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Steady-state and time-resolved fluorescence measurements for studying molecular interactions: interaction of a calcium-binding probe with proteins

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Abstract

The binding of 2-[(2-bis-[carboxymethyl]amino-5-methylphenoxy)-methyl]-6-methoxy-8-bis[carboxymethyl] aminoquinoline, the fluorescent calcium probe Quin2, to serum albumin and several other proteins has been investigated. Changes in fluorescence emission spectra and fluorescence anisotropy revealed interactions between Quin2 and several proteins including human serum albumin, bovine serum albumin, aldolase, phosphoglucose isomerase, glyceraldehyde-3-phosphate dehydrogenase, and alkaline phosphatase. Protein-probe interactions were inhibited by the presence of calcium. Binding was also measured by resonance energy transfer and gel permeation chromatography. Equilibrium binding constants for Quin2 were quantitated by the application of the recently-developed 'SPECTRABIND' program to spectroscopic data (D. Toptygin and L. Brand, Anal. Biochem., 224 (1995) 330–338). Binding of Quin2 to human serum albumin is discussed in terms of the published X-ray crystal structure of human serum albumin (X.M. He and D.C. Carter, Nature, 358 (1992) 209–215).

Keywords: Fluorescence; Fluorescence lifetimes; Quin2; Fluorescence probes; Calcium; Data analysis

1. Introduction

Molecular interactions are often monitored using optical techniques. Methods relying on optical signals from extrinsic or intrinsic fluorophores are advantageous because of their sensitivity and relative ease of use. Fluorescence assays for the study of macromolecular interactions and ligand binding are

Abbreviations: Quin2, 2-[2-bis-[carboxylmethyl]amino-5-methylphenoxy)-methyl]-6-methoxy-8-bis[carboxymethyl] aminoquinoline; EGTA, Ethylenebis (oxynitrilo) tetraacetic acid; BSA, Bovine serum albumin; CPK, Creatine phosphokinase; HSA, Human serum albumin; GPDH, Glyceraldehyde-3-phosphate dehydrogenase; HEPES, N-2-Hydroxyehtyl-piperazine-N'-2-ethane sulfonic acid; MOPS, Morpholino sulfonic acid

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based upon the unique spectroscopic properties, e.g. spectra, lifetimes and quantum yields, exhibited by free and liganded species. Techniques using fluorescent ion-chelating probes have become a powerful tool for measuring pH [1], calcium [2], zinc [3] and magnesium [4]. However, the usefulness of these probes is limited by the specificity of the probe for the molecule of interest without interference from signals attributed to non-specific interactions with other molecules.

One fluorescent ion-chelating probe, Quin2, is employed in the measurement of calcium through the enhanced fluorescence emission upon Quin2-calcium complexation [2]. Previous studies have documented binding of several ion-chelating probes [Fura-2 [5–9], Fura Red [10], Indol [7,11,12], arsenazo III and antipyrylazo III [13,14], MagFura-2 [15]] with endogenous cellular biomolecules, e.g. proteins and several cations, with concomitant changes in spectral characteristics. Likewise, it has been reported that exogenous compounds often used in conjunction with ion indicators alter fluorescence properties and cation-binding ability [16,17].

While the absence of interfering compounds would be ideal, it is not often practical. Since some probe interactions may have a significant relevance when measurements are made on biological systems [18], it is useful to have knowledge of probe behavior in more complex environments to assess the effects on equilibrium binding and spectroscopic characteristics. Additionally, if the concentrations of other species, e.g. Quin2-protein, are high enough, then the apparent concentrations of ions deduced from fluorescence measurements may be misleading. Similarly, if the steady-state spectral characteristics are the same as those of the ion complexes, then the signals may be misinterpreted. Therefore, techniques which can differentiate between spectral contributions of individual complexes could provide a means to overcome these difficulties. Fluorescence properties, e.g. emission and excitation spectra, quantum yields, and fluorescence lifetimes, are highly sensitive to probe environment. Intensity decays have been used to distinguish between free and protein-bound NADH [19] while either time-resolved [5] or steady-state [6,8–14] fluorescence data have reflected binding of molecules, other than calcium, to the calcium-binding probes Fura-2, Indo-1 and others. A combination of steady-state and time-resolved fluorescence measurements offers an excellent way to investigate interactions of these and other probes with multiple ligands. For individual complexes which exhibit enhanced fluorescence intensity without spectral shifts, contributions from each complex may be attainable from altered time-resolved fluorescence characteristics. The overdetermination inherent in time-resolved fluorescence measurements differentiates between binding of the probe to ions and proteins as well as clearly determining the binding constants for each interaction.

This article discusses the application of steady-state fluorescence, nanosecond time-resolved fluorescence, resonance energy transfer, and steady-state emission anisotropy to study interactions of a calcium-chelating probe with multiple ligands. The model system employed in these studies is Quin2, human serum albumin, and calcium. Utilization of a recently-developed computational approach, SPECTRABIND [20], has enabled the determination of binding constants as well as spectral characteristics associated with individual species involved in these interactions.

2. Materials and methods

2.1. Materials

Quin2 and HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid, ultrol grade) were purchased from Calbiochem (San Diego, CA). Calcium calibration buffers were obtained from Molecular Probes (Eugene, OR). Human serum albumin (Fraction V, essentially fatty acid- and globulin-free), bovine serum albumin (Fraction V, essentially fatty acid free), aldolase (Type I, from rabbit muscle), creatine phosphokinase (CPK, Type I, from rabbit muscle), glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle), and alkaline phosphatase (Type III-s from E. coli) were obtained from SIGMA (St. Louis, MO). The source of phosphoglucose isomerase and horse liver alcohol dehydrogenase was Boehringer Mannheim GmbH (Indianapolis, IN). Ethylenebis (oxynitrilo) tetraacetic acid, EGTA, was obtained from Aldrich (Milwaukee, WI). TRIS (ultrapure) was purchased from

United States Biochemical Corporation (Cleveland, OH).

2.2. Calcium titrations

Calcium calibration buffers for titrations contained 10 mM EGTA (or CaEGTA), 10 mM MOPS, pH 7.2, 0.1 M KCl. A stock solution of Quin2 contained 1 mM Quin2, 10 mM TRIS, pH 7.2, 0.1 M KCl. Calcium titrations were performed using the remove/replace method with Ca^{2+}-EGTA buffers to control the free calcium concentration [21]. The final concentration of Quin2 was approximately 15 μM and free calcium concentrations ranged from 0 to 40 μM. Titrations were performed at 20°C in the presence of 0 μM, 3 μM, 5 μM, 15 μM, and 30 μM human serum albumin (HSA). Fluorescence emission spectra were taken at each calcium concentration in the titration. In addition, spectra were obtained on samples containing the following: a)human serum albumin + EGTA, b)human serum albumin + Quin2 + EGTA, c)human serum albumin + Quin2 + Ca^{2+}, d)Quin2 + EGTA, e)Quin2 + Ca^{2+}. A spectrum of a sample with human serum albumin + Ca^{2+} did not differ from that of human serum albumin + EGTA. The concentration of human serum albumin, Quin2, EGTA, and calcium in these samples were 30 μM, 15 μM, 10 mM, and 39.8 μM, respectively. These spectra in conjunction with the five titrations were sufficient for 'SPECTRABIND' analysis.

