Anal. Chem. **2000,** *72,* 227−233

Effects of Ethanol Volume Percent on Fluorescein-Labeled Spinach Apo- and Holocalmodulin

Meagan A. Doody, Gary A. Baker, Siddharth Pandey, and Frank V. Bright*

Department of Chemistry, Natural Sciences Complex, University at Buffalo, The State University of New York, Buffalo, New York 14260-3000

We report the effects of EtOH volume percent (0−70%) on spinach apo- and holocalmodulin that have been site-selectively labeled with fluorescein (F). In these experiments, calmodulin (CaM) has one F reporter group attached to Cys-26, and this site is located immediately adjacent to one of the four primary Ca^{2+}-binding sites (EF hands). The optimum analytical CaM-F sensitivity to Ca^{2+} occurs between ∼10 and 30% EtOH. Our results also show that added EtOH causes changes in CaM and these changes are surprisingly different for apo- and holo-CaM. Apo-CaM-F appears to lose one of its two waters of hydration at ∼20% EtOH and retains one water of hydration between ∼20 and 70% EtOH. In apo-CaM-F, the semiangle that describes the range over which the fluorescein reporter group can precess remains essentially constant (42 ± 2°) between 0 and 70% EtOH. This shows that the fluorescein reporter group precessional freedom in apo-CaM-F is not affected significantly by EtOH. Holo-CaM-F also appears to lose one water of hydration at ∼20−30% EtOH but then appears to denature as the EtOH volume percent increases. The fluorescein reporter group semiangle within holo-CaM-F decreases from 43 ± 1° in neat aqueous buffer to 36 ± 1° at 70% EtOH. This shows that holo-CaM-F is less nativelike and the EF hand "closes down" about the fluorescein reporter group in holo-CaM-F as the EtOH volume percent increases.

Protein structure and stability depend on the physicochemical properties of the local environment that surrounds the biomolecule. For example, one can disrupt protein structure by adding certain cosolvents (e.g., EtOH) or cosolutes (e.g., urea) to water.[1−3] Despite this, proteins and nonaqueous solvents are intentionally brought together. For example, alcohol/water mixtures are commonly used as solvents in electrospray ionization mass spectrometry (ESI-MS) of biomolecules.[4] Alcohol/water microdomains also exist within sol−gel-derived glasses,[5−9] and these glasses are commonly used to immobilize proteins.[10−14]

ESI-MS is a powerful tool for analyzing samples that contain macromolecules[4] and for studying noncovalent complexes without causing excessive dissociation and/or fragmentation.[15−17] However, there are data[1−3,18] to argue that protein conformations/functions may be very different in the types of alcohol/water mixtures that are commonly used in ESI-MS. In fact, there is ESI-MS literature[19−22] showing that some proteins (e.g., calmodulin) are affected by (1) how they are sprayed, (2) the spray solution composition, (3) the specific protein charge state from which the analyst chooses to extract their quantitative information, and (4) the detection mode (negative vs positive ion) that one uses.

Several research groups have been exploring the potential of sol−gel-derived glasses for biosensor development.[9−14,23−30] Ellerby

* Corresponding author. Phone: 716-645-6800, ext. 2162. Fax: 716-645-6963. E-mail: chefvb@acsu.buffalo.edu.

(1) Cascio, M.; Wallace, B. A. *Anal. Biochem.* **1995**, *227*, 90−100.
(2) Zhong, L.; Johnson, W. C., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4462−5.
(3) VanGunsteren, W. F.; Karplus, M. *Nature* **1981**, *293*, 677−8.
(4) Lorenz, S.; Maziarz, E. P.; Wood, T. *Appl. Spectrosc.* **1999**, *53*, 18A−36A.
(5) Sanchez, C.; Ribot, F. *New J. Chem.* **1994**, *18*, 1007−47.
(6) Levy, D.; Esquivias, L. *Adv. Mater.* **1995**, *7*, 120−9.
(7) Shi, Y.; Seliskar, C. J. *Chem. Mater.* **1997**, *9*, 821−9.
(8) Hüsing, N.; Schubert, U. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 22−45.
(9) Ellerby, L.; Nishida, C.; Nishida, F.; Yamanka, S.; Dunn, B.; Valentine, J. S.; Zink, J. *Science* **1992**, *255*, 1113−5.
(10) Avnir, D.; Braun, S.; Lev, O.; Ottolenghi, M. *Chem. Mater.* **1994**, *6*, 1605−14.
(11) Zink, J. I.; Valentine, J. S.; Dunn, B. *New J. Chem.* **1994**, *18*, 1109−15.
(12) Dave, B. C.; Miller, J. M.; Dunn, B.; Valentine, J. S.; Zink, J. I. *J. Sol-Gel Sci. Technol.* **1997**, *8*, 629−34.
(13) Dave, B. C.; Dunn, B.; Valentine, J. S.; Zink, J. I. *ACS Symp. Ser.* **1996**, *622*, 351−65.
(14) Dave, B. C.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Anal. Chem.* **1994**, *66*, 1120A−7A.
(15) Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1991**, *113*, 8534−5.
(16) Loo, J. A. *Mass Spectrom. Rev.* **1997**, *16*, 1−23.
(17) Biemann, K. *Annu. Rev. Biochem.* **1992**, *61*, 977−1010.
(18) Hirota, N.; Mizuno, K.; Goto, Y. *J. Mol. Biol.* **1998**, *275*, 365−78.
(19) Lafitte, J. P.; Grassy, G.; Haiech, J.; Calas, B. *Biochemistry* **1995**, *34*, 13825−32.
(20) Nemirovskiy, O.; Ramanathan, R.; Gross, M. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 809−12.
(21) Hu, P.; Loo, J. *J. Mass Spectrom.* **1995**, *30*, 1076−82.
(22) Hu, P.; Ye, Q.; Loo, J. *Anal. Chem.* **1994**, *66*, 4190−4.
(23) Braun, S.; Rappoport, S.; Zusman, R.; Avnir, D.; Ottolenghi, M. *Mater. Lett.* **1990**, *10*, 1−5.
(24) Crumbliss, A. L.; Stonehuerner, J.; Henkens, R. W.; O'Daly, J. P.; Zhao, J. *New J. Chem.* **1994**, *18*, 327−39.
(25) Brennan, J. D. *Appl. Spectrosc.* **1999**, *53*, 106A−21A.
(26) Ingersoll, C. M.; Bright, F. V. *Anal. Chem.* **1997**, *69*, 403A−8A.
(27) Wang, R.; Narang, U.; Prasad, P. N.; Bright, F. V. *Anal. Chem.* **1993**, *65*, 2671−5.
(28) (a) Jordan, J. D.; Dunbar, R. A.; Bright, F. V. *Anal. Chem.* **1995**, *67*, 2436−43. (b) Gottfried, D. S.; Kagan, A.; Hoffman, B. M.; Friedman, J. M. *J. Phys. Chem. B* **1999**, *103*, 2803−7.
(29) Baker, G. A.; Jordan, J. D.; Bright, F. V. *J. Sol-Gel Sci. Technol.* **1998**, *11*, 43−54.

