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Zinc biosensing with multiphoton excitation using carbonic anhydrase and improved fluorophores

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Abstract. Previously, we had shown that the zinc-dependent binding of certain fluorescent aryl sulfonamide inhibitors could be used with apocarbonic anhydrase II to transduce the level of free zinc as a change in the fluorescence of the inhibitor. While inhibitors such as dansylamide, ABD-M, and ABD-N made possible quantitation of free zinc in the picomolar range with high selectivity, they have only modest absorbance which limits their utility. We describe here the synthesis and properties of two new probes, Dapoxyl™ sulfonamide and BTCS, and their use in zinc biosensing. Dapoxyl sulfonamide exhibits a dramatic increase and blue shift in its emission upon binding to holocarbonic anhydrase II, as well as a 20-fold increase in lifetime: it is thus well suited for quantitating free Zn(II) down to picomolar ranges. The anisotropy of BTCS increases fivefold upon binding to the holoprotein, making this probe well suited for anisotropy-based determination of zinc. BTCS and ABD-N are efficiently excited with two photon excitation using 1.5 ps pulses from a titanium sapphire laser, and exhibit the increased zinc-dependent anisotropy response anticipated on the basis of photoselection. [S1083-3668(00)00201-X]

Keywords: biosensor; zinc; fluorescence anisotropy; carbonic anhydrase; two-photon excitation.

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1 Introduction

For some time, we have been engaged in harnessing the unmatched sensitivity and selectivity of the apocarbonic anhydrase molecule for detection of zinc in complex media. We have found that free zinc ion may be detected at picomolar levels by changes in the fluorescence intensity, anisotropy, wavelength ratio, or lifetime of covalent and noncovalent fluorescent probes.[1–4] The noncovalent probes are typically aryl sulfonamides which act as inhibitors of the enzyme by replacing the zinc-bound water as a fourth ligand, in addition to the three tetrahedrally disposed imidazoles. This inhibitor binding to the enzyme is strongly dependent upon the presence of zinc ion in the active site, and is the source of their utility. In each case, the binding is accompanied by some observable change in the fluorescence of the inhibitor: a change which can quantitate the fraction of enzyme with bound inhibitor. Since the binding of the inhibitors is much tighter to the zinc-containing holoprotein than the apoprotein, at suitable inhibitor concentrations the fraction of enzyme with bound inhibitor is determined by the fraction of enzyme with bound zinc, which is in turn controlled by the zinc concentration and the law of mass action. In most cases, binding of aryl sulfonamides to the protein is enhanced three orders of magnitude by the presence of the metal, in comparison to the apoprotein. Using fluorescent inhibitors such as dansylamide and related ligands such as ABD-M and ABD-N,[3,5] we have demonstrated determination of picomolar free zinc levels using changes in fluorescence observables.

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However, these fluorescent inhibitors are for the most part fairly polar molecules which bind only modestly to the holoprotein. For instance, both dansylamide and ABD-N exhibit K_D's in the range of 0.8 micromolar. Thus while it would be desirable to use low protein and inhibitor concentrations to quantitate low metal ion concentrations, these inhibitors will not be fully bound to the holoprotein at concentrations much below micromolar. Tighter binding inhibitors are thus desirable. The therapeutic utility of carbonic anhydrase inhibitors in treating glaucoma has fueled the search for tight-binding inhibitors, which have been described by several investigators.[6,7] Many of these tighter binding molecules are more hydrophobic, permitting them to interact more strongly with the hydrophobic region of the active site. In our case we also desired a tighter-binding aryl sulfonamide to assure sensitivity to zinc.

Moreover, all the fluorescent aryl sulfonamides described above are fairly weak absorbers: the peak long wavelength (330 nm) absorption maximum for dansylamide is 3300 $M^{-1}cm^{-1}$, whereas for ABD-N and ABD-M it is approximately 8800 $M^{-1}cm^{-1}$ at 410 nm. Clearly, there are many modern fluorophores which exhibit much better extinction coefficients. One important advantage dansylamide and ABD-N have is that when bound to the holoprotein their fluorescence quantum yield goes up dramatically; moreover, their emissions shift, which permits one by judicious choice of wavelength to preferentially observe the bound form in the presence of an excess of free form, enhancing sensitivity and dynamic range. In the case of the visibly excited ABD-N, this