2.3. Gel filtration chromatography

Gel permeation chromatography experiments were done with a Pharmacia Sephadex PD10 (G25 M) column. The column was equilibrated with buffer containing 10 mM TRIS, pH 7.2, 0.1 M KCl, and 0.01 M EGTA or 10 mM TRIS, pH 7.2, 0.1 M KCl, and 0.3 M $CaCl_2$. Samples were applied in 150 μl volume. The void volume was determined to be approximately 3 ml with dextran blue. Several different samples were eluted from the column: Quin2 alone, human serum albumin alone, Quin2 + human serum albumin + EGTA, Quin2 + human serum albumin + $CaCl_2$. Similar elution profiles were obtained for Quin2 alone either in the presence of EGTA or calcium and for human serum albumin in the presence of EGTA or calcium. Samples were made in the buffers described above. The concentrations of Quin2 and human serum albumin were 80 μM and 267 μM, respectively. Fractions collected from the column were diluted into 2 ml and assayed fluorometrically on an SLM 8000 photon-counting spectrofluorometer for the presence protein and Quin2. Two measurements were made on each fraction: $\lambda_{ex} = 295$ nm, $\lambda_{em} = 350$ nm; $\lambda_{ex} = 339$ nm, $\lambda_{em} = 490$ nm, for the detection of protein and Quin2, respectively. The recorded intensities were normalized to a value of one for presentation.

2.4. Steady-state anisotropy measurements

Steady-state anisotropy values were obtained with an SLM 48000 Multiple Frequency Spectrofluorometer (SLM-Aminco, Urbana, IL) in the T-format. Steady-state anisotropy, r, was defined by:

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are intensities obtained with the excitation polarizer oriented vertically and the emission polarizers oriented vertically and horizontally, respectively. The G-factor was defined as:

$$G = \frac{I_{HV}}{I_{HH}} \quad (2)$$

Single point steady-state anisotropy measurements were made on Quin2 in the presence of various proteins using the xenon lamp ($\lambda_{ex} = 339$ nm). The emission path contained KV389 filters. The absorbance of samples used in single point polarization measurements did not exceed 0.1 at 339 nm. The measurements were corrected for any buffer contributions. The sample buffer consisted of 10 mM HEPES, pH 7.05, 10 mM EGTA, 130 mM KCl, 20 mM NaCl. Proteins were dialyzed against this buffer for several hours. The concentration of protein stock solutions were determined using the BioRad modified Lowry assay [22]. The final concentrations of phosphoglucose isomerase, alcohol dehydrogenase, alkaline phosphatase, creatine phosphokinase, glyceraldehyde-3-phosphate dehydrogenase, and bovine serum albumin used in anisotropy experiments were 3.0 mg ml^{-1}, 3.0 mg ml^{-1}, 3.6 mg ml^{-1}, 0.05 mg ml^{-1}, 6.3 mg ml^{-1}, and 0.3 mg ml^{-1}, re-

spectively. The two concentrations of aldolase were 1.1 mg ml^{-1} and 7.0 mg ml^{-1}. The concentration of Quin2 was 100 μM. The sample temperature was maintained by a Neslab Instrument TEQ temperature controller and a PBC4 bath cooler. Experiments with aldolase, creatine phosphokinase, and bovine serum albumin were done at 37°C, while other single point polarization measurements were carried out at 25°C.

Anisotropy measurements of Quin2 titrated with human serum albumin were done at 20°C in a 10 mM TRIS, pH 7.2, 0.1 M KCl, 5 mM EGTA buffer. Samples were excited with $\lambda_{ex} = 339$ nm. The emission path contained KV399 and KV450 filters. At each protein concentration the anisotropy, absorbance, and total steady-state emission intensity were measured. Anisotropy, absorbance, and emission intensity of a sample of human serum albumin (no Quin2) in the same buffer were also measured so that the detectable contribution could be accounted for in the data analysis. The sample intensities were corrected for inner filter effects [23].

The anisotropy data from the titration of Quin2 with human serum albumin was used to calculate the dissociation constant of the protein-probe complex. In order to analyze the data with 'SPECTRABIND' to retrieve binding constants, the data had to be a linear response. Standard SLM software returns anisotropy values which are not linear responses, i.e. the response is not linearly related to the concentration of the detectable species. Therefore, the data had to be converted to its linear components, I_{VV} and I_{VH}, in order to be analyzed. Proper analysis of the data also required weighting the anisotropy values in terms of total intensity of the sample. I_{VV} and I_{VH} were calculated from the combination of anisotropy and total intensity using the following equation:

$$I_{VV} = I_{total}(2r_{ss} + 1)$$

$$I_{VH} = I_{total}(1 - r_{ss}) \quad (3)$$

where I_{total} and r_{ss} are the total steady-state fluorescence intensity and anisotropy. The calculated vertical and horizontal components of the intensity, also corrected for intensity changes with Eq. (3), were used as input responses in the data analysis. The data were analyzed using several different models. Spectroscopically detectable species in the analysis included free Quin2, the Quin2-albumin complex, and free human serum albumin.

2.5. Energy transfer measurements

Fluorescence decay measurements of the tryptophan in human serum albumin were made using a time-correlated single photon counting instrument. A Nd:YAG laser (Spectra Physics Series 3000) synchronously pumped a cavity-dumped dye laser system producing picosecond pulses. The repetition rate was 0.8 MHz with approximately 20 mW power. Frequency-doubled output from the dye laser was used to excite the sample at 295 nm. Single fluorescence photons were detected using a microchannel plate detector. The signal was electronically processed as described previously [25]. The emission path contained a magic angle polarizer (54.7 degrees from vertical) and a monochromator with 16 nm entrance and exit slits. Fluorescence emission was detected at 350 nm. Data collection involved alternating between a sample and a ludox scatterer solution using a sample alternator and a monochromator which were under stepper motor control. A melatonin standard ($\tau = 5.9$ ns) was used to verify the proper operation of the instrument. Sample temperature was maintained using a Neslab TEQ temperature controller and PBC4 bath cooler. Experiments were done at 20°C.

In addition to a lifetime measurement at each Quin2 concentration, the steady-state fluorescence intensity was taken using an SLM 8000 photon-counting spectrofluorometer. The excitation and emission wavelengths were 295 nm and 350 nm, respectively, with 16 nm slits on the monochromators. Excitation and emission polarizers were set at vertical and magic angle (54.7°). Absorbance measurements were taken at each Quin2 concentration to evaluate the effects of inner filtering on the sample fluorescence intensity.