10.1021/ac990551b CCC: $19.00
Published on Web 11/25/1999

et al.[9] raised initial concern about the alcohol that is released as a byproduct from the sol−gel process and its leading to biomolecule denaturation. This particular concern is exacerbated because the water-to-alcohol molar ratio within a typical sol−gel-derived xerogel changes continuously as the xerogel ages and evolves[9] *and* the three-dimensional network within the glass can also influence a protein's dynamics.[28] Despite these concerns, many different biomolecules have been successfully sequestered within sol−gel-derived glasses and they appear to function more or less as they do when they are free in solution.[10−14,25−30]

Calmodulin (CaM, 16.7 kDa, 148 amino acid residues) is a Ca^{2+}-selective protein that regulates a wide range of enzymes that are themselves involved in cellular motility, ion transport, metabolism, and muscle contraction.[20,31−36] CaM contains four primary α-helix−loop−α-helix subdomains known as "EF hands" that bind Ca^{2+}.[22,37−39] CaM is shaped like a dumbbell,[40−42] and the protein has two discrete EF hands located in each of its two globular domains.[31] The four EF hands are designated I, II, III, and IV, respectively, as one moves along the peptide backbone from the protein N− to C termini. The reported Ca^{2+}−CaM binding affinities are in the 5×10^5 to 5×10^6 M^{-1} range.[43] The CaM structure, its metal ion binding properties, and its dynamics have been studied previously by fluorescence,[33,44,45] Raman,[34] circular dichroism,[32] X-ray crystallography,[46,47] nuclear magnetic resonance,[35] and mass spectrometry methods.[19−22,48]

Several ESI-MS reports[19−22] on CaM show evidence for anywhere from zero to six *auxiliary* Ca^{2+}-binding sites in addition to the four known EF-hand-based binding sites within CaM when it is sprayed from alcohol/water solutions. Hu et al.[22] reported on CaM dissolved in water (neat or 2.5 mM ammonium bicarbonate, pH 8) by using negative-ion-mode detection and showed that "if the Ca^{2+} ion concentration is too high (e.g., 5 mM), a majority of the calmodulin molecules bind to four calcium ions, but a small amount of nonspecific Ca^{2+} attachment (e.g., five Ca^{2+} ion binding) can also occur". Interestingly, inspection of the inset to Figure 2b in ref 22 clearly shows mass peaks that are consistent with 5:1 *and* 6:1 Ca^{2+}:CaM complexes when the Ca^{2+} level is at only 1 mM. In a follow-up study, Hu and Loo[21] used negative-ion-mode-detection ESI-MS and showed that "the amount of calcium binding indicated in the ESI mass spectra varies with charge state". These same authors also reported that there was little effect of added MeOH on the ESI mass spectra.

The Hu results are somewhat counter to the work of Milos et al.,[49] which requires the existence of the four primary Ca^{2+}-binding sites (those associated with the four EF hands) *plus* several auxiliary Ca^{2+}-binding sites to properly model the available thermodynamic data. Lafitte et al.[19] attempted to reconcile the differences between the Hu and Milos work and suggested that the absence of any reported "auxiliary sites" in the Hu work could be related to the use of negative-ion-mode detection. Lafitte et al. used positive-ion-mode detection ESI-MS, and they reported that there were six to eight total Ca^{2+} atoms bound to CaM when it was sprayed from a $MeOH/H_2O$ (20/80, v/v) mixture. Nemirovskiy et al.[20] observed the binding of as many as seven Ca^{2+} ions to the noncovalent complex between calcium-activated CaM and melittin by using negative-ion-mode-detection ESI-MS when the complex was sprayed from a $MeOH/H_2O$ (20/80, v/v) solution.

