Improving the Performance of a Sol−Gel-Entrapped Metal-Binding Protein by Maximizing Protein Thermal Stability before Entrapment

Lili Zheng, Kulwinder Flora, and John D. Brennan*,†

Department of Chemistry, Brock University, St. Catharines, ON, L2S 3A1, Canada

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We show that the performance of a sol−gel-entrapped protein can be improved substantially by maximizing the protein thermodynamic stability so that it can withstand the harsh conditions associated with entrapment. Two mutants of the calcium-binding protein oncomodulin were entrapped into optically clear tetraethyl orthosilicate-derived monoliths which were prepared by a two-step sol−gel processing method. The first mutant contained a single tryptophan residue at position 57 of the native CD binding loop in place of the native tryrosine (Y57W), while the second, more stable mutant contained a higher affinity engineered CD binding loop (CDOM33, with a single Trp residue at position 57). The thermodynamic stability of both proteins could be manipulated by adjusting the level of Ca^{2+} present during entrapment. Intrinsic tryptophan fluorescence and Tb^{3+} luminescence (resulting from energy transfer from the Trp residue) were monitored during binding of Tb^{3+} to examine terbium-binding capacity and response times for both entrapped proteins. Tryptophan fluorescence was also used to study the thermal stability of the entrapped proteins at different calcium levels. Improvements in binding ability, thermal stability, and response times were obtained when CDOM33 or Y57W was entrapped with increasing levels of Ca^{2+} present during entrapment.

Introduction

The characteristics of proteins entrapped in glasses formed by the sol−gel processing method have been extensively studied during the past few years. Numerous reports have appeared describing the function,[1] structure,[2] dynamics,[3] accessibility,[4] reaction kinetics,[5] initial stability,[6] and long-term stability[7] of entrapped proteins. These studies have established that, in the majority of cases, entrapped biological molecules retain their characteristic biochemical functionality and remain stable over periods of months. However, in cases where proteins are destabilized by either low pH or high ethanol levels, encapsulation can result in a substantial loss of function[8] and can cause significant structural changes in proteins initially and over time.[2] Encapsulation also increases the response time for the interaction of reagents with biomolecules and makes reaction kinetics complex, mainly as a result of slow diffusion rates for species inside monoliths[2a] combined with electrostatic interactions between charged analytes and the negatively charged silicate matrix.[5]

Poor initial and long-term stability of entrapped proteins combined with increased response times results in serious problems when entrapped proteins are used for biosensor development or other applications which require the interaction of entrapped proteins with external reagents. Hence, the ability to control and optimize these factors is of critical importance. In this

* To whom correspondence should be addressed.

† Present address: Department of Chemistry, McMaster University, Hamilton, Ontario, L8S 4M1, Canada. Tel: (905) 525-9140 (ext. 27033). Fax: (905) 522−2509. E-mail: brennanj@mcmail.cis.mcmaster.ca.

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work, we are interested in examining how the thermodynamic stability of a protein affects its ability to withstand the harsh conditions associated with the sol−gel entrapment procedure (low pH, high ethanol levels), how this correlates with the performance of the entrapped protein (i.e., ability to bind ligand, response time, regenerability, long-term stability), and whether such factors can be controlled and optimized.