The experiment involved titrating a sample containing human serum albumin with Quin2. Human serum albumin was diluted to 2.15 μM in 10 mM TRIS, pH 7.2, 0.1 M KCl, and 5 mM EGTA. Quin2 (1 mM stock in 10 mM TRIS, pH 7.2, 0.1 M KCl) was titrated into the diluted protein solution. The dilution effects of Quin2 additions were accounted

for in the data analysis. The Quin2 concentration ranged from 0–22 μM.

Fluorescence decay data were analyzed by fitting to a model of a sum of exponentials:

$$I(t) = \sum_{i=1}^{n} \alpha_i e^{\frac{-t}{\tau_i}} \quad (4)$$

where α_i and τ_i are the amplitude and lifetime of the i^{th} component. The calculated function, determined using initial guesses for α and τ, was convolved with the measured lamp response. The calculated intensity decay was then compared with the measured sample decay using a non-linear least squares method. The χ^2, weighted residuals and autocorrelation of the residuals were used to reveal systematic deviations in the data [26]. The combination of steady-state intensities and fluorescence decay data were used to calculate the dissociation constant of the human serum albumin-Quin2 complex. The decay curves were normalized to the corrected total intensity prior to analysis with 'SPECTRABIND'.

2.6. Data analysis with 'SPECTRABIND'

Analysis of equilibrium binding data is typically accomplished using the following equation to determine fractional saturation:

$$\bar{\nu} = \frac{F - F_{\min}}{F_{\max} - F_{\min}} \quad (5)$$

where F is the fluorescence intensity at a given ligand concentration, $F_{\min}$ is the fluorescence intensity of free fluorophore (no ligand present), and $F_{\max}$ is the fluorescence intensity when 100% of the fluorophore has bound ligand. Eq. (5) is invalid in the case of multiple equilibria since F may be dependent upon the fraction to which fluorophore is involved in individual ligand binding.

The equilibrium binding data presented here were analyzed using a global approach with the program 'SPECTRABIND' [20]. This program uses spectroscopic data to calculate the concentrations of every spectroscopically detectable species, determine their spectral characteristics and the binding constants based upon a specified binding model. The program is based on a new approach to the analysis of spectroscopic binding data. No assumptions are made about the spectroscopic characteristics of chemical species, i.e. about the shapes of the emission spectra or number of exponentials in the decay curves. The computational approach is based upon the linearity of the spectroscopic response with respect to concentration of individual chemical species:

$$R(x) = \sum_{i=1}^{N_{sp}} C_i S_i(x) \quad (6)$$

where x is an independent spectroscopic variable (wavelength or time), $R(x)$ is the spectral response (spectrum or decay curve), N_{sp} is the number of species, C_i is the molar concentrations of the species i, and $S_i(x)$ is the spectroscopic characteristic of the species i. Since an equilibrium binding study involves several spectra, Eq. (6) can be represented in the matrix form, $\mathbf{R} = \mathbf{CS}$, where the matrix $\mathbf{R}$ contains all the spectra. In the analysis all the elements of the matrixes $\mathbf{C}$ (concentrations) and $\mathbf{S}$ (spectroscopic characteristics) play the role of fitting parameters.

A system of equilibrium equations defines the binding model. The equilibrium binding equations impose constraints on the elements of the concentration matrix $\mathbf{C}$. Additional constraints are placed on the system by delineation of mass conservation:

$$\sum_{i=1}^{N_{sp}} C_{mi} A_{ik} = B_{mk} \quad (7)$$

where B_{mk} is the total concentration of the element k in the sample m, and the coefficient A_{ik} represents the number of times element k is involved in the species i. As with Eq. (6), Eq. (7) can also be represented in the matrix form $\mathbf{CA} = \mathbf{B}$.

This article uses SPECTRABIND to analyze fluorescence intensity, spectra, and decay data obtained during a titration or series of titrations. The series of spectral data are used simultaneously for the data analysis. The number of titrations depends on the number of equilibrium binding equations being solved. In addition to specifying the spectroscopically detectable species, the user also inputs matrix files which contain information on the concentration of ligand and macromolecule, the proposed ligand

binding model, e.g. stoichiometry, and the initial guesses for binding constants. Typical measurements are made on individual species as well as their mixtures obtained through a series of titrations. The values of the binding constants and the spectroscopic characteristics of the species are determined in a non-linear least squares procedure. Data that are not defined linear responses are represented in their linear forms prior to data analysis, e.g. anisotropy was given in terms of I_{VV} and I_{VH}.

SPECTRABIND output includes the binding constants, confidence intervals, and a chi-square for each analysis. The analysis concludes when the program has found the spectroscopic characteristics and concentrations for individual species that result in a minimal chi-square and satisfy the system of equilibrium equations. The chi-square represents the weighted sums of the squared differences between the observed and predicted values:

$$\chi^2 = \sum_{m=1}^{N_{SAM}} \sum_{n=1}^{N_{RES}} \frac{[R_{mn} - (CS)_{mn}]^2}{\sigma^2 R_{mn}} + \sum_{m=1}^{N_{SAM}} \sum_{k=1}^{N_{EL}} \frac{[B_{mk} - (CA)_{mk}]^2}{\sigma^2 B_{mk}} \quad (8)$$

where N_{SAM} is the number of samples, N_{RES} is the number of responses obtained with every sample, e.g. the number of wavelengths or channels, and $\sigma^2 R_{mn}$ and $\sigma^2 B_{mk}$ are the variances calculated individually for every spectroscopic and chemical measurement. Once a minimum is reached, the program also gives the spectroscopic characteristics per unit concentration for each spectroscopically detectable species in the titration, e.g. free ligand, free macromolecule, macromolecule-ligand complex.

3. Results

3.1. Qualitative evidence for protein-probe interactions—Effect of protein on Quin2 fluorescence emission spectra

The criteria for selecting human serum albumin in this model system were its multifunctional binding properties, a published X-ray crystallographic structure, and a greater affinity for small, negatively charged hydrophobic molecules [27,28]. In order to determine if Quin2 was interacting with human serum albumin, a solution of Quin2 was titrated with this protein. Steady-state emission spectra of Quin2 in the presence of increasing amounts of human serum albumin are shown in Fig. 1. To minimize effects of calcium on Quin2 and human serum albumin interactions and fluorescence, EGTA was added at a concentration of 17 mM. The concentration of human serum albumin ranged from 0 to 4.4 μM. Addition of human serum albumin resulted in increased Quin2 fluorescence intensity. At these wavelengths, there is some fluorescence contribution from the protein solution, but this is small (< 2% of the total fluorescence intensity over all wavelengths for 4.4 μM human serum albumin) and therefore does not contribute significantly to the observed enhancement. Unlike the Quin2-Ca^{2+} complex whose emission was slightly red-shifted relative to free Quin2, the presence of protein resulted in a blue-shift of the Quin2 emission spectrum. This blue-shift in the peak observed at these protein concentrations was approximately 10 nm. While the presence of albumin was shown to affect the Quin2 fluorescence emission spectrum, further evidence was needed to confirm the existence of molecular interactions.