Gross and co-workers[48] recently used ESI-MS to perform H/D exchange experiments on apo- and holo-CaM. In apo-CaM, ∼115 protons were exchanged for deuterons in 60 min. However, when CaM was titrated with Ca^{2+} to form holo-CaM, the number of protons that exchanged in 60 min decreased by ∼24. Gross and co-workers argued that the difference in the number of H/D exchanges arose from CaM adopting "a less solvent accessible structure as the protein binds with Ca^{2+}".

Given all the evidence[19−22] to suggest that alcohol affects CaM, our own interest in CaM as a biorecognition element,[45] and the fact that sol−gel-derived xerogels contain varying levels of alcohol,[5−9] we aim to determine how alcohol volume percent actually influences the behavior of a single, known Ca^{2+}-binding site within apo- and holo-CaM when they are dissolved in solution. Toward this end, we report here on the static and time-resolved fluorescence from spinach apo- and holo-CaM that have been site-selectively labeled at cysteine-26 with fluorescein-5-maleimide as a function of EtOH volume percent (0−70%). Fluorescein was chosen as the label (over rhodamine and acrylodan) because our previous work[45] showed that it yielded a species whose emission changed the greatest on going from apo to holo. The fluorescence experiments allow us to determine how added EtOH affects (1) the local microenvironment surrounding the fluorescein reporter group, (2) the global protein mobility (i.e., molar volume or degree of hydration), and (3) the fluorescein's mobility within EF hand I.

THEORY

All dynamic measurements were performed in the frequency domain. The interested reader is directed to refs 50−52 for full

(30) Hartnett, A. M.; Ingersoll, C. M.; Baker, G. A.; Bright, F. V. *Anal. Chem.* **1999**, *71*, 1215−24.
(31) Babu, Y. S.; Bugg, C. E.; Cook, W. J. *J. Mol. Biol.* **1988**, *204*, 191−204.
(32) Crouch, T. H.; Klee, C. B. *Biochemistry* **1980**, *19*, 3692−8.
(33) Gryczynski, I.; Steiner, R. F.; Lakowicz, J. R. *Biophys. Chem.* **1991**, *39*, 69−78.
(34) Seaton, B. A.; Head, J. F.; Lord, R. C.; Petsko, G. A. *Biochemistry* **1983**, *22*, 973−8.
(35) Ehrhardt, M. R.; Urbauer, J. L.; Wand, A. J. *Biochemistry* **1995**, *24*, 2731−8.
(36) Cheung, W. Y. *Science* **1980**, *207*, 19−27.
(37) Lin, Y. M.; Liu, Y. P.; Cheung, W. Y. *J. Biol. Chem.* **1974**, *249*, 4943−54.
(38) Watterson, D. M.; Harrelson, W. G., Jr.; Keller, P. M.; Sharief, F.; Vanaman, T. C. *J. Biol. Chem.* **1976**, *251*, 4501−13.
(39) Wolff, D. J.; Poirier, P. G.; Brostrom, C. O.; Brostrom, A. *J. Biol. Chem.* **1977**, *252*, 4108−17.
(40) Meador, W.; Means, A. R.; Quiocho, F. *Science* **1992**, *257*, 1251−5.
(41) Urbauer, J. L.; Short, J. H.; Dow, L. K.; Wand, A. J. *Biochemistry* **1995**, *34*, 8099−109.
(42) de la Torre, J. G.; Bloomfield, V. A. *Biopolymers* **1977**, *16*, 1779−93.
(43) You, G.; Buccigross, J. M.; Nelson, D. *J. Inorg. Biochem.* **1990**, *38*, 117−25.
(44) Chabbert, M.; Piemont, E.; Prendergast, F. G.; Lami, H. *Arch. Biochem. Biophys.* **1995**, *322*, 429−36.
(45) Watkins, A. N.; Bright, F. V. *Appl. Spectrosc.* **1998**, *52*, 1447−56.
(46) Finn, B. E.; Forsen, S. *Structure* **1995**, *3*, 7−11.
(47) Babu, Y. S.; Sack, J. S.; Greenbough, T. G.; Bugg, C. E.; Means, A. R.; Cook, W. J. *Nature* **1985**, *315*, 37−40.
(48) Nemirovsky, O.; Giblin, D. E.; Gross, M. L. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 711−8.
(49) Milos, M.; Comte, M.; Schaer, J.; Cox, J. *J. Inorg. Biochem.* **1989**, *36*, 11−25.
(50) Jameson, D. M.; Gratton, E.; Hall, R. D. *Appl. Spectrosc. Rev.* **1984**, *20*, 55−106.
(51) Lakowicz, J. R.; Lakczo, G.; Gryczynski, I.; Szmacinski, H.; Wiczyk, W. *J. Photochem. Photobiol., B* **1988**, *2*, 295−311.

details on the exact data acquisition methods and the associated theory.

Fluorescence Intensity Decay Kinetics. The time-resolved decay of fluorescence intensity, $I(t)$, is generally given by[50−52]

$$I(t) = \sum_{i=1}^{n} f_i e^{-t/\tau_i} \quad (1)$$

In this expression f_i represents the fractional intensity of the ith component/species possessing excited-state fluorescence lifetime τ_i.

The average excited-state fluorescence lifetime ($\langle\tau\rangle$) is simply $\sum f_i\tau_i$.

