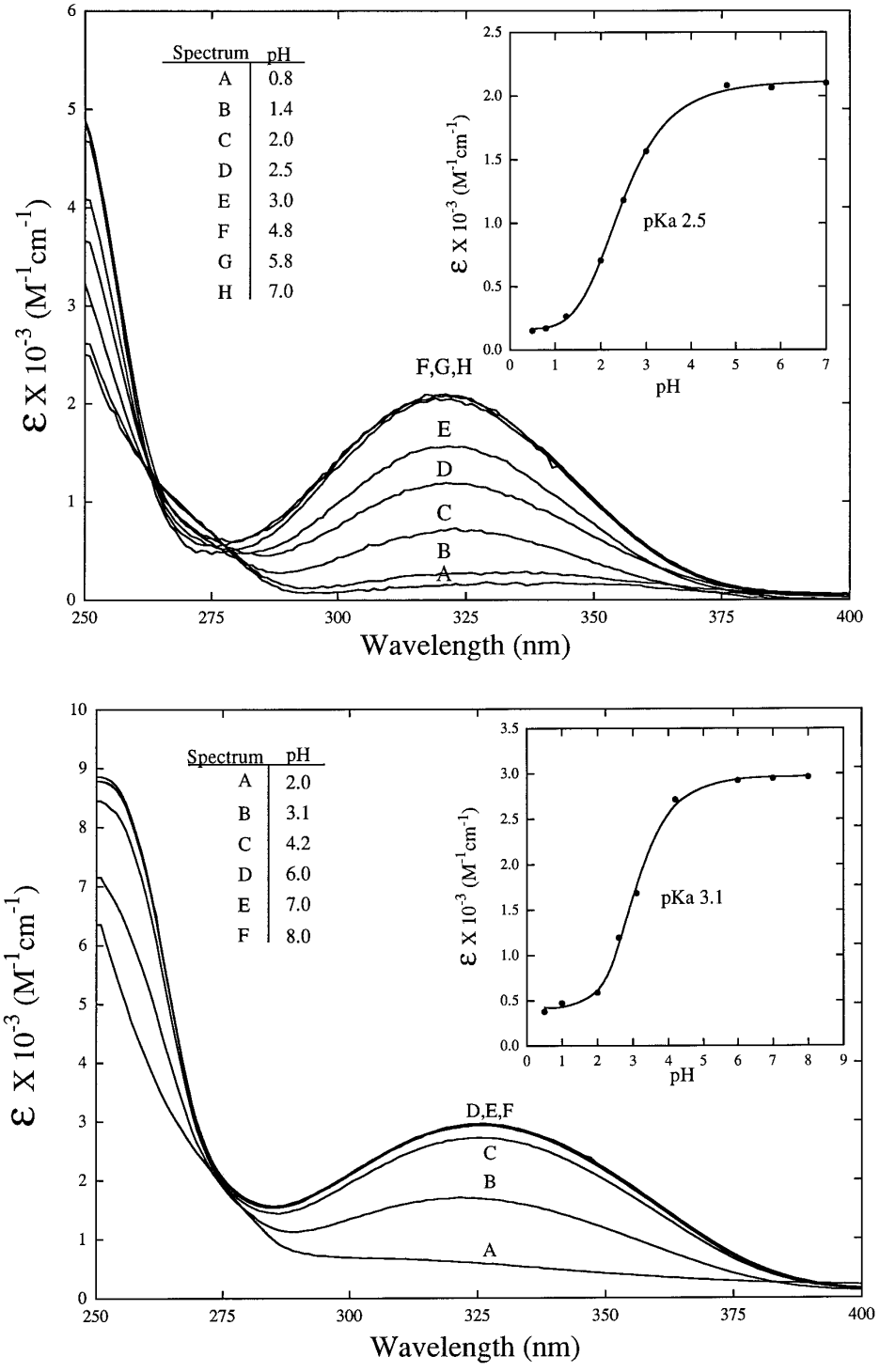


FIG. 1. pH dependence of the absorption spectra. pH titration of Br-ANT-Cys (top) and Br-MANT-Cys (bottom) in water at 22°C. Br-MANT-Cys and Br-ANT-Cys were dissolved into water to give an absorbance of ≤0.5 at 326 nm. Samples were filtered through 0.45-μm Millipore filters. The pH was adjusted individually by adding small amounts (0.5–2.0 μl) of dilute HCl or NaOH, while the sample was stirred in a 3-ml quartz cuvette. The pH was monitored using an Ingold combination glass electrode and digital pH meter. The absorbance spectra from 250 to 400 nm were recorded using a Varian 2200 UV–visible spectrophotometer interfaced with a Varian DS-15 data station. The apparent pK_a values (insets) were estimated by fitting the data using the four-parameter logistic equation (Sigma Plot, Jandel Scientific) $f(x) = \dfrac{a - d}{1 + \left(\dfrac{x}{c}\right)^b} + d$, where a and d are the asymptotic maximum and minimum values, respectively; b is the slope parameter; c is the pK_a; x is the pH; and $f(x)$ is the molar extinction coefficient at 323 nm for Br-ANT (top) and at 326 nm for Br-MANT (bottom).