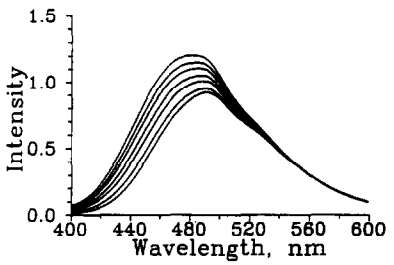

Fig. 1. Effect of human serum albumin on the steady-state emission spectra of Quin2. Emission spectra were taken for Quin2 in the presence of 17 mM EGTA at protein concentrations ranging from 0–4.4 μM. The concentration of Quin2 was 34 μM. All data were obtained at 20°C. The buffer contained 10 mM TRIS, pH 7.2, 0.1 M KCl, and 5 mM EGTA. The excitation wavelength was 325 nm.

3.2. Chromatographic study of protein-probe interactions

Since it was of interest to obtain evidence for binding by a different method, a chromatographic

technique similar to that of Bancel et al. [11] was used to study Quin2-protein interactions. In this study, several samples were applied to a size exclusion column: human serum albumin + EGTA; human serum albumin + Ca^{2+}; Quin2 + EGTA; Quin2 + Ca^{2+}; human serum albumin + Quin2 + EGTA; human serum albumin + Quin2 + Ca^{2+}. The first two samples were used to determine the void volume of the column which is equivalent to the retention time of human serum albumin. Quin2 + EGTA and Quin2 + Ca^{2+} were used as references for the elution of probe alone. The last two samples were used to provide information as to which form of Quin2 was interactive with the albumin. The elution profiles for the human serum albumin and Quin2 references are shown in Fig. 2(A) where human serum albumin (60 kDa) eluted in the void volume (○) and Quin-2 eluted at approximately 8.5 ml (□). The presence of calcium did not affect the elution of either human serum albumin alone or Quin2 alone (data not shown).

The remaining panels in Fig. 2 show the elution patterns for samples containing both human serum albumin and Quin2. The bottom two panels reflect samples eluted in the absence(B) and in the presence(C) of calcium. In the absence of calcium, Quin2 fluorescence was only detected in the void volume and coincided with the observed protein fluorescence, indicating that Quin2 co-eluted with the protein. The elution pattern for human serum albumin and Quin2 in the presence of calcium resembled the individual mobilities of protein and probe alone, i.e. human serum albumin was detected in the void volume whereas Quin2 was detected at approximately 8.5 ml. Thus addition of calcium to this sample abolished the co-elution of Quin2 with human serum albumin.

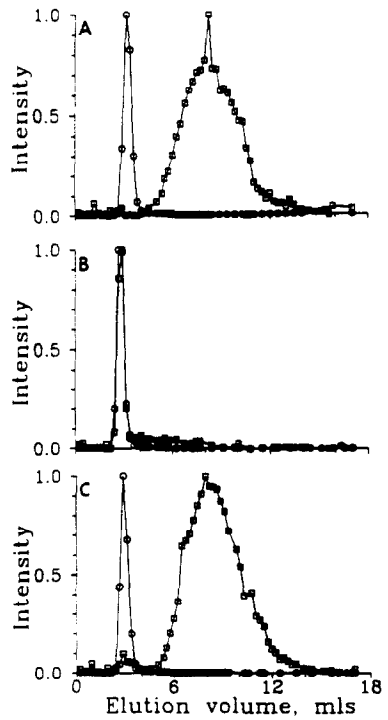

Fig. 2. Effect of human serum albumin on gel permeation chromatography of Quin2. Panel (A) depicts the elution profile of either human serum albumin alone (○) or Quin2 alone (□). Panel (B) is the elution profile of a solution of Quin2 and human serum albumin in the presence of 60 mM EGTA. Panel (C) shows the elution profile of a solution of Quin2 and human serum albumin in the presence of 0.3 M $CaCl_2$. Fluorescence of human serum albumin (o, dotted line) was detected by exciting the eluant at 295 nm and measuring emission at 350 nm. Quin2 (□, solid line) was detected with an excitation and emission pair of 340 nm and 490 nm. The concentrations of Quin2 and human serum albumin were 70 μM and 262 μM, respectively.

3.3. Steady-state fluorescence anisotropy

Steady-state fluorescence anisotropy was also utilized as an independent measure of the existence of protein-Quin2 interactions. Table 1 gives the fluorescence anisotropy values of Quin-2 in the presence of several proteins. To avoid confusion, all measurements were made in the presence of EGTA unless addition of calcium was specified. Steady-state fluorescence anisotropy depends on both hydrodynamic and spectral parameters including the fluorescence decay time of the fluorophore [24]. The anisotropy values for free Quin2 and its calcium complex were 0.036 and 0.006, respectively; the decrease in the anisotropy upon calcium binding is mainly due to the increase in the fluorescence decay time.

Other samples represented in Table 1 are Quin2 in the presence of various proteins. It should be pointed out, that the numbers in Table 1 reflect intensity-weighted average anisotropy resulting from the contributions of the free and protein-bound forms of

Table 1
Effect of various proteins on Quin2 steady-state anisotropy [a]

Sample	Anisotropy(r)
EGTA	0.036 ± 0.001
Ca^{2+}	0.006 ± 0.001
Phosphoglucose isomerase	0.070 ± 0.001
Alcohol dehydrogenase	0.053 ± 0.002
Alkaline phosphatase	0.089 ± 0.004
CPK	0.037 ± 0.001
Aldolase (1.1 mg/ml)	0.035 ± 0.001
BSA	0.089 ± 0.001
GPDH + EGTA	0.052 ± 0.001
GPDH + Ca^{2+}	0.005 ± 0.001
Aldolase (7.0 mg/ml) + Egta	0.067 ± 0.002
aldolase (7.0 mg/ml) + Ca^{2+}	0.006 ± 0.001

[a] $\lambda_{ex} = 339$ nm; Emission path contained KV399 filters. Sample temperature was 25°C except for measurements with CPK, aldolase, and BSA which were done at 37°C. All samples contained Quin2 at 100 μM. The concentration of phosphoglucose isomerase, alcohol dehydrogenase, alkaline phosphatase, CPK, BSA, and GPDH were 3.0 mg ml^{-1} (24 μM), 3.0 mg ml^{-1} (36 μM), 3.6 mg ml^{-1} (45 μM), 0.05 mg ml^{-1} (0.6 μM), 0.3 mg ml^{-1} (4 μM), and 6.3 mg ml^{-1} (26 μM), respectively. The concentrations of aldolase were 1.1 mg ml^{-1} (7 μM) and 7.0 mg ml^{-1} (45 μM).

Quin2. Since we did not determine the fractions of free and bound Quin2 in each case, the anisotropies of individual chemical species cannot be calculated from the data in Table 1. Even in the cases where 100% of the Quin2 is bound to a protein, the steady-state anisotropy can have a low value if the binding is flexible. Accordingly, the anisotropy values in Table 1 are of limited value except that they indicate which proteins bind Quin2 under given conditions.