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property is very desirable for imaging zinc fluxes in tissues by fluorescence microscopy, because the unbound fluorophore contributes less to the background. In the case of ABD-N, observing the emission on the blue side at 540 nm reduces the free probe background to a negligible level.[5]

The increase in quantum yield of dansylamide and ABD-N is perforce accompanied by a corresponding increase in lifetime. For these fluorophores which exhibit zinc-dependent binding this is an advantage for lifetime-based sensing, which relies upon the difference in lifetimes between the free and bound forms.[8,9] However, a large increase in lifetime upon binding to the macromolecule is something of a disadvantage for *anisotropy*-based sensing, as the signal is based essentially on the ratio of lifetime to rotational correlation time of the free and bound forms using the Perrin equation

$$r = r_0 / 1 + (\tau / \theta_c),$$

where r is the observed anisotropy, r_0 is the anisotropy in the absence of rotational diffusion, τ is the lifetime, and θ_c is the rotational correlation time. As an example, we may compare the utility of ABD-M and ABD-N as anisotropy probes and lifetime probes. In the free form they are rather similar with respect to quantum yield and lifetime, but bound ABD-N has a higher apparent quantum yield and a longer average lifetime than ABD-M ($\langle\tau\rangle = 4.98$ ns for bound ABD-N vs 1.53 ns for bound ABD-M). For a protein CA's size, in fact it is desirable to have a much shorter lifetime to maximize the anisotropy of the protein-bound form. In this regard the 4 ns lifetime of Elbaum's original fluorescein-based anisotropy probe was much better, although this probe had other deficiencies.[7] The desirability of a short lifetime in this case is in contradistinction to probes for anisotropy-based sensing which are attached to macromolecules and rely on quenching to reduce the lifetime.[1] In that case a lifetime similar to the rotational correlation time is desirable.

We also sought probes which could be effectively used in multiphoton fluorescence experiments, particularly using two-photon excitation from the titanium sapphire laser. Since the brilliant innovation by Webb and his colleagues of two photon-excited fluorescence microscopy, this field has grown dramatically.[10] In particular, multiphoton fluorescence microscopy has demonstrated the high resolution and optical sectioning capability of scanning confocal microscopy, but with much reduced bleaching of the specimen. From a sensing viewpoint multiphoton excitation also offers the prospect of greater dynamic range in anisotropy-based sensing[11] reduced background fluorescence, and the potential for interrogating sensor transducers in refractory matrices such as whole blood.[12] Our recent results in imaging zinc release from the hippocampus in response to electrical stimulus suggest that multiphoton excitation of these probes may prove of value in imaging experiments. Clearly, it would be desirable for any probe to be excitable by multiphoton excitation.

Consequently, we sought fluorescent aryl sulfonamides with enhanced absorbance and shorter lifetimes than our current probes, particularly with a view to their use for imaging zinc fluxes in cell and tissue specimens.

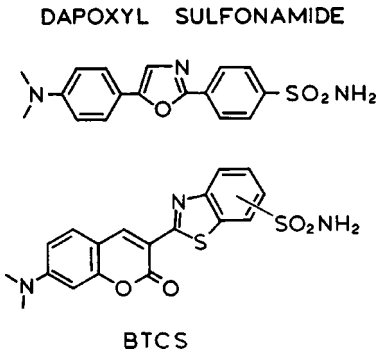

Fig. 1 Structures of BTCS and Dapoxyl sulfonamide.

