Fluorophores processing a 6-acetyl-2-dimethylaminonaphthalene moiety show fluorescence that is extremely sensitive to solvent polarity (Weber, G., and Farris, F. J. (1979) Biochemistry 18, 3075–3078). We have synthesized and characterized 6-acryloyl-2-dimethylaminonaphthalene (Acrylodan) which selectively labels thiol moieties in proteins. The quantum yield of this agent is markedly enhanced after reaction with thiols, and as expected, the fluorescent derivatives are very sensitive to dipolar perturbation from their environments. The usefulness of Acrylodan in the study of "hydrophobic" domains, conformational changes, and dipolar relaxation processes in proteins is demonstrated by measurements of fluorescence spectra and lifetimes of a mercaptoethanol adduct dissolved in different solvents and of adducts of this agent with parvalbumin, troponin C, papain, and carbonic anhydrase.

Fluorescent molecules, whose spectra or quantum yields are markedly sensitive to dipolar influences in their environments, are widely used as reporter probes in the study of putative "hydrophobic pockets" in proteins and to demonstrate conformational changes in macromolecules. Such probes are obviously most useful when small perturbations cause large changes in fluorescence. For this reason the fluorophore Prodan described by Weber and Farris (1) is an especially promising probe since it displays a very large shift in emission wavelength maximum from water to hexane as solvents. As Weber and Farris (1) and McGregor and Weber (2) have pointed out, the marked sensitivity of Prodan derives from the large dipole moment developed in the excited state as a consequence of facile charge delocalization between the 2-dimethylamino moiety and the carbonyl group in the naphthalene's 6-position. One would therefore anticipate that such a fluorophore would truly be a polarity sensor.

Other 6-acetyl-2-dimethylaminonaphthalene derivatives show spectral responses to change in solvent polarity that are essentially identical with those of Prodan. Consequently, they have been used in the study of dipolar relaxation phenomena in proteins and membranes (3). However, all the 6-acetyl-2-dimethylaminonaphthalene derivatives that are commercially available interact noncovalently, which limits their possible usefulness.

We report here the synthesis and characterization of a covalently reacting agent: Acrylodan. This reagent reacts efficiently and selectively with thiols at pH 7.0 in a manner similar to that of aromatic vinyl agents (4).

EXPERIMENTAL PROCEDURES
Preparation of Acrylodan from Prodan—The method of Reich et al. (6) was used for the preparation of ad unsaturated ketone in the conversion of Prodan to Acrylodan.

Twenty milliliters of tetrahydrofuran (distilled from CaH2) were added to a two-neck 50-ml flask equipped with an N2 inlet and a dropping funnel. An N2 atmosphere was established, and the flask was cooled to −78 °C. 0.92 ml (6.0 mmol) of diisopropylamine was added, followed by 3.64 ml of a 1.65 m solution of n-butyllithium in hydrocarbon solvent (6.0 mmol). After 15 min, 1.135 g (5.0 mmol) of Prodan were added in 20 ml of tetrahydrofuran. After an additional 30 min, a solution of 6.0 mmol of PhSeBr (prepared from 0.94 g of PhSeSePh in 10 ml of tetrahydrofuran and 0.16 ml of Br2) was added rapidly. Subsequently, the reaction was quenched in 150 ml of 10% Na2S04 and 30 ml of water and extracted twice with ether. The combined ether extracts were washed with 10% Na2S04 solution, followed by washing with 5% NaHC03 solution, followed by NaHC03 solution. The mixture was stirred at room temperature for 90 min. The reaction mixture was then poured into 200 ml of 2% NaHCO3 solution and extracted twice with ether. The combined ether extract was washed again with 5% NaHCO3 solution, followed by 10% Na2S04 solution. The ether phase was dried over anhydrous Na2S04, filtered, and evaporated to dryness to yield 1.2 g of a yellow solid.

The oil was dissolved in 100 ml of methanol. To the solution was added 15 ml of H2O, 0.42 g of NaHCO3 (5 mmol), and 2.15 g (10 mmol) of NaI2. The mixture was stirred at room temperature for 90 min. The reaction mixture was then poured into 200 ml of 5% NaHCO3 solution and extracted twice with ether. The combined ether extract was washed again with 5% NaHCO3 solution, followed by 10% Na2S04 solution. The ether phase was dried over Na2S04, filtered, and taken to dryness to yield 1.2 g of a yellow solid. The product was recrystallized from 95% EtOH to yield 1.0 g of Acrylodan, melting point 200 °C.
the excitation wavelengths used. While there are clear limitations to
this approach, it provides a valuable tool for studying protein conformation.

Materials for Biomedical Experiments—Parvalbumin and troponin C were prepared by previously published methods (6, 7). Carbonyl anhydrase B was the gift of Dr. Guntam Sanjay, Department of Pharmacology, University of Florida, and papain was purchased from Boehringer Mannheim. All other chemicals were analytical reagent grade from commercial sources.