The biomolecule we have chosen for this study is the Ca^{2+}-binding protein oncomodulin (OM).[9] Several single tryptophan mutants of this protein are available,[10,11] allowing the structure and function of the protein to be monitored using fluorescence spectroscopy. The stability of several mutant OM proteins was recently reported to be dependent on the loading of metal ions,[12] the type of metal ion bound (Ca^{2+} or Tb^{3+}),[13] and the affinity of the protein for the metal ion.[12,13] On the basis of these studies, two mutants were chosen for encapsulation into sol−gel-derived matrixes: Y57W, which has a Trp replacing a tyrosine residue at position 57 of the CD loop,[10] and CDOM33, which has a modified CD loop, prepared by insertion of a 12 amino acid sequence with a significantly higher affinity for Ca^{2+} than the native CD or EF loop and with a Trp in position 7 of the binding loop (amino acid position 57).[11] These two mutants of the protein were chosen for several reasons. First, there are significant changes in the intensity and spectral characteristics of the Trp residue upon protein denaturation which can be used to examine changes in the structure of the protein upon entrapment.[13] Second, the placement of the Trp residue at position 57 of the CD binding loop allows for efficient transfer of excitation energy to bound Tb^{3+} by the electron exchange mechanism,[14,15] resulting in two terbium luminescence peaks at 490 and 545 nm. The ratio of Trp fluorescence to Tb^{3+} luminescence provides a sensitive method for examining the binding capacity and binding kinetics of the entrapped proteins under different entrapment conditions. Finally, the Y57W mutant is less stable that the CDOM33 mutant at similar levels of Ca^{2+};[13] however, the stability of both proteins can be manipulated by adjustment of the Ca^{2+} concentration in solution.[13] The ability to adjust the protein stability before entrapment allows for a study of the effects of initial protein stability on the structure, function, and long-term stability of the protein after entrapment and aging. Establishing a link between these factors is fundamentally important to the development of optimized entrapment protocols and will result in improvements in devices which utilize entrapped proteins.

Experimental Section

Chemicals. All protein samples were donated by Dr. A. G. Szabo of the University of Windsor and were used without further purification. Details of the preparation and purification procedures have been described elsewhere.[16] Tetraethyl orthosilicate (TEOS, 99.999+%), terbium(III) chloride (hexahydrate, 99.9%), dipicolinic acid (DPA, 99%), and trichloroacetic acid (TCA, 99+%) were supplied by Aldrich (Oakville, ON). Anhydrous calcium chloride (99+%) was obtained from Fisher scientific. Polymethacrylate fluorimeter cuvettes (transmittance curve C) were obtained from Sigma (Oakville, ON). All water was twice distilled and deionized to a specific resistance of at least 18 MΩ cm using an Elga "Prima" 5-stage water purification system. All other chemicals were of analytical grade and were used without further purification.

Procedures. *Preparation of Protein-Doped Sol−Gel Slides.* All proteins were dissolved in 10 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) buffer containing 100 mM KCl at pH 7.2. The concentration of protein was determined by UV−vis absorbance measurements using $\epsilon_{280} = 6900\ M^{-1}\ cm^{-1}$ for both proteins.[10,11] Holoproteins (metal-loaded) were prepared by adding a 3-fold excess of Ca^{2+} or Tb^{3+} to the proteins using 100 μM stock solutions of $CaCl_2$ or $TbCl_3$ which were prepared in PIPES buffer. The exact concentration of the Tb^{3+} stock solution was determined by a dipicolinic acid titration.[17]

Hydrolyzed TEOS solutions were prepared by sonicating a mixture of 4.5 mL of TEOS, 1.4 mL of water, and 100 μL of 0.1 N HCl for 1 h at ambient temperature until the mixture became clear. The silane solution was then stored at −20 °C for 7−10 days to ensure complete hydrolysis.[2a] Storage at this temperature minimized changes in solution composition after hydrolysis. A volume of 300 μL of the hydrolyzed TEOS solution (at 0 °C) was rapidly mixed with an equal volume of PIPES buffer (10 mM, pH 7.2, with 100 mM KCl, 10 μM of holoprotein, 3−100-fold excess of calcium or 3-fold excess of both Tb^{3+} and Ca^{2+} versus protein) at a temperature of 0 °C and immediately placed into a disposable acrylate cuvette (1 cm path length) which was then sealed with Parafilm and placed on its side until gelation occurred (normally about 5−10 min). A small hole was punched in the Parafilm and the sol−gel-derived slides were aged at 4 °C for 25 days. At this point, the slides were rehydrated, as described previously,[2a] and stored in sealed cuvettes to prevent further drying. The dimensions of the aged, protein-doped glass slides were 15 mm × 8 mm × 0.2 mm.