2 Experiment

2.1 Syntheses of Dapoxyl Sulfonamide and BTCS

Dapoxyl™ sulfonyl chloride was a product of Molecular Probes, Eugene, OR (Cat No. D-10160) and was used without further purification. Dapoxyl sulfonamide (Figure 1) was synthesized from the sulfonyl chloride and a tenfold molar excess of aqueous ammonium hydroxide in DMF for one hour. The product precipitated from the neutralized aqueous phase and was collected by centrifugation. Absorbance ($\epsilon_{365} = 22\,000\ \mathrm{M^{-1}\,cm^{-1}}$), excitation, and emission spectra (Figure 2) were similar to those published on Molecular Probes' website (⟨http://www.probes.com⟩) for the ethylamine adduct. BTCS [Figure 1, 3-(2-benzothiazoyl)-7-diethylaminocoumarin sulfonamide] was synthesized similarly from the analogous sulfonyl chloride (Lambda Fluorescence, Pleasant Gap, PA, Catalog No. D-015). The sulfonyl chloride has recently been unavailable in the U.S.; it may be possible to obtain it from the parent firm, Lambda Probes and Diagnostics, Graz, Austria. The reaction product was further purified by reverse-phase HPLC on a C_4-silica gel column eluted with 10 mM ammonium acetate and a gradient of 0%–90% acetonitrile. The excitation and emission spectra of BTCS (ϵ_{466}

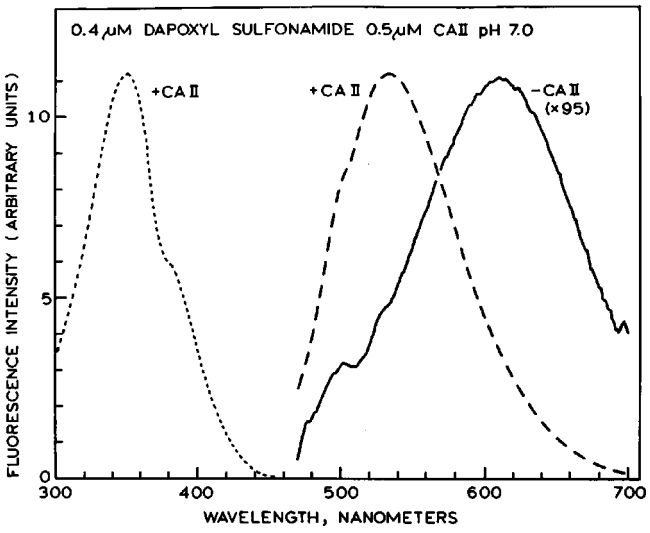

Fig. 2 Normalized excitation (·····) and emission spectra of Dapoxyl sulfonamide in the absence (—) and presence (- - - - -) of holocarbonic anhydrase II.

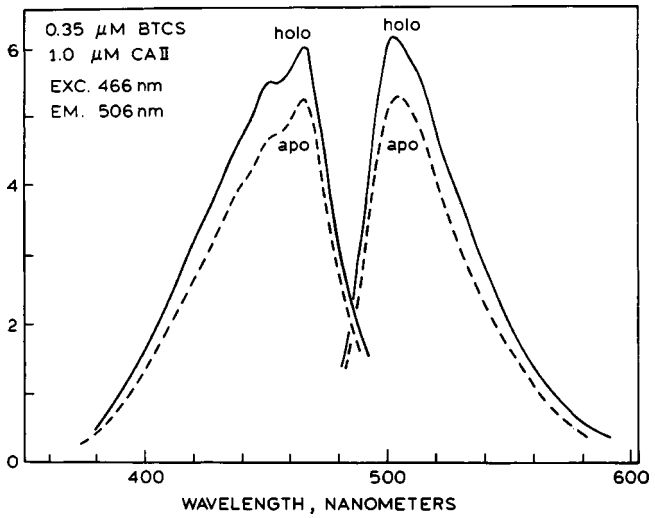

Fig. 3 Normalized excitation (peak at 466 nm) and emission (peak at 504 nm) spectra of BTCS in the presence of apo- (- - - -) and holocarbonic anhydrase (—).

$= 36\,000\ M^{-1}\,cm^{-1}$) in the presence and absence of holo-CA are depicted in Figure 3. Both Dapoxyl sulfonamide and BTCS are only sparingly soluble in aqueous solutions near neutrality, and typically they were introduced to aqueous samples in small volumes of DMF.