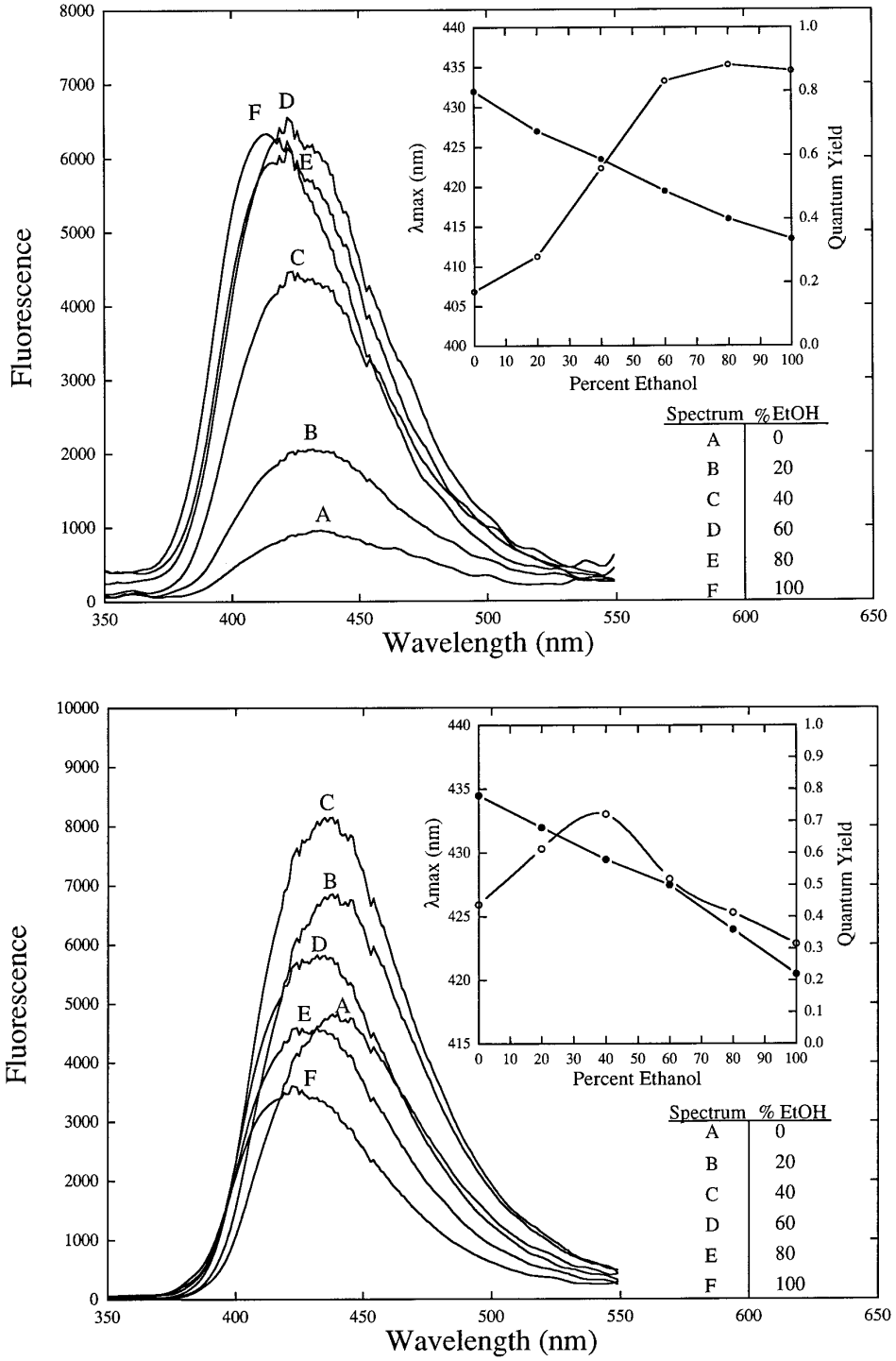


FIG. 2. Corrected emission spectra of Br-ANT-Cys (top) and Br-MANT-Cys (bottom) in ethanol water mixtures. Data were collected with the excitation polarizer (calcite prism) at 55° from vertical and the emission polarizer at 0° from vertical. Excitation and emission slit widths were set at 4 nm. Correction factors were obtained by calibrating the emission monochrometer and photomultiplier with a standard tungsten light source. (Insets) The wavelength at maximum emission intensity from the corrected emission spectra (λ_{max}, ●) is plotted with relative quantum yields (○) versus percentage ethanol concentration. Relative quantum yields were determined, using quinine sulfate in 0.1 N H_2SO_4 as a standard (quantum yield, 0.70 at 25°C), by the relationship of (28). A Varian 2200 UV–visible spectrophotometer was used to measure the absorbance in 1-ml cuvettes. Samples were diluted to an absorbance of 0.015 at 326 nm for all quantum yield measurements.

TABLE 1

Fluorescence Properties of Br-ANT-Cys and Br-MANT-Cys

Solvent	Br-ANT-Cys		Br-MANT-Cys	
	Wavelength[b] (nm)	Quantum yield[c]	Wavelength[b] (nm)	Quantum yield[c]
Ethanol	413	0.86	424	0.31