Pore Size Analysis. Pore size analysis was performed on samples prepared with varying levels of Ca^{2+} present during entrapment using a Coulter SA3100 surface area/pore size analyzer. Before analysis, the monoliths (either with or without encapsulated protein) were crushed to a fine powder and outgassed at 100 °C for 12 h to remove air and bound water from the surface of the powder. The pressure was measured as nitrogen was adsorbed and desorbed at a constant temperature of −196 °C. From the desorption branch of the resulting isotherm, the average pore size and distribution of sizes was determined using the BJH (Barrett, Joyner, and Halenda) calculation.[18]

Measurement of Fluorescence Spectra. Fluorescence emission spectra of protein-doped monoliths were collected using instrumentation and procedures which are described elsewhere.[2a] Emission spectra were collected from 305 to 450 nm for Ca^{2+}-loaded proteins and from 305 to 570 nm for Tb^{3+}-loaded proteins with excitation at 295 nm. All spectra were collected in 1 nm increments at a rate of 180 nm min^{-1}. Samples were maintained at a temperature of 20 ± 0.2 °C (except where otherwise stated) using a Neslab R110 recirculating water bath. Appropriate blanks were subtracted from each sample

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and the spectra were corrected for deviations in emission monochromator throughput and photomultiplier tube response.

Binding Capacity and Response Time Studies. Protein-doped slides which were prepared with varying levels of Ca^{2+} present in the gelation buffer were placed in a 1 cm^2 quartz cuvette. The sample was excited at 295 nm and the fluorescence intensity at 545 nm was recorded at 5 s intervals using a 4.99 s integration time immediately after 2.0 mL of a terbium solution (0.1 mM to 5.0 mM) was added. The change in intensity was recorded until the signal reached a plateau. The response time was determined from the point where a plateau in the signal intensity occurred. The binding and kinetic data reported is the average obtained from three samples.

Tb^{3+}-Binding Curves. To determine Tb^{3+}-binding constants for entrapped proteins, it was necessary to evaluate the extent of Tb^{3+} binding to the silicate matrix to obtain a reliable value for the concentration of free Tb^{3+} in solution. The degree of Tb^{3+} binding to the matrix was determined by incubating approximately 0.2 g of intact sol−gel-derived glass slides overnight in 2.0 mL of 0.1 mM Tb^{3+} or 1.0 mM Tb^{3+}. The glass was then removed and the concentration of free Tb^{3+} in solution was determined by titrating with a 0.5 mM solution of dipicolinic acid in PIPES buffer. The Tb^{3+} concentration values were compared to those obtained from solutions which were not incubated with the glass to provide an estimate of Tb^{3+} binding per gram of glass.

To obtain Tb^{3+}-binding curves, monoliths were prepared and aged with varying levels of excess Ca^{2+} present in the gelation buffer, as described above. The aged monoliths (25 days old) were rehydrated with 1.5 mL of a buffer solution containing a 3-fold or 50-fold molar excess of Ca^{2+}. The affinity of Tb^{3+} binding to the entrapped proteins was examined by titrating the protein with a 10 mM stock solution of Tb^{3+} in PIPES buffer and collecting a fluorescence spectrum at each point until no further spectral changes were observed. The spectra were integrated over the Trp emission band and the Tb^{3+} peak centered at 545 nm, and the ratio of intensities (Tb^{3+}:Trp) was determined and converted to a percent signal enhancement. Normalized signal enhancement was plotted against the ratio of total concentration of Tb^{3+} to the concentration of binding competent protein to generate binding curves.

Scatchard analysis of Tb^{3+} binding was done to obtain equilibrium binding constants for proteins which were able to fully bind Tb^{3+} (i.e., no denatured fraction). In this case, the fluorescence intensity was first converted to the fraction of binding sites filled (f) according to the equation

$$f = \frac{F - F_{min}}{F_{max} - F_{min}} \tag{1}$$

where F is the measured fluorescence at a given concentration of Tb^{3+}, F_{min} is the fluorescence intensity in the absence of Tb^{3+}, and F_{max} is the maximum fluorescence intensity obtained in the presence of excess Tb^{3+}. The fraction of sites filled was multiplied by the protein concentration to determine the amount of Tb^{3+} bound, and this value was in turn used to calculate the free concentration of Tb^{3+} at each point during the titration (assuming that no binding of Tb^{3+} to the glass occurred, as indicated by the DPA titration). The concentration of Tb^{3+} bound was divided by the protein concentration to obtain the average number of Tb^{3+} ions bound per macromolecule (ν). Finally, $\nu/[Tb^{3+}]_{free}$ was plotted against ν and the equilibrium binding constants (K) were obtained from the Scatchard equation

$$\frac{\nu}{[Tb^{3+}]_{free}} = -K\nu + KN \tag{2}$$

where N is the number of binding sites.