Fluorescence Labeling—For labeling of the proteins, stock solutions of Acrylodan in either acetone or dimethyl formamide were prepared. The proteins were dissolved in a buffered aqueous solution at pH 7.0 (e.g. 150 mM KCl, 20 mM MOPS) and the reagent added to 2-5 mM excess (final concentration) over that of the protein; protein concentrations generally were 10^-6 M. The reaction was allowed to proceed at 4 °C for 2-12 h, and the progress of the reaction was easily followed by the development of blue-green fluorescence. In general, reactions with Acrylodan proceeded relatively rapidly; 2-4 h of reaction at 4 °C were usually sufficient to achieve maximum labeling. The proteins were separated from excess reagent either by dialysis or gel filtration on a Bio-Gel P-2 column.

2-ME-Acrylodan was synthesized by addition of mercaptoethanol to a solution of the reagent in CH3CN. The reaction was allowed to proceed at room temperature for 4 h, whereupon the acetone was evaporated off under a stream of nitrogen and the product purified by high pressure liquid chromatography.

Measurement of Absorption and Fluorescence Properties—Absorption spectra were recorded on a Cary 219 spectrophotometer. Fluorescence spectra were recorded on an SLM 4800 fluorometer, and technical spectra were then corrected for grating transmission and detector response. Fluorescence lifetimes were measured either on the SLM 4800 instrument or an SLM subnanosecond phase fluorometer by use of the cross-correlation method described by Spencer and Weber (8). For these lifetime measurements, a solution of 1,4-bis[2-(5-phenyloxazo)-1benzene in ethanol (r = 1.35 msc) was used as a reference to minimize wavelength-dependent effects in the phototube response, and a polarizer was placed in the emission path and oriented to appropriate magic angles for either vertically polarized or unpolarized excitation to eliminate effects on r due to Brownian rotation. For measurement of lifetime across fluorescence emission bands, wavelengths were selected with 5 nm band pass interference filters centered on the wavelength of interest. Anisotropies were measured as described by Weber and Bulajouzian (9).

Corrected fluorescence emission spectra of the Acrylodan derivative were used to estimate the fluorescence quantum yields of these derivatives. The quantum yields determined were all relative to that of quinine sulfate dissolved in 1.0 M sulfuric acid which has been measured by Melhuish (10) and found to be 0.56 for λex = 365 nm. The quantum yield of the Acrylodan adducts was calculated from the relation

\[ \phi_e = \frac{F(\lambda_{max}, A_{max})}{F(\lambda_{max}, A_0)} \]

where \( \phi_e \) and \( \phi_0 \) refer to the quantum yields of the adduct and quinine sulfate, respectively; \( F(\lambda_{max}, A_{max}) \) and \( F(\lambda_{max}, A_0) \) refer to the areas under the corrected emission spectra (with all the instrumental conditions held the same for measurements of both spectra); \( A_{max} \) and \( A_0 \) refer to the absorbances of the adduct and quinine sulfate, respectively, at the excitation wavelengths used. While there are clear limitations to the accuracy of this simple method, the errors are not large and do not materially affect the interpretation of our data.

RESULTS AND DISCUSSION

Acrylodan itself exhibits only a very low fluorescence quantum yield even when dissolved in nonpolar aprotic solvents; we attribute this to deactivating effects of the double bond conjugated to the carbonyl group. However, thiol adducts of Acrylodan are brightly fluorescent. The absorption spectra and fluorescence of the mercaptoethanol adduct of Acrylodan are very similar to that of Prodan (1) in terms of both the λmax of absorption and emission maxima in various solvents, and the sensitivity of emission to solvent polarity (Table I). (The molar extinction coefficients for Acrylodan in ethanol at λmax of 387 nm are 14,600 M^-1 cm^-1 and 6,200 M^-1 cm^-1 at 290 nm in water, the molar extinction at 360 nm is 12,900 M^-1 cm^-1.) These values may be compared with those of Prodan given by Weber and Farris (1) of 18,400 and 3,300 M^-1 cm^-1 at 360 nm and 294 nm, respectively, for ethanol solutions and 14,500 M^-1 cm^-1 for aqueous solutions of Prodan at 355 nm.) This similarity was clearly demonstrated by use of a Lippert plot (1, 11) of the corrected Stokes shift (in wave numbers) plotted against the orientational polarizability (\( \Delta \)) of solvents. The thioether moiety of the adduct therefore does not significantly perturb the fundamental fluorescence properties of the 6-acyl-2-dimethylaminonaphthalene moiety.