Ethanol–water (80)[a]	416	0.87	428	0.41
Ethanol–water (60)	419	0.85	431	0.52
Ethanol–water (40)	424	0.55	434	0.73
Ethanol–water (20)	427	0.27	436	0.61
Water	432	0.16	440	0.42
Buffer (pH 7.5)[d]	—	—	440	0.42
Ethyl acetate	408	0.05	421	0.09
ρ-Dioxane	406	0.40	420	0.19
DMF	407	0.52	419	0.07

[a] The numbers in parentheses indicate the percentage (v/v) of ethanol in ethanol–water mixtures.
[b] The maximum wavelength of the corrected emission spectrum (see Fig. 2 for details).
[c] Relative quantum yields (see Fig. 2 for details).
[d] 10 mM Tris–Cl.

single exponential decay with lifetime denoted by τ_1 in Table 2. The differences in average lifetimes observed for different ethanol concentrations paralleled the changes in the quantum yields (Table 1).

Excitation polarization spectra of Br-ANT-Cys and Br-MANT-Cys. Excitation polarization spectra of Br-ANT-Cys and Br-MANT-Cys in 90% glycerol at 2°C are shown in Fig. 3. The spectra of the two compounds are similar, but Br-MANT showed more complex behavior corresponding to the longest wavelength transition. The high limiting polarization values between 330 and 375 nm offer a wide range of excitation wavelengths appropriate for rotational studies.