Decay of Fluorescence Anisotropy. The time-resolved fluorescence anisotropy decay, $r(t)$, of a fluorescently labeled macromolecule provides direct information on the macromolecule's "global" rotational reorientation dynamics and the fluorescent reporter group's local motion.[50−52] Previous work on labeled proteins shows that $r(t)$ is often well described by[53,54]

$$r(t) = \sum_{i=1}^{2} r_0(\beta_i e^{-t/\phi_i}) \quad (2)$$

where r_0 represents the limiting fluorescence anisotropy in the absence of any fluorophore rotational reorientation, β_1 is the fractional contribution to the total anisotropy decay arising from the faster rotational reorientation time, ϕ_1, and β_2 is the fractional contribution to the total anisotropy decay that is associated with the global protein rotational reorientation time, ϕ_2. The true rotational reorientation time for the fluorescent probe when it is attached to a macromolecule is given by[54]

$$\phi_{\text{local}} = 1/(1/\phi_1 - 1/\phi_2) \quad (3)$$

One can also assign a semiangle, θ, to the range of angles over which the fluorophore can precess during its excited-state fluorescence lifetime:[53,55]

$$\theta = \cos^{-1}[{}^1/_2((8\beta_2{}^{1/2} + 1)^{1/2} - 1)] \quad (4)$$

If the fluorophore precessional motion becomes totally restricted, θ will approach zero and the observed anisotropy decay will become dominated by the protein global motion. If the fluorescent reporter group moves independently of the protein global motion, θ will increase toward 90° and the anisotropy decay will be dominated by the faster rotational reorientation time.

EXPERIMENTAL SECTION

Reagents. The following chemicals were used: fluorescein-5-maleimide (F5M, Molecular Probes); 3-morpholinopropanesulfonic acid (MOPS), ethylene glycol bis(β-aminoethyl) ether *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), essentially salt-free, lyophilized powder phosphodiesterase 3′:5′-cyclic nucleotide activator (calmodulin, CaM) from spinach (Sigma); calcium chloride, sodium chloride, dimethylformamide (DMF) (Aldrich); and ethanol (200 proof, Quantum Chemical Corp.). All reagents were of the highest purity available from the indicated vendors, and they were used as received. Aqueous solutions were prepared from doubly distilled−deionized water (Millipore).

Preparation of Fluorescein-Labeled CaM. The site-selective labeling of spinach CaM with F5M has been described elsewhere.[45] Throughout the remainder of this paper we use "CaM-F" to denote spinach CaM that has been site-selectively labeled at Cys-26 with F5M.

Sample Preparations. All samples for study contained 2 μM CaM-F dissolved in a 10 mM pH 7.0 MOPS buffer containing 0.5 M NaCl and 2 mM EGTA with varying volume percentages of EtOH. Apo- and holo-CaM-F samples were prepared immediately prior to our performing the experiments. Holo-CaM-F was prepared by taking the apo-CaM-F samples and adding 20 mM $CaCl_2$. Steady-state fluorescence studies revealed that all samples reached equilibrium within 5 min of our adding EtOH, remaining constant during an experiment.

Fluorescence Measurements. Steady-state measurements were carried out with an SLM 8100 spectrofluorimeter using a Xe arc lamp as the excitation source. The excitation and emission spectral band-passes were 4 nm for all experiments. All spectra were blank-corrected, and the blank contributions never exceeded 1% of the sample fluorescence. For all steady-state fluorescence experiments, we acquired the entire emission profile from 460 to 600 nm, while exciting at 450 nm, and we integrated the area under the emission profile. The fluorescein emission profiles did not exhibit any significant spectral shift as EtOH was added.

Frequency-domain fluorescence experiments were performed on an SLM 48000 MHF spectrofluorimeter. The excitation source was a CW argon ion laser (Coherent model Innova 90-6) operating at 488.0 nm. An interference filter (Oriel) was placed in the excitation beam path to minimize extraneous plasma tube superradiance from reaching the emission detector. Emission was observed through a 515 nm long pass filter (Oriel). The blanks under these experimental conditions were always less than 1% of the sample fluorescence. The electro-optic modulator (Pockels) was operated at a 5 MHz repetition rate. Data were acquired at 5 MHz intervals up to 150 MHz. We typically acquired 10−20 complete data sets per sample, and at least three discrete samples were prepared and studied under each set of conditions. All results are reported as the mean for a given series of measurements. Error bars represent one standard deviation from the mean value. We used fluorescein dissolved in 0.1 N NaOH ($\tau_{\text{ref}} = 4.00$ ns) and rhodamine 6G dissolved in neat ethanol ($\tau_{\text{ref}} = 3.85$ ns) as our reference lifetime standards. Under our experimental conditions, the recovered CaM-F decay times were independent of the reference fluorophore. The sample temperature was 22 ± 1 °C.

(52) (a) Bright, F. V.; Betts, T. A.; Litwiler, K. S. *Crit. Rev. Anal. Chem.* **1990**, *21*, 389−405. (b) Bright, F. V. *Appl. Spectrosc.* **1995**, *49*, 14A−9A.

(53) Wang, R.; Sun, S.; Bekos, E. J.; Bright, F. V. *Anal. Chem.* **1995**, *67*, 149−59.

(54) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Plenum Press: New York, 1983; Chapter 6.

(55) Munro, I.; Pecht, I.; Stryer, L. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 56−60.

(56) Haugland, R. P., Ed. *Handbook of Fluorescence Probes and Research Chemicals*, 6th ed.; Molecular Probes: Eugene, OR, 1996.

(57) Sjöback, R.; Nygren, J.; Kubista, M. *Spectrochim. Acta, Part A* **1995**, *51*, L7−L21.