All the proteins labeled in this study contained a single thiol group, and the absorbance of each derivatized protein was in keeping with a fluorophore:protein labeling ratio of < 1. Two of the labeled proteins, parvalbumin and troponin C, were subsequently reduced and carboxamidomethylated, and tryptic peptides were prepared. These were subjected to high pressure liquid chromatography on a reverse phase (C18 Bondapak) column, and the tryptic peptides were detected with both a UV detector (at 206 nm) and a fluorescence detector (λex = 380, λem > 470 nm). For both Thn-C and parvalbumin, only a single fluorescent peptide was found.

Both the absorption and fluorescence spectra of the protein-Acrylodan adducts were significantly different from those of the 2-ME-Acrylodan derivative; the absorption maxima of the parvalbumin and Thn-C derivatives were 374 and 383 nm, respectively, compared to 390 nm for the 2-ME-Acrylodan adduct dissolved in a buffered aqueous solution at pH 7.0. Similar blue shifts in the absorption spectra (relative to the absorption spectrum of either the 2-ME adduct or of Prodan in water) were found in the papain and carbonic anhydrase adducts.

The papain-Acrylodan adduct had the most blue shifted fluorescence emission spectrum (λmax was 491 nm); Table I; the fluorescence emission of carbonic anhydrase and parvalbumin (in the absence of Ca2+) was rather more red shifted, and troponin C (in the absence of Ca2+) had the highest wavelength of emission of the four proteins (Table I). Both parvalbumin and troponin C bind divalent metal ions and are known from CD spectral data to undergo substantial alteration in conformation when Ca2+ binds (12, 13). It is also known that when Ca ions bind to these proteins, their CD spectra change concomitantly with fluorescence of probes attached to their respective thiol residues (14-16). The characteristics of the spectra of these proteins in the presence and absence of Ca2+, respectively, are given in Table I; they show that in both proteins, Ca2+ causes a shift in the emission toward the red, and there is a concomitant decrease in fluorescence yields. When the Thn-C-Acrylodan adduct is dissolved in 6 M guanidine hydrochloride (i.e. the protein is completely

**Fig. 1. Structure of Acrylodan.** The NMR assignments of the hydrogen atoms denoted by subscripts A, B, and C are given in the text.

\[ F. G. Prendergast, unpublished data. \]
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Table I

| Solvent      | Emission (λmax) | Bandwidth | Quantum Yield (Φ) | τ0 (nsec) | τm (nsec) |
|--------------|----------------|-----------|-------------------|-----------|-----------|
| Water        | 540 2344        | 0.18      | 1.28 1.65         |           |           |
| Dioxane      | 435 3068        | 0.83      | 2.47 2.92         |           |           |
| Acetonitrile | 468 3223        | 0.78      | 3.35 3.61         |           |           |
| Ethanol      | 502 3006        | 0.79      | 3.24 3.56         |           |           |
| Methanol     | 513 2967        | 0.57      | 3.28 3.41         |           |           |
| Parvalbumin  | 498 3875        | 0.70      | 3.50 4.15         |           |           |
| Parvalbumin + Ca | 511 3078    | 0.62      | 1.87 3.11         |           |           |
| Parvalbumin + D$_2$O + Ca | 510 3180  | 0.72 2.20 | 3.75 3.82 |           |           |
| Troponin C   | 510 3460        | 0.62      | 3.35 4.0          |           |           |
| Troponin C + Ca | 517 3071       | 0.50      | 1.95 3.25         |           |           |
| Papain       | 491 3411        | 0.71      | 4.07 4.33         |           |           |
| Carbonic anhydride | 501 3800  | 0.63      | 3.19 3.59         |           |           |

* These are emission wavelength maxima taken from corrected emission spectra.
* These are the band width of the various emission spectra at half-band height.
* Φ are the quantum yields of the Acrylodan adducts and were measured as described in the text.
* The fluorescence lifetimes τ0 and τm represent the fluorescence lifetimes determined by phase delay and relative modulation, respectively, as described by Spencer and Weber (8). These lifetimes were measured with a modulation frequency of 30 MHz for each lifetime measurement, the entire emission was captured (although Rayleigh and Raman scattering were eliminated) by use of appropriate Schott KV (cut-on) filters in the emission path.

denatured), the fluorescence emission λmax shifts to 532, from which we infer that the fluorophore has been completely exposed to the solvent.