Opposed to the effect of calcium on the anisotropy of Quin2, addition of phosphoglucose isomerase, alcohol dehydrogenase, alkaline phosphatase, and bovine serum albumin resulted in the increased anisotropy values of 0.070, 0.053, 0.089, and 0.089, respectively. In contrast, creatine phosphokinase and aldolase (1.1 μM) did not affect Quin2 anisotropy significantly. However, at higher concentrations of aldolase (7 mg ml^{-1} in the absence of calcium), there were distinct changes in the anisotropy of Quin2 to a value of 0.067. In some cases, increasing the concentration of protein resulted in new anisotropy values, e.g. aldolase (7.0 mg ml^{-1}) which could not be accounted for by the effect of protein concentration on the viscosity of the medium. Upon addition of Quin-2 to horse liver alcohol dehydrogenase, an increased anisotropy followed by precipitation was observed. It has been suggested that removal of Zn^{2+} from alcohol dehydrogenase causes aggregation of the protein [29].

Fig. 3 depicts the titration of Quin2 with human serum albumin both in the absence (○) and in the presence (□) of calcium. When the titration was performed in the presence of protein (absence of calcium), the anisotropy of Quin2 increased in a saturable and significant manner, suggesting that protein-probe interactions are occurring. In contrast, the titration of the Quin2-Ca^{2+} complex with human serum albumin showed only a small enhancement. Human serum albumin by itself exhibits some fluorescence with excitation at 325 nm which may be due to a chromophore tightly associated with the albumin as some molecules, e.g. pyridoxal phosphates, are known to do; applications of techniques such as dialysis, charcoal, or SM2 hydrophobic beads [28] usually do not result in their removal. The anisotropy associated with this fluorescence was found to be 0.258. As human serum albumin was titrated into the Quin2-Ca^{2+} solution, the fraction of the visible fluorescence due to the albumin component increased and thus resulted in the small increase in the anisotropy observed in the Quin2-Ca^{2+} complex. These data are in agreement with both the results obtained with gel chromatography and Quin2 fluorescence emission spectral changes.

Since the effects of human serum albumin on Quin2 fluorescence parameters appears to be calcium-dependent, further qualitative experiments were done to assess the effect of calcium on the interaction of Quin2 with other proteins. The results of such effects are depicted in Table 1 for several proteins. In the absence of calcium, addition of glyceraldehyde-3-phosphate dehydrogenase and aldolase to Quin2 resulted in increased anisotropy values, supporting the existence of probe-protein interactions. However, addition of calcium to Quin2-protein samples resulted in a decrease in anisotropy the value associated with the Quin2-Ca^{2+} complex (0.006). The results which were obtained with glyceraldehyde-3-phosphate dehydrogenase and aldolase were qualitatively similar to those of human serum albumin.

3.4. Quantitation of equilibrium binding-steady-state anisotropy studies for the determination of K^D

In an attempt to determine the dissociation constant of the protein-probe complex, several titrations such as that shown in Fig. 3 were done with the addition of human serum albumin to Quin2. Data were analyzed using the program 'SPECTRABIND' described in Materials and Methods. Use of the 'SPECTRABIND' analysis method indicated that the data were consistent with two equal and independent binding sites for Quin2 per molecule of human serum albumin. Criteria for fitting the data with this model included the chi-square value, confidence intervals, and the recovered concentrations for spectroscopically detectable species in each sample in the titration. For purposes of the calculation, the protein was treated as two subunits with one Quin2 molecule binding per subunit. Human serum albumin exhibits a wide range of binding constants and stoichiometries for various ligands. However, He and Carter [27] proposed two major ligand binding sites on human serum albumin. Analysis of the isothermal binding data resulted in a $\chi^2 = 1.8$ and an intrinsic binding constant of $K_d = 7.5\,\mu M$. Using steady-state anisotropy values, the binding of Quin2 to one site on human serum albumin could not be resolved from binding to the other site. Other binding models did not fit the data adequately and resulted in significantly higher chi-square values. For example, the model of 1:1 binding of Quin2 per molecule of human serum albumin gave a chi-square of 47.5.

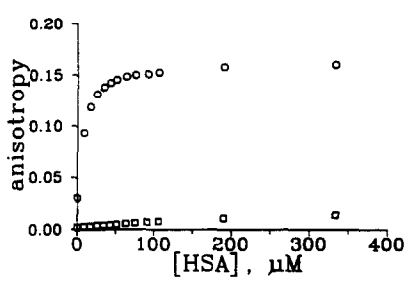

Fig. 3. Effect of human serum albumin on the steady-state anisotropy of Quin2. Data shown represent the titrations of Quin2 with human serum albumin in the presence of EGTA (o) or calcium (□). The concentrations of Quin2, EGTA, and Ca^{2+} were 100 μM, 83 mM, and 17 mM, respectively. The titrations were done at 20°C with $\lambda_{ex} = 339$ nm as described in Section 2.

3.5. Resonance energy transfer measurements

Energy transfer provides another method for evaluating interactions between Quin2 and proteins. In order for energy transfer to occur, the fluorescence emission of the donor (Trp in human serum albumin) must overlap with the absorption of the acceptor (Quin2). Since energy transfer is a non-radiative process by which the excited state is depopulated, it appears as a quench of fluorescence emission of the tryptophan in human serum albumin.

Table 2 shows the results of a typical titration of human serum albumin by Quin2 using steady-state and time-resolved fluorescence methods. The sample intensity and lifetime at each Quin2 concentration are given. As the concentration of Quin2 increased, the fluorescence intensity of human serum albumin decreased. The data required three exponentials to obtain an adequate fit with the χ^2 for individual data curves ranging from 1.0 to 1.2. The lifetimes of human serum albumin alone were 0.4 ns, 2.4 ns, and 7.0 ns with absolute alphas of 0.08, 0.13, and 0.15, respectively. As Quin2 was added to human serum albumin, α_3, τ_2, and τ_3 decreased and the value of α_1 increased slightly. The observed decrease in the

Table 2
Effect of Quin2 on Trp emission and lifetimes in human serum albumin [a]

[Quin2], μM	α_1	τ_1	α_2	τ_2	α_3	τ_3	$\langle\tau\rangle$ [b,c]	I_{ss}
0	0.08	0.4	0.13	2.4	0.15	7.0	3.9	1.37
0.8	0.09	0.4	0.13	2.4	0.15	6.9	3.7	1.33
2.5	0.08	0.4	0.13	2.5	0.13	6.9	3.7	1.23
5.8	0.10	0.4	0.13	2.3	0.11	6.6	3.2	1.08
13.8	0.11	0.3	0.13	2.1	0.09	6.1	2.6	0.85
21.5	0.12	0.3	0.12	2.0	0.07	5.9	2.2	0.70

[a] $\lambda_{ex} = 295$ nm; $\lambda_{em} = 350$ nm. Pre-exponential terms are given as absolute alphas. I_{ss} refers to the steady-state fluorescence intensity.