(58) Zhao, Z.; Shen, T.; Xu, H. *Spectrochim. Acta, Part A* **1989**, *45*, 1113−6.

(59) Leonhardt, H.; Gordon, L.; Livingston, R. *J. Phys. Chem.* **1971**, *75*, 245−9.

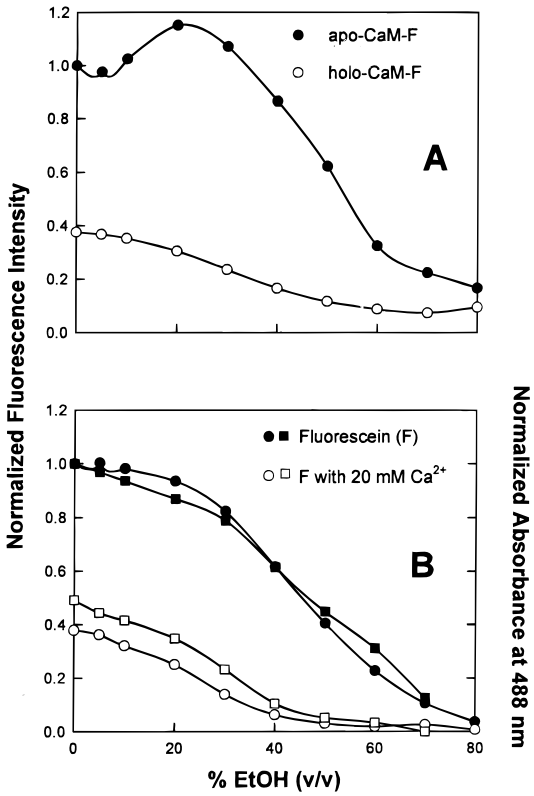

Figure 1. Effects of EtOH volume percent and Ca^{2+} on the electronic absorbance and fluorescence from free fluorescein (F) and fluorescein-labeled CaM (CaM-F): (A) normalized fluorescence results for apo- and holo-CaM-F; (B) normalized fluorescence (●, ○) and absorbance at 488 nm (■, □) results for free fluorescein. The curves between points are merely aids for the eye.

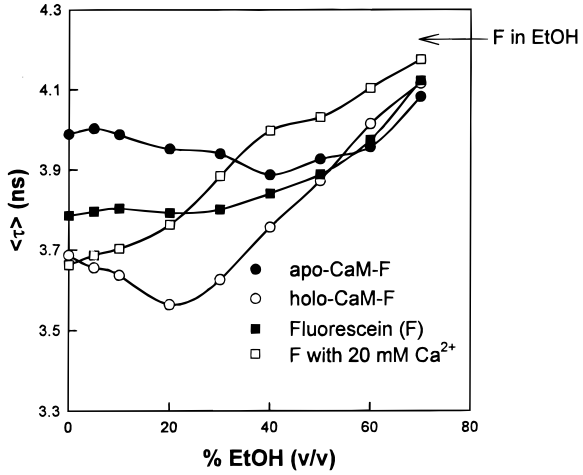

Figure 2. Effects of EtOH volume percent on the average excited-state fluorescence lifetime ($\langle\tau\rangle$) for CaM-F and free fluorescein in the absence and presence of Ca^{2+}. The excited-state fluorescence lifetime of free fluorescein dissolved in neat EtOH is denoted by the arrow. The curves between points are merely aids for the eye.

RESULTS AND DISCUSSION

Steady-State Absorbance and Fluorescence. Our fluorescent probe is selective for free thiols,[45,56] and there is only one free cysteine residue in spinach CaM at position 26 in EF hand I.

Figure 1A illustrates the effects of added EtOH on the fluorescein reporter group fluorescence in apo- and holo-CaM-F. These results show that apo-CaM-F always exhibits a greater fluorescence relative to holo-CaM-F at all EtOH volume percentages studied here. This suggests that Ca^{2+} associates with CaM-F and effects some degree of selective conformational change[45] even when the CaM-F is dissolved in 70% EtOH. These results also show that the maximum CaM-F sensitivity (difference between apo- and holo-CaM-F fluorescences) to Ca^{2+} occurs between ~10 and 30% EtOH.

We explored the EtOH-induced decrease in the CaM-F emission in more detail by investigating (Figure 1B) the steady-state absorbance (squares) and fluorescence (circles) of free fluorescein (F) dissolved in ethanol/buffer mixtures with and without added Ca^{2+}. In solution, fluorescein can exist as a dianion, a monoanion, a neutral species, and/or a cation depending on the solvent pH, ionic strength, and dielectric constant.[57−61] Previous work has also shown that the colorless leuco form of fluorescein is favored in neat EtOH.[58] Inspection of the data presented in Figure 1B shows that the emission tracks the fluorescein absorbance, suggesting that the added EtOH primarily leads to an increase in the leuco dye formation which results in a concomitant decrease in the fluorescein absorbance and emission. The decrease in the fluorescein emission/absorbance on adding Ca^{2+} suggests that there may also be some type of selective interaction between Ca^{2+} and the probe. The latter issue was not explored further.