Thermal Denaturation Studies. A total of 2.0 mL of buffer solution was placed into the cuvettes containing the monolith. The solution was purged with nitrogen for 15 min and the cuvette was immediately capped to minimize the amount of dissolved oxygen, which was shown to cause irreversible alterations in the fluorescence signals from thermally unfolded oncomodulin.[13] The temperature was raised in ca. 3 °C increments, starting at 20 °C and going to 95 °C, and then lowered to 20 °C to check reversibility. The temperature of the solution in a second cuvette which was also present in the four-sample turret was measured directly using a thermistor probe (Hanna Instruments model 9043A) to account for loss of heat through the Tygon tubing connecting the sample holder and the water bath. Initially, the samples were allowed to equilibrate for 5, 10, 20, or 30 min at each temperature, at which point a fluorescence spectrum was collected from the sample and from an appropriate "no protein" blank. Spectra obtained at 10, 20, or 30 min equilibration times were identical, thus a 10 min waiting time was selected for most experiments. The corrected emission spectra were integrated over the entire emission band. The integrated intensity was normalized to the intensity at the starting temperature (usually 20 °C) and was plotted against temperature to generate the unfolding curve.

The unfolding enthalpy change ($\Delta H°_{un}$) and entropy change ($\Delta S°_{un}$) were calculated by fitting the unfolding curve to the following equation[19]

$$F_T = \frac{(F_{0N} + s_N T) + (F_{0U} + s_U T)\exp\{-\Delta H°_{un}/RT + \Delta S°_{un}T/RT\}}{1 + \exp[-(\Delta H°_{un}/RT + \Delta S°_{un}T/RT)]} \tag{3}$$

where F_T is the fluorescence at a given temperature T, F_{0N} and F_{0U} are the fluorescence intensity of the native state and unfolded state at a given reference temperature (20 °C), s_N and s_U are the temperature dependence of the fluorescence for the native and denatured states (slope of the pre- and post-unfolding regions, respectively), and R is the gas constant. The transition temperature (T_{un}) was calculated by dividing $\Delta H°_{un}$ by $\Delta S°_{un}$.[19] The free energy change for unfolding ($\Delta G°_{un}$) was determined by using eq 4, with a reference temperature (T_r) of 20 °C

$$\Delta G°_{un}(T_r) = \Delta H°_{un} - T_r\Delta S°_{un} + \Delta C_{p,un}[(T_r - T_{un}) - T_r \ln(T_r/T_{un})] \tag{4}$$

where $\Delta C_{p,un}$ is the differential heat capacity for unfolding and accounts for the temperature sensitivity of the entropy and enthalpy terms on going from T_r to T_{un}.

To compare the thermal stability results with previous studies of oncomodulin stability in solution[13] and to determine the effects of metal ion concentration, the thermal stability was examined for encapsulated Y57W and CDOM33 with no extra calcium added in either the gelation buffer or surrounding buffer, with a 50-fold molar excess of calcium in the gelation buffer (with respect to the protein concentration) but no calcium in the surrounding buffer, and with a 50-fold molar excess of calcium in both the gelation and surrounding buffer solutions.