[b] $$\langle\tau\rangle = \frac{\sum_{i=1}^{n} \alpha_i \tau_i}{\sum_{i=1}^{n} \alpha_i}$$

[c] The changes in this lifetime at increasing concentrations of Quin2 most likely reflect changing proportions of two lifetimes which were not resolved by the data analysis.

average lifetime of the tryptophan of human serum albumin as the concentration of Quin2 was increased is also consistent with the quenching of the intensity by Quin2.

Energy transfer data were then used to determine the binding constant and binding stoichiometry of human serum albumin and Quin2. Data analysis was accomplished with the program 'SPECTRABIND' which is described in Section 2. As with the fluorescence anisotropy data, the energy transfer data were best fit for a model of two equal and independent Quin2 sites per one human serum albumin molecule. The dissociation constant was determined to be 4 μM. This value is in good agreement with that obtained using emission anisotropy data.

As was described earlier, results from data analysis with the program SPECTRABIND include the spectroscopic characteristics of all spectroscopically detectable species. Fig. 4 shows nanosecond decay output from 'SPECTRABIND'. These decays represent theoretical decays of human serum albumin alone (A) and of the Quin2-saturated human serum albumin (B). Comparison of Fig. 4 with individual decays obtained at various Quin2 concentrations revealed that the human serum albumin in these experiments was not saturated with Quin2. The average lifetime of human serum albumin could decrease further at even higher Quin2 concentrations. Energy transfer measurements corroborated the existence of interactions between Quin2 and human serum albumin. As with data presented earlier, calcium abolishes energy transfer (data not shown). These results are consistent with the results of column chromatography which suggests that the presence of calcium disrupts protein-probe interactions. The implication of this type of interaction is significant in that protein-probe interactions would interfere with the ability of Quin2 to bind to calcium.

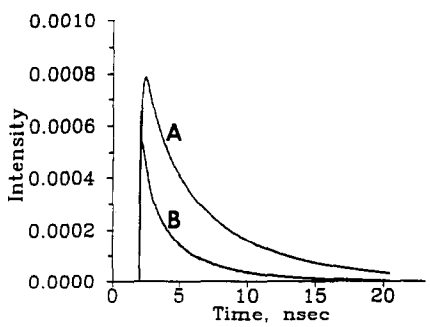

Fig. 4. Theoretical fluorescence decay curves of human serum albumin(A) and the human serum albumin-Quin2 complex(B). Data were analyzed using SPECTRABIND for 2:1 binding of Quin2 to human serum albumin. Curve (B) depicts the decay when all the Quin2 sites are saturated. Intensities represent intensity per unit concentration of species. The program SPECTRABIND is described in Section 2.

3.6. Effect of protein-probe interactions on calcium binding

Other data in these studies suggest that Quin2 binding to proteins is a calcium-dependent phenomenon. It has been postulated that protein-probe interactions may require the four negatively charged carboxyl groups of Quin2. If this were assumed to be true, the presence of proteins could alter the cation-binding ability of the probe. Any effects would be dependent upon the protein-probe affinity as well as their concentrations.

To further study the effects of Quin2 interactions with protein on cation binding capability, Quin2 titrations with calcium were performed at various protein concentrations. Emission spectra taken at each calcium concentration for two different titrations are shown in Fig. 5. Panel (A) depicts the emission spectra during a titration in the absence of protein. Panel (B) shows the emission spectra obtained on a Quin2 titration with calcium in the presence of 15.3 μM human serum albumin. The peak of emission is about 492 nm for free Quin2 in the absence of protein. As was shown earlier, the presence of protein alters the emission properties of Quin2 such that the emission spectra have distinctly different characteristics than those shown in panel (A). In the presence of human serum albumin and 0 μM calcium, the intensity of free Quin2 is greater than that for free Quin2 in the absence of protein and 0 μM calcium. As the concentration of calcium increased, the intensity of Quin2 in the presence of protein increased and the spectrum shifted from a peak of about 455 nm to 492 nm. There was also an isoemissive point at about 455 nm. In the absence of

protein (panel A), addition of calcium causes only a small red shift in the spectrum (about 5 nm) and increases the intensity. Upon reaching saturation of Quin2 with calcium, the intensity and peak emission wavelength are the same in the absence and in the presence of human serum albumin.

Titration data similar to those shown in Fig. 5 were collected at five concentrations of human serum albumin. In these titrations, two different complexes were being formed: (i) Quin2-Human serum albumin; (ii) Quin2-Ca^{2+}. The data from all five concentrations were analyzed for binding constants using the program 'SPECTRABIND' as is described in Section 2. As with the anisotropy and energy transfer data, the model of Quin2 binding to human serum albumin was 2:1. Using 'SPECTRABIND', the dissociation constant of the Quin2-Ca^{2+} and the Quin2-HSA complexes were 74 nM and 8.5 μM, respectively, with a χ^2 of 1.1. The spectroscopically detectable species used in the analysis were free Quin2, Quin2-Ca^{2+}, Quin2-Human serum albumin, and free human serum albumin. Fig. 6 depicts the species-associated emission spectra recovered from 'SPECTRABIND' including the spectroscopically detectable species: free Quin2, Quin2-Ca^{2+} complex, Quin2-Human serum albumin complex, free human serum albumin.

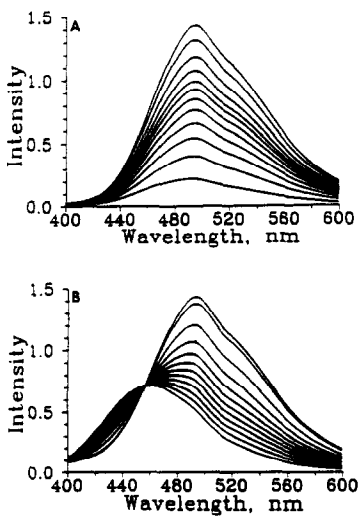

Fig. 5. Effect of protein-probe interactions on the calcium-binding capacity of Quin2. Panel (A) shows the steady-state emission spectra obtained on a titration of Quin2 with calcium in the absence of human serum albumin. Panel (B) was generated from a titration of Quin2 with calcium in the presence of 15.3 μM HSA. The concentration of calcium ranged from 0–40 μM. The concentration of Quin2 was 10 μM. The titration was performed at 20°C as is described in Section 2.

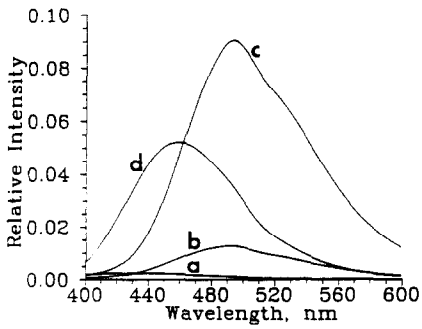

Fig. 6. Theoretical steady-state emission spectra of HSA(a), Quin2(b), the Quin2-calcium complex(c), and the Quin2-HSA complex(d). These spectra were generated from SPECTRABIND by analyzing data from titrations of Quin2 with calcium in the presence of five different concentrations of HSA. The analysis approach is described in Section 2. The intensities represent intensities per unit concentrations of the species given.