Time-Resolved Fluorescence Intensities. The time-resolved intensity decay kinetics for apo- and holo-CaM-F are well-described[62] by a double-exponential-decay law at all EtOH loadings studied here. These results are in line with previous work from our laboratory on CaM-F dissolved in neat buffer.[45] Figure 2 summarizes the effects of EtOH volume percent on the average excited-state fluorescence lifetime ($\langle\tau\rangle$) of free fluorescein in the absence (■) and presence (□) of Ca^{2+} and of apo- (●) and holo-CaM-F (○). The imprecision in the measured $\langle\tau\rangle$ values is on the order of 0.03 ns. These results show that the fluorescein $\langle\tau\rangle$ in the absence of Ca^{2+} (■) remains constant until we reach ~40−50% EtOH; $\langle\tau\rangle$ then increases above 40−50% EtOH and moves toward the $\langle\tau\rangle$ for fluorescein dissolved in neat EtOH (arrow, "F in EtOH"). The $\langle\tau\rangle$ for free fluorescein in the presence of Ca^{2+} (□) begins about 0.1 ns below the value for free fluorescein without Ca^{2+}; it then steadily increases as the EtOH volume percent increases and also approaches the $\langle\tau\rangle$ for fluorescein dissolved in neat EtOH. The apo-CaM-F $\langle\tau\rangle$ (●) decreases slightly between 0 and ~50% EtOH. Above ~60% EtOH, however, there is a clear increase in the apo-CaM-F $\langle\tau\rangle$ toward the value for fluorescein dissolved in neat EtOH. The $\langle\tau\rangle$ for holo-CaM-F (○), which is 0.3 ns shorter than that for apo-CaM-F in neat buffer to begin with, decreases by 0.2 ns between 0 and 20% EtOH. Above ~20% EtOH, $\langle\tau\rangle$ increases toward the value for fluorescein dissolved in neat EtOH.

The results of these time-resolved intensity decay experiments show that the fluorescein reporter group in CaM-F senses a more

(60) Martin, M. M. *Chem. Phys. Lett.* **1975**, *35*, 105−11.
(61) Commission on Electroanalytical Chemistry. *Pure Appl. Chem.* **1998**, *70*, 1419−22.
(62) Beechem, J. M.; Gratton, E.; Ameloot, M.; Knutson, J. R.; Brand, L. *Topics in Fluorescence Spectroscopy*; Plenum Press: New York, 1991; Vol. 2, Chapter 5.

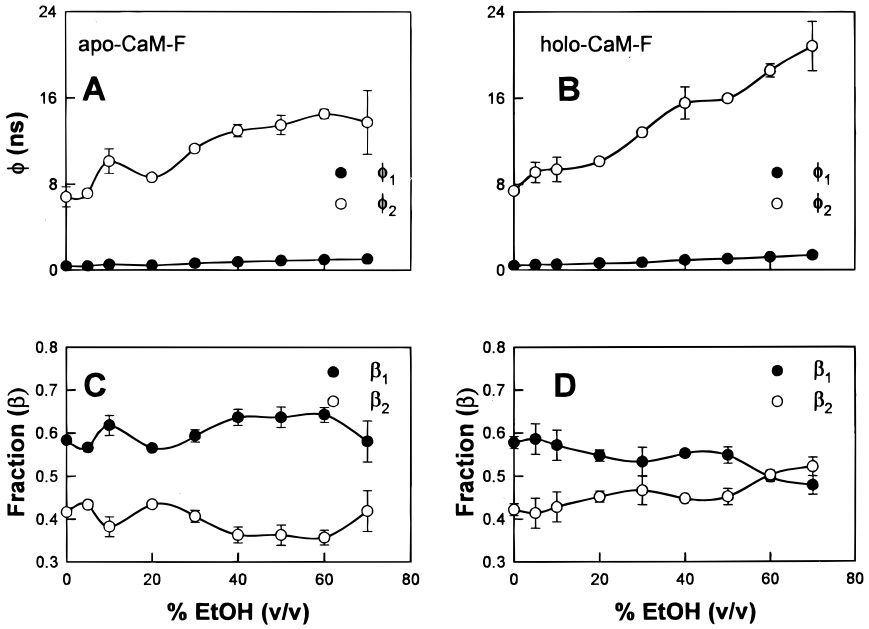

Figure 3. Effects of EtOH volume percent on the rotational reorientation dynamics of apo- (A, C) and holo-CaM-F (B, D). The curves between points are merely aids for the eye.

EtOH-like environment as we increase the EtOH volume percent. However, the pathways taken toward this "EtOH-like" environment are very different for the fluorescein reporter groups in apo- and holo-CaM-F. On the basis of the previous work on fluorescein dissolved in liquids[57−61] and our earlier work on CaM-F dissolved in neat buffer,[45] we can see that the differences in the $\langle\tau\rangle$ vs EtOH profiles (Figure 2) arise from changes in the fluorescein group pK_a. For example, in aqueous buffer without EtOH, the fluorescein pK_a is greater in holo-CaM-F than in apo-CaM-F.[45] As we increase the EtOH volume percent, the pK_a difference, which is related to $\Delta\langle\tau\rangle$, increases between apo- and holo-CaM-F and appears to peak near ∼20% EtOH. From an analytical perspective, this result shows that the optimum condition for producing the greatest Ca^{2+} sensitivity with CaM-F is not a purely aqueous environment but is one that contains ∼10−30% EtOH. This assessment is also supported by the steady-state emission data presented in Figure 1A; however, the reason for this sensitivity increase (a change in the fluorescein reporter group pK_a) is *not* at all evident from the steady-state results. As we increase the EtOH volume percent above ∼20−30%, the pK_a difference systematically decreases and the pK_a values for the fluorescein reporter group in apo- and holo-CaM-F become essentially equivalent above ∼50% EtOH.