Selectivity of reaction of Br-ANT and Br-MANT with protein thiols. In general, haloacetates are known to react preferentially with thiol groups on proteins under physiological pH conditions (4). To test for labeling stoichiometry and selectivity of the probes described here, we performed the experiment described in Table 3. The regulatory light chain of smooth muscle myosin contains a single cysteine residue. The protein was reacted with a fourfold excess of either Br-ANT or Br-MANT at pH 7.9. Disulfide bond formation under these conditions could not be detected by native gel analysis (data not shown). The unreacted thiol concentration was then determined by using DTNB (16). This indicated that all of the protein thiols had reacted. If this labeling was restricted to thiols, then three equivalents of unreacted Br-ANT or Br-MANT would be expected to remain. The remaining equivalents of Br-ANT and Br-MANT were determined after reaction with excess L-cysteine and backtitration of the unreacted L-cysteine with DTNB. As shown in Table 3, the calculated amounts of unreacted Br-ANT and Br-MANT were as predicted for a selective and stoichiometric reaction with the single thiol group of the protein.

Fluorescence lifetimes of labeled model protein. The lifetimes of the regulatory light chain labeled with Br-ANT and Br-MANT are shown in Table 4. The fluorescence decay was best fit to three exponentials. However, one of the lifetimes is not shown in Table 4 as it comprised $<7\%$ of the fractional intensity and was short. We attribute this to a small amount of free probe contaminating the labeled protein sample.

DISCUSSION

The synthesis and purification of the two fluorescent derivatives was relatively uncomplicated, with reasonable yields of stable products from readily available starting materials. These probes were found to be thermally stable at or below room temperature in all solvents (see Table 1) tested (data not shown). Although not charged at neutral pH, these compounds are capable of forming multiple hydrogen bonds and were found

TABLE 2

Lifetimes of Br-ANT-Cys and Br-MANT-Cys[a]

Solvent	Br-ANT-Cys				Br-MANT-Cys			
	τ_1 (ns)	f_1[d]	τ_2 (ns)	χ^2	τ_1 (ns)	f_1[d]	τ_2 (ns)	χ^2
EtOH	6.6 ± 0.1	0.97 ± 0.01	1.4 ± 0.1	2.0	5.1 ± 0.1	1.00	—	2.0
EtOH (80)[b]	6.9 ± 0.1	0.97 ± 0.01	2.4 ± 0.3	1.4	7.3 ± 0.1	0.99 ± 0.01	0.0 ± 0.4	1.7
EtOH (60)	6.4 ± 0.1	0.95 ± 0.01	2.0 ± 0.2	2.4	7.9 ± 0.1	0.96 ± 0.01	0.7 ± 0.1	3.5
EtOH (40)	4.3 ± 0.1	1.00	—	5.9	8.1 ± 0.1	0.97 ± 0.01	0.8 ± 0.1	3.6
EtOH (20)	2.6 ± 0.1	0.94 ± 0.01	20 ± 6	2.0	7.9 ± 0.1	0.98 ± 0.01	1.6 ± 0.2	1.1
Buffer[c]	1.3 ± 0.1	0.83 ± 0.01	15 ± 2	2.3	7.8 ± 0.1	0.88 ± 0.01	4.0 ± 0.2	3.0

[a] Lifetimes (τ_1 and τ_2) obtained by fitting frequency-domain data to a two-exponential decay model (see Results).
[b] The numbers in parentheses indicate the percentage (v/v) of ethanol in ethanol–buffer mixtures.
[c] 50 mM ammonium bicarbonate, pH 7.9, 0.1 mM EGTA, and 0.1 mM EDTA.
[d] f_1 represents the fractional fluorescence intensity associated with τ_1.