2.2 Other Materials and Methods

Human carbonic anhydrase II (recombinant) was a generous gift of Dr. Carol Fierke of Duke University, and the relatively inexpensive bovine carbonic anhydrase II was obtained from Sigma. The zinc was removed as previously described by treatment with dipicolinate. Special care was taken to avoid contamination with zinc and other transition metals, and zinc ion buffers were formulated from MOPS, and Bicine or NTA as described elsewhere.[3] Fluorescence spectra and anisotropies were determined on a Spectronics AB-2 spectrophotofluorometer; the former are uncorrected. Fluorescence lifetimes were determined on an ISS K2 multifrequency phase fluorometer using the 442 nm line (30 mW) of a Kimmon HeCd laser (for BTCS) or the ultraviolet lines (100 mW) of a Spectra-Physics model 2065-7S argon ion laser (for dapoxyl sulfonamide) essentially as previously described.[13] Two-photon excitation was obtained from a Spectra-Physics Tsunami mode-locked titanium: sapphire laser which produced 880 mW average power at 780 nm with pulses emitted at 80 MHz with duration of 1.5 ps. ABD-N was synthesized in our laboratory by a procedure to be described elsewhere (Thompson et al., in preparation). Other reagents were reagent grade or better and used without further purification.

3 Results

3.1 Dapoxyl Sulfonamide

Dapoxyl sulfonamide, like dansylamide, exhibits a dramatic increase and blue shift in its fluorescence intensity upon binding to holocarbonic anhydrase, in comparison to its emission free in solution Figure 2. In particular, emission of the bound form shifts from a peak at 605 to 530 nm, and increases about 90-fold, compared to the free form. The increase and large shift suggest that the fractions of Dapoxyl sulfonamide in the free and bound forms (and thus the zinc concentration) may be determined by measuring the intensity, or the ratio of fluorescence intensities on the blue and red sides of the emission band. This is in fact the case: zinc-dependent intensity and intensity ratios are depicted in Figure 4. The dramatic increase in intensity at 535 nm is apparent, as well as the tenfold increase in 535/685 nm intensity ratio. The advantages of intensity ratios for accuracy in quantitating concentrations of metal ions such as calcium are by now widely appreciated.[14] These intensity changes permit the affinity of holocarbonic anhydrase for Dapoxyl sulfonamide to be determined as 0.13 μM (data not shown). The intensity increase of Dapoxyl sulfonamide is also accompanied by a large increase in fluorescence lifetime. We measured the frequency-dependent phase angles and demodulation of dapoxyl sulfonamide free in solution, in the presence of holocarbonic anhydrase II, and in the presence of sonicated unilamellar vesicles of DOPC/cholesterol 3:1 (0.1 μM). The results of these experiments are

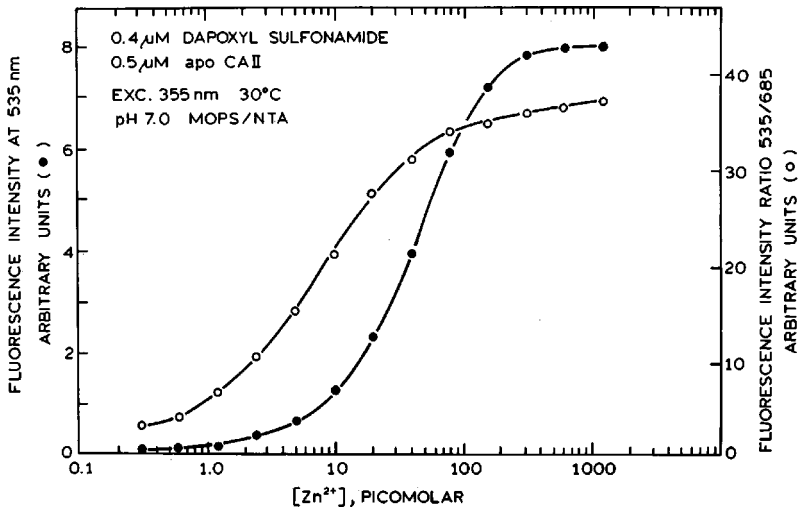

Fig. 4 Fluorescence emission intensities at 535 nm (●) and intensity ratios (535/685 nm) (○) as a function of zinc concentration.