Rotational Reorientation Dynamics. The fluorescence anisotropy decays for apo- and holo-CaM-F are well described[62] by an anisotropic rotor model with two discrete rotational reorientation times at all EtOH loadings studied. Figure 3 summarizes the effects of EtOH volume percent on the time-resolved anisotropy decay parameters for apo- (Figures 3A,C) and holo-CaM-F (Figures 3B,D). Parts A and B of Figure 3 present the recovered rotational reorientation times (ϕ) for apo- and holo-CaM-F, respectively, while parts C and D show the fractional contribution (β) associated with each rotational reorientation time. Several aspects of these data merit additional discussion. First, there is a steady increase in ϕ_1, the faster rotational reorientation time, for apo- and holo-CaM-F as the EtOH volume percent increases. Second, there is a systematic increase in ϕ_2 for apo- and holo-CaM-F as we increase the EtOH volume percent; however, the relative increase is greater for holo-CaM-F. Third, in apo-CaM-F, the β terms associated with each rotational reorientation time remain essentially constant as the EtOH volume percent is changed. Finally, the β terms for holo-CaM-F change by ∼30% as we increase the EtOH volume percent from 0 to 70%. Together, these data suggest that increasing EtOH volume percent affects holo-CaM-F more than apo-CaM-F (vide infra). This result is somewhat surprising, given that holo-CaM-F is reportedly less solvent accessible.[48]

To explore how the addition of EtOH might influence the overall size and shape of CaM-F, we began by assuming that added EtOH dehydrated and/or denatured CaM. The basis for this assumption is that monohydric alcohols reportedly lead to peptide/protein backbone desolvation, strengthening of hydrogen bonds, stabilization of helical structures, and/or denaturation, depending on the actual alcohol volume percent.[63] On the basis of this assumption, we used the global CaM rotational reorientation time, ϕ_2, in concert with the Debye−Stokes−Einstein expression (eq 5) to estimate how added EtOH might change the overall

$$V = \phi RT/\eta \qquad (5)$$

CaM-F molar volume.[54] In this expression, ϕ represents the experimentally measured global CaM rotational reorientation time, η is the bulk solvent viscosity, V is the volume of the rotating unit, and T is the sample Kelvin temperature. The actual η values at each EtOH volume percent were obtained from the literature.[64]

Figure 4 illustrates the effects of Ca^{2+} and EtOH volume percent on the apparent CaM-F molar volume. In addition to the

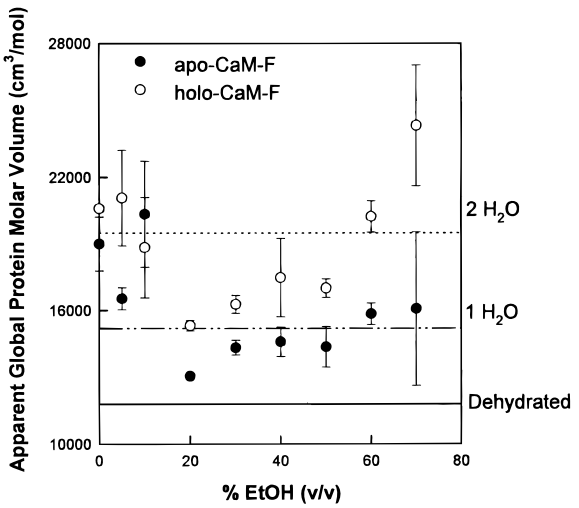

Figure 4. Apparent global protein molar volumes for apo- (●) and holo-CaM-F (○) as a function of EtOH volume percent. Known values for the CaM molar volume with two (···), one (··—··), and zero (—) hydration shells of water are shown for comparison.

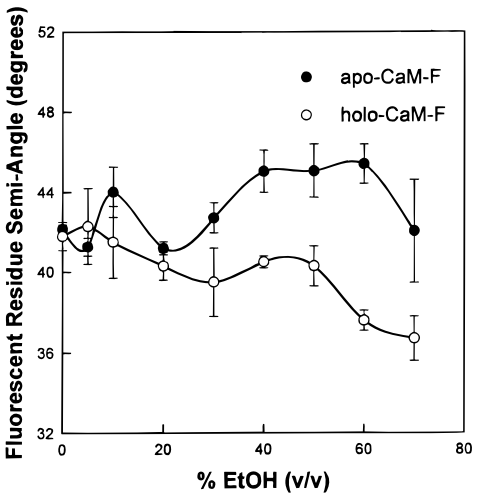

Figure 5. Effects of EtOH volume percent on the fluorescein reporter group semiangle within CaM-F for apo- (●) and holo-CaM-F (○). The curves between points are merely aids for the eye.

experimental data (points), we also present horizontal lines that denote the predicted CaM molar volume[65,66] when the protein is completely dehydrated (—), is hydrated with a single layer of H_2O molecules (··—··), and is hydrated with two layers of H_2O molecules (···). Although there may be other explanations for the data presented in Figures 3 and 4, if we use the results presented in Figure 4 as a point of discussion, we can make several interesting observations. Apo- and holo-CaM-F appear to exist initially with two waters of hydration. As the EtOH volume percent increases, both forms of CaM-F appear to lose one water of hydration and/or collapse such that their molar volume decreases. This dehydration/collapse peaks or is complete for apo- and holo-CaM-F by ~20% EtOH. Between ~20 and 70% EtOH, apo-CaM-F appears to exist with a single layer of water molecules. In contrast, over the same EtOH volume percent range, holo-CaM-F appears to either rehydrate, superhydrate (both seem unlikely), or systematically denature. The apparent holo-CaM-F molar volume also appears to be 3000−8000 cm^3/mol greater than that of apo-CaM-F across the EtOH volume percent range studied. This result suggests that holo-CaM-F, if it collapses on itself, cannot pack as well as apo-CaM-F when EtOH is added.