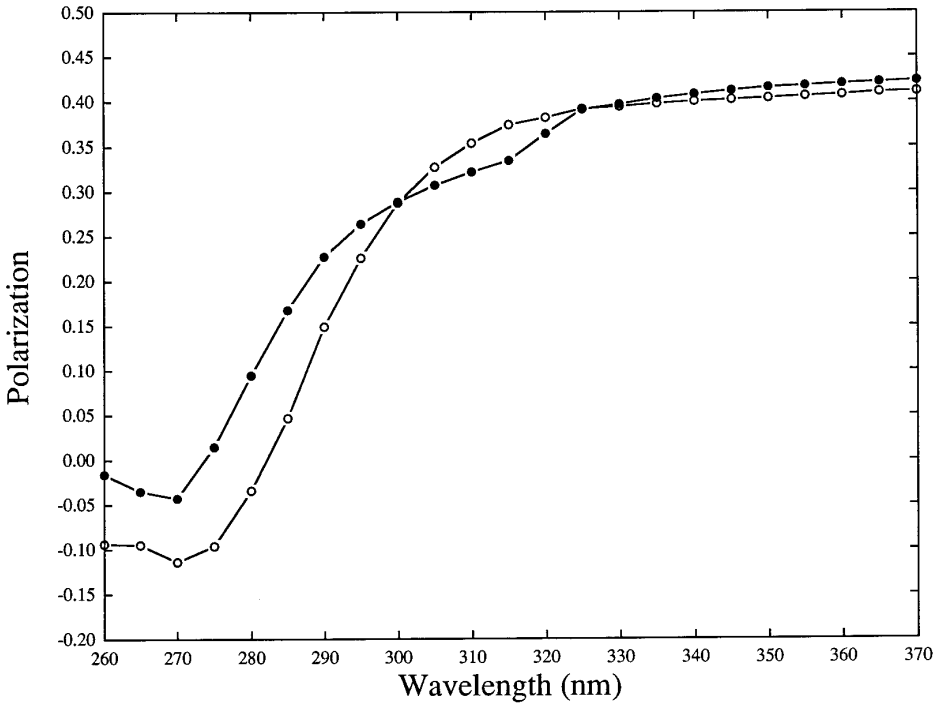


FIG. 3. Excitation polarization spectra of Br-ANT-Cys (○) and Br-MANT-Cys (●) in 90% glycerol at 2°C. The spectra were recorded point by point by varying the excitation wavelength. Data were acquired with the photomultipliers in the T-format except between 290 to 330 nm, which are affected by the Woods anomaly (29) in which the L-format was used. A Schott 389-nm cutoff filter was used to select for emission wavelengths above 389 nm and to exclude the exciting light.

to be readily soluble in neutral buffered solutions (our experiments tested up to 0.3 mM), allowing for modification of proteins without exposure to high concentrations of organic solvents. Stock solutions of 5 mM in MeOH were found to be useful for protein-labeling studies.

Two considerations guided our decision to synthesize two analogous probes. First, the length of the leash between the protein and the fluorophore is a feature which may become important in rotational and quenching studies. The leash length is about 0.72 nm for Br-ANT and about 0.91 nm for Br-MANT. Second, the two probes were expected to have different fluorescence properties due to the methyl-substituted nitrogen of Br-MANT versus the unsubstituted nitrogen of Br-ANT. Although Br-MANT emitted at a longer wavelength than Br-ANT as expected (Table 1) (7, 8), the difference between the two was only 8 nm, compared to a 14- to 17-nm difference observed for corresponding

TABLE 3

Stoichiometry and Selectivity of Regulatory Light-Chain Labeling

Probe	RLC20 initial (measured)[a]	Free probe added (measured)[b]	Unreacted free probe (calculated)[c]
Br-ANT	1.0 ± 0.1	4.0 ± 0.1	3.2 ± 0.2
Br-MANT	1.0 ± 0.1	4.0 ± 0.1	3.1 ± 0.2

[a] The sulfhydryl concentration due to the regulatory light chain was determined using DTNB (see Materials and Methods) and normalized to 1.0 equivalent for each experiment.

[b] The equivalents of free probe added were calculated using the respective molar extinction coefficients (see Results).

[c] The equivalents of unreacted probe were determined by adding six equivalents of L-cysteine to the reaction mixture and determining the amount of unreacted L-cysteine remaining after 12 h reaction on ice. The equivalents of unreacted probe were backcalculated from these data.