4. Discussion

Protein-probe interactions have been shown to occur with other calcium-chelating dyes, e.g. Fura-2 and Indo-1, resulting in changes in their steady-state fluorescence properties [5–12]. Resulting from the physical nature of human serum albumin and its wide range of ligand binding, it was suspected that Quin2 would also interact with it. In particular, human serum albumin is known to bind many small, aromatic, carboxylic acids to two sites on the protein with approximately equal binding constants and with analogous binding chemistry [27]. Binding of Quin2 would result in emission spectra which are distinct from that of free Quin2 in solution because of the asymmetric distribution of hydrophobic amino acid residues in the binding crevices for carboxylic acids. The blue shift in the peak of fluorescence emission of Quin2 as well as increased intensity in the presence of protein are indicative of interactions between Quin2 and human serum albumin. Both the blue shift

in the emission and intensity enhancement have been reported for other calcium-binding probes in the presence of protein [9,11]. An increase in quantum yield and blue shifts in emission spectra suggest movement of the probe into a more hydrophobic environment. Quantum yield generally increases as the polarity of the solvent or probe environment decreases due to a reduced rate of nonradiative decay. The nature of the probe-protein interactions may also constrain the excited state of the probe, thereby increasing the probability of fluorescence.

Bancel et al. [11] postulated that only the free form of the calcium-chelating probe Indo-1 could interact with bovine serum albumin because of the availability of the negative charge on the carboxyl groups for electrostatic interaction with positive charges on the protein. The gel chromatography results for Quin2 presented in Fig. 2 in this article are similar to those obtained by Bancel et al. [11] for Indo-1 interactions with bovine serum albumin. Quin2 elution from the column in the absence of protein was that expected for a molecule of its size. However, the addition of human serum albumin resulted in its co-elution in the void volume. This observation could only be explained by the fact that non-covalent probe-protein interactions forced Quin2 off the column at a much faster rate than would be expected. Results from changes in emission spectra are in agreement with these column studies.

Addition of calcium to samples containing Quin2 and human serum albumin resulted in an elution profile much like that of Quin2 alone. This suggested that the presence of calcium disrupted Quin2-protein interactions. The high affinity of the probe for calcium would result in the formation of a probe-Ca^{2+} complex. Occupation of the carboxyl groups by this cation would alter the overall molecular charge and interfere with the ability of the probe to form a complex with the protein. Since addition of calcium appeared to abolish probe-protein interactions, this suggests that Quin2-protein interactions may be occurring through the carboxyl groups. Both data presented here and those of Bancel et al. [11] support the existence of these types of interactions between the calcium-chelating probes and albumins. This contrasts with the earlier theory of Konishi et al. [6] who proposed that Fura-2, an analogous calcium-chelating probe, even when bound to other proteins could still bind to calcium. While this does not appear to be true for Quin2 and the series of proteins studied, formation of the calcium-Quin2 complex may enhance interactions with proteins not yet determined. Calcium can also be bound to the human serum albumin, possibly preventing Quin2-albumin interactions. However, the affinity of calcium for albumin is about four orders of magnitude lower than that for Quin2: approximately 9×10^2 M^{-1} as compared to 1.7×10^7 M^{-1} [28]. Thus, since Quin2 has a high affinity for calcium, it is likely that the calcium is primarily bound by Quin2 rather than protein.

Fluorescence polarization is a property which is dependent upon the rotational diffusion of the fluorophore and the fluorescence lifetime. For a fluorophore that has exponential intensity decay and can be approximately considered as a spherical rotator, the steady-state anisotropy is:

$$r = \frac{r_o}{1 + \frac{\tau}{\phi}} \tag{9}$$

where r is the observed steady-state anisotropy, r_o is the anisotropy at $t = 0$, and ϕ is the rotational correlation time. The motions of the fluorophore, in turn, are dependent upon the viscosity of the solvent as well as the size and shape of the fluorophore. The emission anisotropy values obtained for both free Quin2 and its calcium complex reflect values for rapidly tumbling, small molecules, i.e. 0.036 and 0.006 respectively. Binding of calcium by Quin2 resulted in a greater depolarization of the fluorescence emission than for free Quin-2. The lower value for the Quin2-Ca^{2+} complex reflects its longer decay time as compared with that of free Quin2 [30].

The existence and extent of fluorescence anisotropy changes observed with each protein differed and depended on a variety of factors, such as the proportion between protein-bound and free Quin2 as well as the flexibility of the linkage between the protein and the fluorophore (Eq. (9) is not applicable when the linkage is flexible). The anisotropy of several of the Quin2-protein complexes indicate that it rotates more like a macromolecule. While τ_{ave} of Quin2 in the presence of albumin is longer (data not shown), the increased emission anisotropy values suggest that the rotational diffusion is constrained significantly, and the latter effect dominates over the decrease in

anisotropy caused by the increased lifetime. The results in Table 1 indicate that the presence of albumins, phosphoglucose isomerase, alkaline phosphatase, glyceraldehyde 3-phosphate dehydrogenase can affect the emission anisotropy of Quin2. The numbers in Table 1 do not represent the anisotropies of pure Quin2-protein complexes because of possible contributions from free Quin2. Nevertheless, the anisotropy data demonstrate that Quin2 may interact with intracellular proteins as well as albumin with significant changes in the fluorescence properties of Quin2.

Data demonstrating that the emission anisotropy of Quin2 in the presence of protein decreases to that of the Quin2-Ca^{2+} complex upon addition of calcium suggests that abolition of probe-protein interactions occur in a calcium-dependent manner. Since Quin2 interactions with calcium occur through the carboxyl groups, this suggests that these groups are unavailable for protein interactions. One may argue that calcium may bind to the protein rather than Quin2 with the resultant release of Quin2. However, in this case, the observed anisotropy values would be closer to that of free Quin2 (0.036) rather than to that of the Quin2-Ca^{2+} complex (0.006). Further, if the Quin2-Ca^{2+} complex were binding to human serum albumin, then there would have been large systematic deviations in the analysis of titration, emission anisotropy, and energy transfer data, resulting in a statistically higher χ^2. Likewise, the dissociation constant of the protein-probe complex obtained from these data is in good agreement with those from anisotropy and energy transfer data.

The binding constant derived from emission anisotropy measurements taken during titrations of Quin2 with human serum albumin is consistent with the proposed model for human serum albumin, i.e. two major ligand binding sites for aromatic carboxylic acids. The inability to resolve differences between the two binding sites for Quin2 on human serum albumin supports the belief that these sites are chemically and physically similar. Since the emission anisotropy is dependent upon the lifetime and rotational correlation time of Quin2, large differences in the two probe environments would be reflected in unique effects on the anisotropy of Quin2.