Reversibility and Regenerability Studies. Tb^{3+} was allowed to bind to CDOM33 which was entrapped in a sol−gel-derived matrix using a 50-fold molar excess of Ca^{2+} in the gelation buffer. Following complete binding of the Tb^{3+}, the slides were rinsed with 10 mM PIPES buffer (first without and then with 25 mM Ca^{2+}) several times to remove excess unbound terbium (12 h per rinse cycle). The decrease in Tb^{3+} luminescence was used to measure of the reversibility of binding. To test regenerability, a volume of 250 μL of 40% (m/v) trichloroacetic acid (TCA) was added to the slides in 2.0 mL of PIPES buffer and left for 5 min. The slides were then rinsed with distilled water and then with 10 mM PIPES buffer containing 25 mM Ca^{2+} a minimum of five times (12 h per rinse cycle). Tb^{3+} luminescence was used to confirm regeneration.

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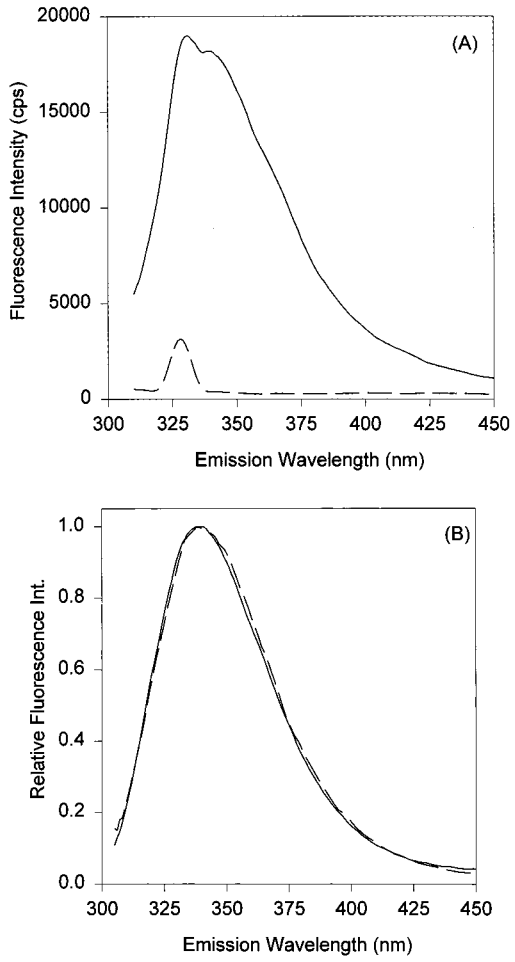

Figure 1. (A) Raw emission spectra of sol−gel-derived monolithic glass slides containing entrapped CDOM33 (solid line) and no protein (dashed line). (B) Normalized emission spectra of CDOM33 in solution (solid line) and entrapped in a sol−gel-derived glass slide (dashed line).

Results and Discussion

Fluorescence Spectroscopy of Entrapped Proteins. Details regarding the measurement of tryptophan fluorescence from proteins entrapped into sol−gel-derived monoliths were described previously.[2a] Figure 1a shows the raw data obtained for an emission scan of entrapped CDOM33 and of the corresponding blank. The spectra were obtained from samples which had 10 μM of the protein entrapped (solid line) or no protein entrapped (dashed line). The emission spectra of the sample provide a signal on the order of 2×10^4 counts per second (cps) with minimal spectral noise (ca. 20 cps). The blank shows background counts of approximately 300 cps, mainly due to a small amount of light scattering. Both the sample and blank show a peak at 326 nm due to Raman scattering from the aqueous solution surrounding the monolith. The limit-of-detection using the acquisition parameters outlined in the Experimental Section was 0.2 μM of entrapped CDOM33. This concentration is far below that commonly used for absorbance-based studies of entrapped proteins and leads to a major savings in the amount of protein used.

Figure 1b shows the normalized emission spectra of CDOM33 in solution and when entrapped. Both spectra have been corrected for background signals and for wavelength deviations in monochromator throughput and photomultiplier tube response. Both the free and entrapped protein have essentially identical emission spectra. This provides strong evidence that the protein is not denatured during entrapment and indicates that the internal environment within the silicate matrix has a dipolarity which is similar to that of an aqueous solution, in agreement with previous reports.[2a]