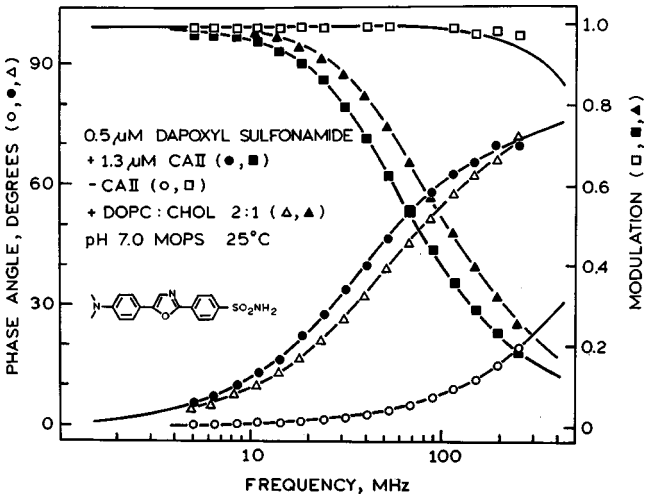

Fig. 5 Frequency-dependent phase shifts (△, ●, ○) and modulations (■, □, ▲) for Dapoxyl sulfonamide free in solution (○, □), bound to DOPC: cholesterol vesicles (▲, △), and bound to holocarbonic anhydrase (●, ■).

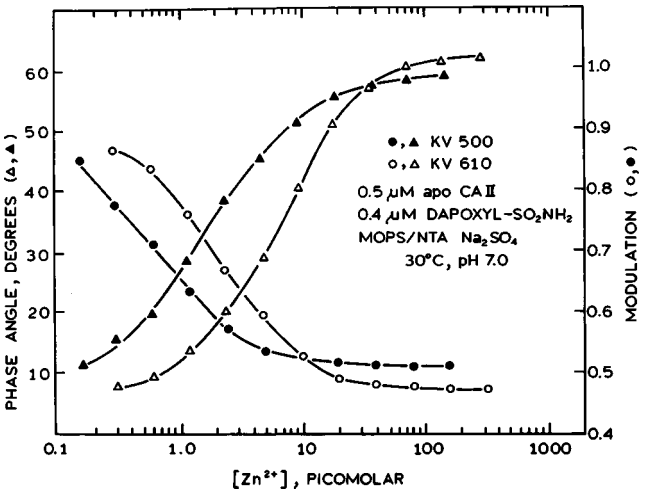

Fig. 6 Phase shifts (▲, △) and modulations (●, ○) of Dapoxyl sulfonamide as a function of zinc concentration, with the emission observed through KV 500 (●, ▲) or KV 610 (△, ○) barrier filters.

depicted in Figure 5; the values of lifetime and fractional intensity derived from fitting these data to one- and two-exponential models are listed in Table 1. The 20-fold increase in fluorescence lifetime accompanying Dapoxyl sulfonamide binding to the protein is unsurprising in view of the intensity increase, since we do not anticipate static quenching of the free probe *per se*. As we, Lakowicz, Wolfbeis, and others have shown, quantitating analytes by changes in fluorescence lifetime offers several advantages.[15–17] Thus, we measured the zinc dependence of phase and modulation at 100 MHz as a function of free zinc concentration; the results are depicted in Figure 6. The changes in phase and modulation are large enough to be very useful: the phase increases from 7 to 62 deg on the red side of the emission band, with the modulation change being commensurate. These large changes make it readily apparent that Dapoxyl sulfonamide can easily be used for lifetime-based sensing.

The hydrophobic nature of Dapoxyl sulfonamide suggested that it might bind to phospholipid bilayers such as that in the cell membrane. In fact it does, exhibiting fluorescence in the presence of DOPC/cholesterol unilamellar vesicles which closely mimics its emission bound to carbonic anhydrase in color and lifetime (Table 1 and Figure 5). When added to a rat brain hippocampal slice (results not shown) the dye stained a variety of cell membranes. Our preliminary results suggest that Dapoxyl sulfonamide partitions into membranes reversibly, as it will desorb from the membrane to bind to holocarbonic anhydrase. This suggests that Dapoxyl sulfonamide will bind to holocarbonic anhydrase present in the cell. By comparison ABD-N does not apparently penetrate the cell. Finally, we found that Dapoxyl sulfonamide can be readily excited by picosecond infrared light pulses from the titanium sapphire laser at 800 nm; however, since its one-photon anisotropy response to changes in zinc ion concentration is modest, its two-photon response is small as well.