TABLE 4

Lifetimes of Labeled Regulatory Light Chain[a]

Probe	τ (ns)	Fractional intensity	χ^2
Br-ANT	10.6 ± 0.1	0.564 ± 0.002	2.2
	4.8 ± 0.1	0.365 ± 0.002	
Br-MANT	9.2 ± 0.1	0.742 ± 0.002	5.7
	6.2 ± 0.3	0.243 ± 0.002	

[a] Experiments were performed at 25°C in 50 mM ammonium bicarbonate (pH 7.9), 0.1 mM EGTA, 0.1 mM EDTA. The protein concentration was 5.1 μM (0.1 mg/ml).

nucleotide analogs. Similarly, the differences in the maximum absorption wavelength, between Br-ANT and Br-MANT, were not as pronounced as was found for the corresponding nucleotide analogs (7, 8). However, these probes, with absorption maxima in the region of 323–326 nm and extinction coefficients in the 2000–3000 M^{-1} cm^{-1} range, can serve as acceptors for energy transfer from tryptophan (13).

Both analogs showed a constant absorption spectrum (Fig. 1) and constant quantum yield (data not shown) over a pH range found to be most useful for protein studies (pH 5–8). Similarly, both probes showed the expected blue shift in emission maximum upon decreasing solvent polarity (Fig. 2). It appears that Br-ANT would be the preferred probe for monitoring the polarity of the environment, because it showed a simple relationship between quantum yield and solvent polarity (Fig. 2, top, and Table 1). A maximum quantum yield at intermediate EtOH concentrations was observed for Br-MANT (Fig. 2, bottom, and Table 1). This behavior has been previously noted with *N*,*N*-diethylaminoethyl 2-(*N*-methylamino)benzoate–HCl (26) and with derivatives of GDP (T. Hazlett, personal communication), all of which contain the methyl anthraniloyl functional group. Although this suggests that this property is intrinsic to the fluorophore, the explanation for this unexpected behavior will remain obscure until further studies are conducted that consider the complicated effects of solvent composition upon these probes. The lifetimes of the probes (Table 2) generally parallel the quantum yields (Table 1) in different solvent compositions.

Protein studies that utilize extrinsic fluorophores often require stringent site-selective labeling. Both Br-MANT and Br-ANT selectively label the single cysteine residue (cys-108) in the RLC20 of smooth muscle myosin (Table 3). This reactivity is expected as these compounds are similar to other haloacetyl derivatives that are known to react selectively with thiols in proteins (4, 25).

It was of interest to examine the fluorescence lifetimes of the two new probes attached to a cysteine residue of a model protein. We selected the regulatory light chain of smooth muscle myosin because it contains a single cysteine residue in an exposed position on the protein (using the skeletal light chain as a structural homologue (27)). As can be seen in Table 4, the decay for both fluorophores was found to fit well to a double-exponential model, with the longer lifetime comprising the greater fractional fluorescence intensity. The physical basis of the two components is not known. We can rule out labeling at multiple residues from the data in Table 3. A two-component decay was also observed for AEDANS-labeled protein (10). It is notable that the lifetimes were longer when the probes were covalently bound to a model protein (Table 4) compared to the values of Br-ANT-Cys and Br-MANT-Cys in 100% EtOH (Table 2). The observed lifetimes for labeled protein (Table 4) are well suited to studies of the rotational dynamics of proteins in this molecular weight range (M_r 19,600).

It is of interest to compare the spectral and chemical properties of these new anthranoyl probes with other sulfhydryl-reactive probes. Br-ANT and Br-MANT are unique in that they contain only a single aromatic ring and are neutral at physiological pH. They have similar absorption and emission maxima to the small negatively charged salicylate fluorophore, but have longer lifetimes (3). The popular probe, 1,5-IAEDANS (25), with relatively long lifetimes has two rings, is negatively charged, and emits at approximately 100-nm-longer wavelengths than the anthraniloyl probes reported here. In summary, the unique combination of the chemical and spectral properties of Br-ANT and Br-MANT, combined with their ease of synthesis, suggests that these probes will be a useful addition to the family of fluorescent probes.