Terbium-Binding Ability of Entrapped CDOM33 and Y57W OM. *No Extra Calcium Added.* The Tb^{3+}-binding ability of CDOM33 and Y57W OM which was entrapped with no Ca^{2+} present in the gelation buffer was initially tested by adding 2.0 mL of a 0.1 mM Tb^{3+} solution to a cuvette containing the monolith. A small terbium peak at 545 nm (<0.5% of the full signal for the protein in solution) was observed after 1.5 h for both proteins. The internal volume of the monolith after aging, determined from cryogenic nitrogen adsorption experiments, was ca. 60 μL, while the pore diameter was 6.5 ± 1.1 nm and was independent of the level of Ca^{2+} present during gelation. Since the volume of the protein solution was 300 μL before mixing with the silane solution, the local concentration of the entrapped protein increases by approximately 5-fold, to a final value of 50 μM. If the concentration of unbound terbium inside the matrix is the same as in the surrounding solution (i.e., no Tb^{3+} binds to the silicate), the local concentration of terbium inside the matrix should be ca. 2 times that of the entrapped protein, resulting in complete binding of Tb^{3+} by the protein. The lack of a large Tb^{3+} luminescence signal indicated that (1) a substantial fraction of protein had denatured, (2) a large fraction of the Tb^{3+} was associated with the negatively charged silicate matrix, lowering the concentration of free terbium ions in the surrounding solution enough to prevent binding, or (3) the binding constant (K) of the entrapped protein is significantly lower than that of the free protein.

To examine the possibility of protein denaturation by ethanol during entrapment, the Trp emission spectra of holoproteins CDOM33 and Y57W oncomodulin in solution were monitored during the addition of ethanol. The results are shown in Figure 2. Titrations of CDOM33 and Y57W OM resulted in significant changes in the spectral properties of both proteins with the presence of >30% ethanol, confirming that ethanol caused structural changes in the proteins which were consistent with denaturation. The ethanol content of freshly prepared monoliths has been determined to be about 35% (v/v), on the basis of fluorescence studies using pyranine[20] and 7-azaindole.[21] Hence, the level of ethanol present during entrapment is sufficient to destabilize oncomodulin and thus reduce the binding constant of the protein. It should be noted that the destabilization of Y57W is greater than is observed for CDOM33 (as shown in Figure 2), consistent with expectations based on stability studies of the proteins in solution.[13]

Addition of more concentrated Tb^{3+} solutions (1.0, 3.0, or 5.0 mM) was done to further investigate the effects of Tb^{3+} interactions with the silicate matrix. A 1.0 mM solution resulted in a higher terbium signal (27 ± 2% of maximum for CDOM33, 10 ± 1% of maximum for

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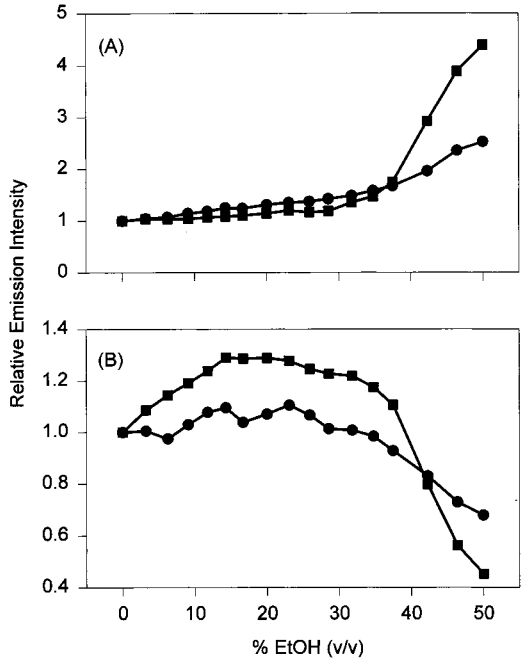

Figure 2. Response of Y57W (■) and CDOM33 (●) oncomodulin to the presence of ethanol in solution. The samples contain a 3:1 ratio of Tb^{3+} to protein. Panel A shows the effect of ethanol on the Trp emission. Panel B shows the effect of ethanol on Tb^{3+} luminescence. Error bars fall within the size of the symbols.