3.2 BTCS

Unlike Dapoxyl sulfonamide, BTCS exhibits little change in its fluorescence spectrum, intensity (Figure 3), or lifetime Table 1 in the presence of holocarbonic anhydrase compared with free in solution. Thus it is not terribly useful as an intensity, intensity ratio, or lifetime probe. However, the negligible change in lifetime (Table 1) makes BTCS an excellent anisotropy probe. In particular, the fluorescence anisotropy of BTCS in the presence of apocarbonic anhydrase exhibits a

Table 1

Sample[a]	τ_1	f_1	τ_2	f_2	χ^2
Dapoxyl sulfonamide+holo-CA	3.80±0.05	0.94	0.49±0.04	0.06	10
Dapoxyl sulfonamide	0.22±0.01	1.00			4.0
BTCS+holo-CA	2.81±0.05	0.93	1.35±0.29	0.07	0.9
BTCS	2.63±0.11	0.90	1.65±0.56	0.10	1.3
BTCS+DOPC/Cholesterol 3:1	2.56	1.00			0.9

[a] "Sample" refers to the experimental sample, τ_i is the lifetime of the component i in nanoseconds, f_i is the fractional intensity of the component i, and χ^2 is the reduced chi squared, a measure of the goodness of fit.

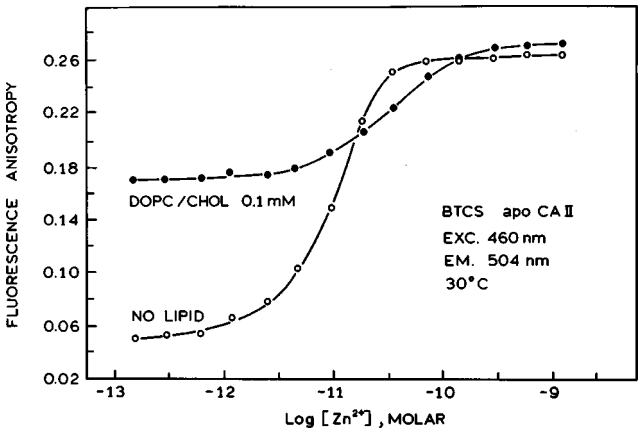

Fig. 7 Fluorescence anisotropies of BTCS plus apocarbonic anhydrase as a function of free zinc in the absence (○) and presence (●) of 0.1 mM DOPC/cholesterol vesicles.

fivefold increase (from 0.05 to 0.25) as the binding site is saturated by increasing concentrations of free zinc ion in the picomolar range (Figure 7). Moreover, BTCS also reversibly binds to phospholipid bilayers with a small change in lifetime (Table 1), but a substantial jump in anisotropy. We believe this binding is in fact a partitioning into the bilayer, as exhibited by many other nonpolar molecules. An important feature of the anisotropy is that the anisotropy of BTCS bound to holocarbonic anhydrase is 50% higher than that of BTCS bound to the membrane (Figure 7), suggesting that emission from BTCS bound to carbonic anhydrase inside cells could be resolved from that bound to the cell membrane. Moreover, this difference might be imaged by fluorescence anisotropy microscopy.[18] Experiments are ongoing to test this proposition. Finally, BTCS (like Dapoxyl sulfonamide) readily undergoes two photon excitation (Figure 8). In particular the two-photon excitation anisotropy displays the expected increase over the anisotropy obtained with one-photon excitation.[11]

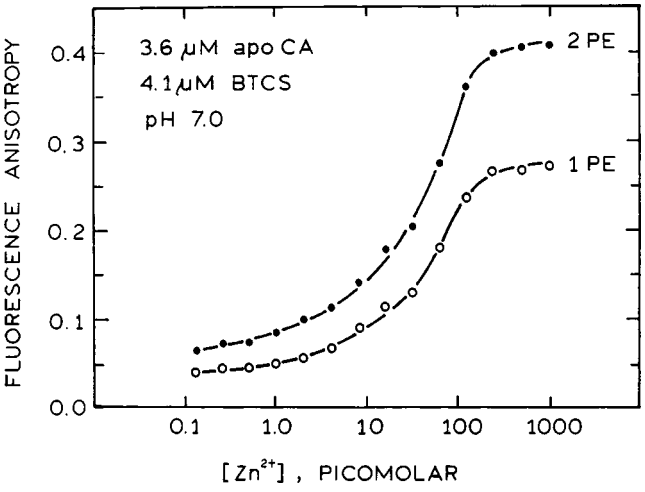

Fig. 8 Fluorescence anisotropy of 4.1 μM BTCS with 3.6 μM apobovine CA as a function of free Zn(II) concentration with one photon excitation at 460 nm (○) and two photon excitation at 780 nm (●).