Resonance energy transfer measurements also provide evidence for two separate Quin2 binding sites on human serum albumin. This was detected by the asymmetry in the location of the Trp residue on human serum albumin and the effects of Quin2 on the fluorescence decay parameters of this Trp residue. Changes in both the steady-state and dynamic properties of tryptophan provide evidence for resonance energy transfer between the tryptophan of human serum albumin and Quin2. Analysis of the results shown in Table 2 indicates that the decrease in average lifetime and steady-state fluorescence intensity is due not only to a decrease in τ_3, but also a decrease in the absolute alpha associated with τ_3. This is consistent with two mechanisms by which the fluorescence of the tryptophan in human serum albumin is quenched. According to the X-ray crystal structure presented by He and Carter [27], the tryptophan is located in one of the proposed major ligand binding sites. One type of quenching may be the result of Quin2 binding in this major ligand binding site which would place it in close proximity with the tryptophan residue. In this case, the energy transfer rate which would be very high would result in a very short decay time reflected as an apparent decrease in alpha 3. In fact, the theoretical decay curves from 'SPECTRABIND' analysis shown in Fig. 4 support this. The peak intensity of the decay in Fig. 4 (B) is lower than that of decay Fig. 4 (A). This is most likely because of the rapid quenching of the tryptophan in human serum albumin by a Quin2 molecule which is located in the major binding site. The second type of quenching, probably resulting from Quin2 binding to a site distant from the single Trp, would result in a decrease in the long lifetime of human serum albumin. The appearance of this new decay constant could not be resolved either by global or single curve analysis. These data are consistent with the existence of two thermodynamically equivalent but spectroscopically distinguishable binding sites. This model is further supported by the 'SPECTRABIND' analysis (vide infra).

Data presented in this article for Quin2 binding to human serum albumin are consistent with the X-ray crystallographic structure of human serum albumin and binding of other ligands to human serum albumin. The proposed ligand binding sites of human serum albumin contain mostly hydrophobic residues which shield ligands from the aqueous environment [28]. The observed chromatic shifts of Quin2 fluores-

cence emission and intensity enhancement as well as the gel filtration chromatography data suggest protein-probe interactions. The lack of observed changes for the Quin2-Ca^{2+} complex in the presence of serum albumin may be explained by several reasons: (i) binding of the Quin2-Ca^{2+} complex to albumin has no significant effect on the fluorescence; (ii) binding of the Quin2-Ca^{2+} complex to albumin is much weaker than that of free Quin2.

Knowing the dissociation constant of all the complexes, it was possible to calculate the apparent constant for the Quin2-Ca^{2+} complex in the presence of any concentration of human serum albumin. This calculation was performed for the titrations shown in Fig. 4. At a concentration of 15.3 μM human serum albumin, the apparent affinity of Quin2 to calcium decreased 2.8 times, the dissociation constant increased from 74 nM to 208 nM. The implications of these measurements extend beyond studies of protein-probe complexes. Konishi et al. [6] reported that nearly 80% of Fura2, a calcium-binding probe which also contains the tetracarboxylate structure, may be bound to macromolecules in cells. The effects of such interactions have been addressed for Fura2 [31]. Generally, intracellular ion concentrations are determined using known dissociation constants. The constants are determined in buffers which mimic the intracellular milieu for ionic strength, pH, and cation content. Literature values generally do not account for effect of proteins on the dissociation constant. If the probe-protein interactions are not taken into consideration, then the apparent calcium concentration measured in the protein soup inside cells may be distinctively lower than the actual value.

In addition to studying how Quin2 may interact with proteins and what effects those interactions have on calcium-binding, these studies demonstrate how the combination of steady-state and time-resolved fluorescence techniques as well as a new computational approach can be used to define molecular interactions. With the emergence of new generations of cation probes, non-specific interactions must be investigated. The techniques presented here offer an approach to understanding probe behavior in complex systems.

References

[1] J.E. Whitaker, R.P. Haugland and F.G. Prendergast, Anal. Biochem., 194 (1991) 330.
[2] R.Y. Tsien, Biochem., 19 (1980) 2396.
[3] C.J. Frederickson, E.J. Kasarskis, D. Ringo and R.E. Frederickson, J. Neurosci. Meth., 20 (1987) 91.
[4] B. Raju, E. Murphy, L.A. Levy, R.D. Hall and R.E. London, Am. J. Physiol., 256 (1989) C540.
[5] S.M. Keating and T.G. Wensel, Biophys. J., 59 (1991) 186.
[6] M. Konishi, A. Olson, S. Hollingworth and S.M. Baylor, Biophys. J., 54 (1988) 1089.
[7] L.A. Blatter and W.G. Wier, Biophys. J., 58 (1990) 1491.
[8] S.M. Baylor and S. Hollingworth, J. Physiol. (Lond.), 403 (1988) 151.
[9] F. Bancel, J.-M. Salmon, J. Vigo, T.Vo-Dinh and P. Viallet, Anal. Biochem., 204 (1992) 231.
[10] N. Kurebayashi, A.B. Harkins and S.M. Baylor, Biophys. J., 64 (1993) 1934.
[11] F. Bancel, J.-M. Salmon, J. Vigo and P. Viallet, Cell Calcium, 13 (1992) 59.
[12] H. Ikenouchi, G.A. Peeters and W.H. Barry, Cell Calcium, 12 (1991) 415.
[13] J. Maylie, M. Irving, N.L. Sizto and W.K. Chandler, J. Gen. Physiol., 89 (1987) 41.
[14] S.M. Baylor, S. Hollingworth, C.S. Hui and M.E. Quinta-Ferreira, J. Physiol. (Lond.), 377 (1986) 89.
[15] T.W. Hurley, M.P. Ryan and R.W. Brinck, Am. J. Physiol., 263 (1992) C300.
[16] L. Järlebark and E. Heilbronn, Eur. J. Pharmacol., 225 (1992) 75.
[17] R. Brandes, V.M. Figueredo, S.A. Camacho, A.J. Baker and M.W. Weiner, Biophys. J., 65 (1993) 1983.
[18] J. Wages, Jr., B. Packard, D. Walbridge and L. Brand, J. Cell Biol., 103 (1986) 514 A.
[19] J.R. Lakowicz, H. Szmacinski, K. Nowaczyk and M.L. Johnson, Proc. Natl. Acad. Sci., 89 (1992) 1271.
[20] D. Toptygin and L. Brand, Anal. Biochem., 224 (1995) 330.
[21] D.G. Moisescu and H. Pusch, Pflug. Arch., 355 (1975) R122.
[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
[23] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Plenum, New York, 1983, pp. 44–45.
[24] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Plenum, New York, 1983, pp. 134–136.
[25] M. Badea and L. Brand, Meth. Enz., 61 (1979) 378.
[26] A. Grinvald and I.Z. Steinberg, Anal. Biochem., 59 (1974) 583.
[27] X.M. He and D.C. Carter, Nature, 358 (1992) 209.
[28] T. Peters, Jr., Advances in Protein Chemistry, 37 (1985) 161.
[29] C.J. Spangler, Ph.D. Thesis, Johns Hopkins University, Baltimore, Maryland (1990).
[30] K.M. Hirshfield, D. Toptygin, B.S. Packard and L. Brand, Anal. Biochem., 209 (1993) 209.
[31] W.N. Ross, Biophys. J., 64 (1993) 1655.