From these data it would appear that in all these proteins the 6-acyl-2-dimethylaminonaphthalene moiety attached to the thiol has been sequestered in hydrophobic pockets (to use common parlance), whence the blue shifts in absorption and emission maxima. We infer that the observed red shift in emission wavelength when Ca binds to either parvalbumin or troponin C indicates a Ca$^{2+}$-induced configurational change in the protein which causes extrusion of the fluorophore further into the solvent. Additional information on the dipolar character of the fluorophore binding domains may be gleaned from examination of the half-widths of the emission spectra. An increase in emission spectral half-widths (relative to the half-width of emission of the fluorophore in nonviscous solvents) indicates incomplete dipolar relaxation of the environment around the fluorophore (1, 2). In nonviscous solvents, where dipolar relaxation (solvent reorientation) times are faster than the rate of fluorescence emission, the spectrum of a fluorophore displays a minimum spectral half-width. Not surprisingly, therefore, the half-widths of the 2-ME-Acrylodan adduct in various solvents are not markedly different (Table I). In contrast, the spectral half-band widths for all the proteins (except the complexes of Ca$^{2+}$ with labeled parvalbumin and troponin C) are generally several hundred wave numbers greater than the fluorescence of the 2-ME-Acrylodan adducts dissolved in solvents, especially water. This finding is consonant with that of Weber and Farris (1) for Prodan bound to bovine serum albumin, and indicates incomplete dipolar relaxation around the fluorophore. The half-widths observed for the parvalbumin-Ca and Tn-C-Ca complexes are indicative of enhanced dipolar relaxation rates relative to those observed for the Ca-free proteins. This is in agreement with our earlier inference that Ca$^{2+}$ induces an extrusion of the fluorophore into bulk solvent (see above). There is also an obvious difference in the domains of the fluorophores bound to parvalbumin and Tn-C, respectively, despite the known structural homologies between the two proteins (11). Further and unequivocal evidence of dipolar relaxation was obtained by demonstration of progressive increase of τ, decrease in steady state anisotropies, and inversion of τ0 and τm (τ measured by phase and modulation, respectively) as the fluorescence emission spectrum is traversed from the blue to the red edge, and finally by differential phase fluorometry by use of the criteria of Lakowicz and Czerk (17, 18).

In most fluorescently labeled proteins, the location of the fluorophore and hence its physical relation to the protein's amino acid side chains and degree of exposure to solvent are not known with certainty. It is therefore difficult at best to make judgments as to the factors contributing to the apparent polarity of the binding pocket and to the apparent dipolar relaxation reported by the probe.

To assess the usefulness and the sensitivity of the fluorescence of Acrylodan adducts in investigating the molecular basis of dipolar relaxation processes, we measured the effects of D$_2$O on the fluorescence of parvalbumin-Acrylodan. The results are given in Fig. 2. The data show that for the parvalbumin-Acrylodan adducts in D$_2$O, the emission of the Ca-free protein is red shifted relative to that in water (by 240 cm$^{-1}$) and the fluorescence intensity and half-band width slightly increased. When calcium is added, both spectra shift to the red, but the emission spectra in H$_2$O and D$_2$O are now identical, i.e. half-band widths differ little, if at all. However, quantum yields of the parvalbumin-Ca complex in D$_2$O are about 15\% greater than in H$_2$O and the fluorescence lifetimes are correspondingly longer. (Measurements of CD spectra showed protein conformation to be identical in H$_2$O and D$_2$O solutions.) These are but preliminary data, but they do suggest that solvent molecules have ready access to the fluorophore and that the apparent dipolar relaxation derives, at least in part and probably in good measure, from the effects of solvent molecules. This ought not to be surprising since water molecules adsorbed to proteins may have relaxation times similar to the time required for fluorescence emission, i.e. are in the range 1-5 nsec (19). Small deuterium "isotope" effects such as those observed here could then derive from slower relaxation times of protein-bound D$_2$O molecules (compared to H$_2$O) or slower proton exchange rates with the fluorophore. Either way, the solvent molecules probably would play a major if not the dominant role in determining apparent binding site polarity and dipolar relaxation. The role of solvent will be more directly assessed through use of differential phase fluorometry (20) and phase suppression spectroscopy (21).

Finally, Dodiuk and Kosower (22) and Dodiuk et al. (23) have asserted that fluorescent probes cannot yield information regarding binding site polarity (cf. Ref. 24). Their com-

![Fig. 2. Corrected fluorescence emission spectra of the Acrylodan adduct of parvalbumin in buffered solutions containing either H$_2$O (-- --) or D$_2$O (——). A and B depict the parvalbumin in the absence of Ca$^{2+}$ and C and D the parvalbumin adduct in the presence of Ca$^{2+}$.](http://www.jbc.org/Downloadedfrom)
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ment relates to fluorophores such as anilinonaphthalenesulfonates and toluidinonaphthalenesulfonates in which the fluorescence properties are determined by both intramolecular configuration (of substituents that can undergo torsional motions) and the dipolar properties of the probes’ environment. However, there are no flexible substituents on the 6-acyl-2-dimethylaminonaphthalene group that will significantly influence its fluorescence. The spectral shifts observed when this fluorophore is placed in different solvents (1, 2) therefore truly reflect changes in polarity.

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