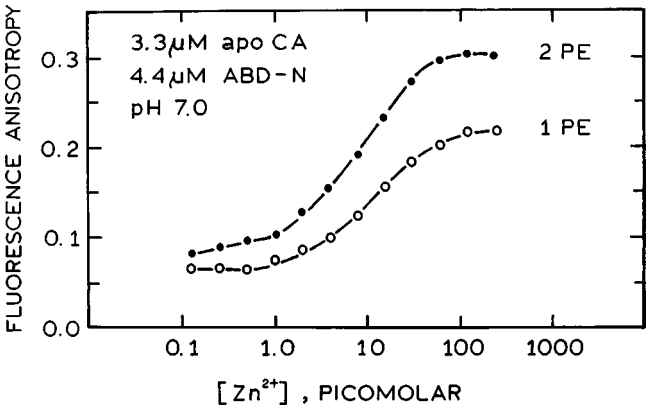

Fig. 9 Fluorescence anisotropy of 4.4 μM ABD-N and 3.3 μM apobovine CA as a function of free Zn(II) concentration with one photon excitation at 420 nm (○) and two photon excitation at 780 nm (●).

3.3 ABD-N

We also determined the suitability of ABD-N for two-photon excitation, and for measurement of zinc concentration by fluorescence anisotropy with two-photon excitation. While ABD-N free has a very modest quantum yield, ABD-N when bound to the protein has a substantial quantum yield[5] and may be readily excited with two-photon excitation. Moreover, it exhibits a significant increase in anisotropy upon binding to the holoprotein, and in the presence of apoenzyme and varying Zn concentrations the anisotropy becomes Zn dependent (Figure 9). When excited with two photons the anisotropy difference becomes more marked, as expected.[11] ABD-N is relatively hydrophilic and evinces little tendency to bind to lipid bilayers, and is thus in a sense complementary to BTCS and Dapoxyl sulfonamide in this regard. We note that very similar results to those obtained with human CA II were obtained with bovine CA II, which is significantly less expensive.

4 Discussion

What are the implications of these new probes, and the ability to observe them with multiphoton excitation? First and foremost, the greater absorbance of Dapoxyl sulfonamide and BTCS make them usable at lower concentrations than ABD-N, ABD-M, or dansylamide. Moreover, their tighter binding to the holoprotein (results not shown) also makes them usable at lower concentrations. As is evident from the foregoing data, BTCS performs well as an anisotropy probe, whereas Dapoxyl sulfonamide is best as an intensity, intensity ratio, or lifetime probe. The lipid solubility of BTCS and Dapoxyl sulfonamide make them cell penetrant, but they also fluoresce when bound to membranes. In this regard they are complementary to ABD-M and ABD-N, which are relatively polar and do not penetrate the cell. These probes offer the prospect of intracellular monitoring of zinc levels at very low levels. While BTCS is readily excited with visible light sources, the peak excitation of Dapoxyl sulfonamide is in the near UV; fortunately, it should be excitable by the new blue diode lasers.

We also demonstrate here that zinc levels may be determined by two photon-excited anisotropy, the first example we

know of where this technique has been used for determination of an analyte. The virtues of two photon excitation for microscopy[10] and for determination of analytes in highly scattering, absorbing media such as whole blood[12] are becoming well established. We and others have already established the virtues of anisotropy-based sensing for analytes such as antigens and metal ions, including insensitivity to variations in excitation strength, probe concentration and optical properties, as well as facile (achromatic) imaging in the microscope.[18] The fact that two-photon excitation creates by photoselection an intrinsically higher limiting anisotropy gives increased dynamic range, and from an anisotropy imaging standpoint, provides improved contrast. Thus we believe that the marriage of two-photon excitation with anisotropy-based sensing offers significant advantages for many research applications, especially those involving imaging through the microscope.

Acknowledgments

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