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REFERENCES

1. Fairclough, R. H., and Cantor, C. R. (1978) *Methods Enzymol.* 48, 347–379.
2. Eftink, M. R. (1991) *Methods Biochem. Anal.* 35, 127–205.
3. Haugland, R. P. (1983) *in* Excited States of Biopolymers (Steiner, R. F., Ed.), pp. 29–58, Plenum, New York.
4. Means, G. E., and Feeney, R. E. (1971) *Chemical Modification of Proteins*, Holden-Day, San Francisco.
5. Brune, M., Hunter, J. L., Corrie, J. E. T., and Webb, M. R. (1994) *Biochemistry* 33, 8262–8271.
6. Corrie, J. E. (1994) *J. Chem. Soc. Perkin Trans.* 1, 2975–2982.
7. Hiratsuka, T. (1982) *J. Biol. Chem.* 257, 13354–13358.
8. Hiratsuka, T. (1983) *Biochem. Biophys. Acta* 742, 496–500.
9. Cremo, C. R., Neuron, J. M., and Yount, R. G. (1990) *Biochemistry* 29, 3309–3319.
10. Rosenfeld, S. S., Xing, J., Rener, B., Lebowitz, J. J., Kar, S., and Cheung, H. C. (1994) *J. Biol. Chem.* 269, 30187–30194.
11. Hazlett, T. L., Moore, K. J., Lowe, P. N., Jameson, D. M., and Eccleston, D. F. (1993) *Biochemistry* 32, 13575–13583.
12. Hagag, N., Birnbaum, E. R., and Darnall, D. W. (1983) *Biochemistry* 22, 2420–2427.
13. Haugland, R. P., and Stryer, L. (1967) *in* Conformation of Biopolymers (Ramachandron, G. N., Ed.), Vol. 1, pp. 321–335, Academic Press, New York.
14. Knipping, G., Hermetter, A., and Hubmann, M. R. (1993) *J. Biochem.* 25, 505–511.
15. Wu, P., and Brand, L. (1994) *Anal. Biochem.* 218, 1–13.
16. Anderson, G. L., and Wetlaufer, D. B. (1975) *Anal. Biochem.* 67, 493–502.

17. Gratton, E., Jameson, D. M., and Hall, R. D. (1984) *Annu. Rev. Bophys. Bioeng.* 13, 105–124.
18. Jameson, D. M., Gratton, E., and Hall, R. D. (1984) *Appl. Spectros. Rev.* 20, 55–106.
19. Spencer, R. D., and Weber, G. (1970) *J. Chem. Phys.* 52, 1654–1663.
20. Facemyer, K. C., and Cremo, C. R. (1992) *Bioconjugate Chem.* 3, 408–413.
21. Facemyer, K. C., Tibeau, M. R., and Cremo, C. R. (1993) *in* Techniques in Protein Chemistry IV, pp. 187–192, Academic Press, San Diego.
22. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fugimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* 150, 76–85.
23. Penefsky, H. S. (1977) *J. Biol. Chem.* 252, 2891–2899.
24. Clark, R. H., and Wagner, E. C. (1944) *J. Org. Chem.* 9, 55–67.
25. Hudson, E. N., and Weber, G. (1973) *Biochemisty* 12, 4154–4161.
26. Trzos, W., and Reed, J. K. (1981) *FEBS Lett.* 127, 196–200.
27. Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G., and Holden, H. M. (1993) *Science* 261, 1–58.
28. Parker, C. A., and Reese, W. T. (1960) *Analyst (London)* 85, 587–600.
29. Jameson, D. M. (1984) *in* Fluorescein Hapten: An Immunological Probe (Voss, E. W., Jr., Ed.), pp. 23–48, CRC Press, Boca Raton, FL.