Figure 5 illustrates the effects of EtOH volume percent on the fluorescein reporter group semiangle in apo- and holo-CaM-F. These results show that the semiangle associated with apo-CaM-F remains constant (42 ± 2°) over the entire EtOH volume percent range studied. This result suggests that the addition of EtOH does not affect the ability of the fluorescein reporter group within EF hand I to precess at all in apo-CaM-F. The semiangle associated with holo-CaM-F, in contrast, decreases from 43 ± 1 to 36 ± 1° as the EtOH volume percent is increased between 0 and 70%. This decrease suggests that the amino acid residues that surround Cys-26 close down around the fluorescein reporter group in holo-CaM-F as we increase the EtOH volume percent.

CONCLUSIONS

Alcohols are known to affect proteins and other biomolecules.[1−3,63] By using a site-selective labeling strategy, we have investigated the effects of added EtOH on the local and global dynamics of the local microenvironment surrounding EF hand I within apo- and holo-CaM-F. The results of these experiments illustrate several points. First, the addition of EtOH modulates the fluorescein dianion and leuco dye equilibria and this is manifested by changes in the fluorescein absorbance and emission spectra as well as the excited-state fluorescence lifetimes. Second, the optimum sensitivity of CaM-F to Ca^{2+} is exhibited between ~10 and 30% EtOH, which is a manifestation of the difference between the pK_a values of the fluorescein reporter group in apo- and holo-CaM-F. Third, the CaM protein backbone limits the extent to which EtOH can interact with the fluorescein residue in CaM-F. Fourth, apo-CaM-F is not affected by added EtOH as much as holo-CaM-F. In one interpretation of these data, apo-CaM-F appears to lose one water of hydration between 0 and 20% EtOH and remain in this hydration state between 20 and 70% EtOH. The precessional semiangle that describes the fluorescein residue mobility within apo-CaM-F does not change over the EtOH volume percent range studied, indicating that the *local* protein structure surrounding the fluorescein reporter group is not affected to any significant extent in apo-CaM-F as the EtOH volume percent is increased. In holo-CaM-F, there is also some evidence for protein dehydration between 0 and 20% added EtOH, which is akin to the case of apo-CaM-F; however, as the EtOH volume percent is increased above 20% EtOH, there is evidence for holo-CaM-F denaturation. The fluorescein residue semiangle

(63) (a) D'Auria, S.; Nucci, R.; Rossi, M.; Bertoli, E.; Tanfani, F.; Gryczynski, I.; Malak, H.; Lakowicz, J. R. *J. Biochem.* **1999**, *126*, 545−52. (b) Ueda, I.; Yoshinda, T. *Chem. Phys. Lipids* **1999**, *101*, 65−79. (c) Shimizu, S.; Shimizu, K. *J. Am. Chem. Soc.* **1999**, *121*, 2387−94. (d) Kentsis, A.; Sosnick, T. R. *Biochemistry* **1998**, *37*, 14613−22. (e) Kamatari, Y. O.; Konno, T.; Kataoka, M.; Akasaka, K. *Protein Sci.* **1998**, *7*, 681−8. (f) Cowan, D. A. *Comp. Biochem. Physiol.* **1997**, *118*, 429−38. (g) Buck, M.; Schwalbe, H.; Dobson, C. M. *Biochemistry* **1995**, *34*, 13219−32. (h) Shiraki, K.; Nishikawa, K.; Goto, Y. *J. Mol. Biol.* **1995**, *245*, 180−94. (i) Schonbrunner, N.; Wey, J.; Engels, J.; Georg, H.; Kiefhaber, T. *J. Mol. Biol.* **1996**, *260*, 432−45. (j) Thomas, P. D.; Dill, K. A. *Protein Sci.* **1993**, *2*, 2050−65.
(64) Lide, D., Ed. *CRC Handbook of Chemistry and Physics*, 73rd ed.; CRC Press: Boca Raton, FL, 1992.
(65) Small, E. W.; Anderson, S. R. *Biochemistry* **1988**, *27*, 419−28.
(66) Yao, Y.; Schöneich, C.; Squier, T. C. *Biochemistry* **1994**, *33*, 7797−810.

in holo-CaM-F decreases significantly as the EtOH volume percent is increased. This suggests that the local protein structure surrounding the fluorescein reporter group is closing down around the fluorescein reporter group in holo-CaM-F as the EtOH volume percent increases. These particular results are somewhat surprising, given that recent H/D exchange experiments[48] argue that holo-CaM is less solvent accessible compared to apo-CaM. We suggest that the differences between our work and that of Gross and co-workers[48] arise because our fluorescence experiments report predominantly on the local microenvironment surrounding the fluorescein reporter group (cysteine-26) whereas the H/D exchange experiments report on all the protein residues that can be accessed by the solvent (H_2O/D_2O).

ACKNOWLEDGMENT

Financial support for this work was provided by the National Science Foundation and the Office of Naval Research.

Received for review May 25, 1999. Accepted October 18, 1999.

AC990551B