Y57W) in a shorter period of time. Addition of 3.0 mM Tb^{3+} increased the response of CDOM33 slightly to 29 ± 1% but had no effect on the signal from Y57W. The higher level of Tb^{3+} (5.0 mM) resulted in no further improvement in binding capacity for either protein. In a second set of experiments, the aged monoliths were soaked in a 3.0 mM calcium solution before addition of the 1.0 mM terbium solution to further examine the effects of analyte−matrix interactions on the binding capacity of the entrapped proteins. Under these conditions, there was again about 30% of the maximum terbium signal obtained for CDOM33 and 10% of the maximum signal obtained for Y57W compared to the values obtained in solution. This value did not increase when the level of Tb^{3+} was increased to 3.0 or 5.0 mM. These results indicated that between 70% and 90% of the protein was irreversibly denatured when the standard sol−gel entrapment method was used, with the less stable mutant being denatured to a greater extent.

Dipicolinic acid titrations were done to examine Tb^{3+} binding to the silicate matrix. These experiments indicated that less than 5% of a 0.1 mM solution of Tb^{3+} bound, while there was effectively no binding observed from a 1.0 mM solution of Tb^{3+} in which sol−gel-derived slides had been incubated (<1%). These results indicated that the interaction of Tb^{3+} with the glass matrix was not significant, and thus the amount of free Tb^{3+} available for binding to the entrapped protein should be similar to that available in solution. Hence, the decreased ability of the entrapped protein to bind Tb^{3+} must be due to a combination of partial protein denaturation and a decrease in the binding constant for Tb^{3+} binding. The changes in the binding constants for the entrapped proteins are described in more detail below.

Effects of Entrapment with Excess Calcium. CDOM33 and Y57W OM were entrapped with a 5-, 10-, 20-, 30-, 40-, 50-, 60-, 80-, and 100-fold excess Ca^{2+} in the gelation buffer (with respect to the protein concentration). The relative terbium-binding capacity of these entrapped proteins was tested by adding 2.0 mL of a 1.0 mM terbium solution. There was a significant increase in the terbium signal as the concentration of entrapped calcium increased, as shown in Figure 3. The results indicate that CDOM33 was able to retain full reactivity when there was between 40- and 60-fold excess calcium present. This result is important for two reasons: first, it shows that all of the protein was entrapped in a functional form; second, it shows that the protein was fully accessible to analyte. Increasing the calcium level to values greater than 60-fold with respect to the protein concentration caused the binding capacity of CDOM33 to decrease slightly. The decrease in binding capacity was consistent with the onset of protein aggregation at high calcium levels during encapsulation.[22]

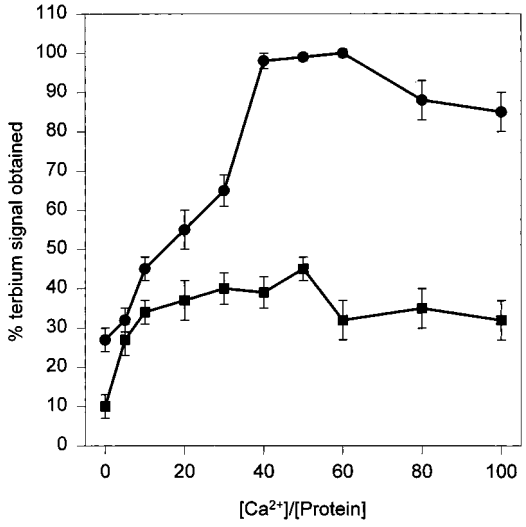

Figure 3. Tb^{3+}-binding capacity for Y57W (■) and CDOM33 (●) oncomodulin entrapped with varying levels of Ca^{2+} present in the gelation buffer.

Y57W also showed an increase in terbium-binding ability when the calcium level in the gelation buffer was increased. However, in this case the highest terbium signal was observed to be only 45% of that obtained in solution when a 30−50-fold molar excess of calcium was present. Addition of a larger amount of Tb^{3+} had no effect on the magnitude of binding. The terbium-binding ability of Y57W decreased slightly when the calcium level increased beyond a 60-fold molar excess with respect to the protein, in agreement with the results obtained for CDOM33.

To further examine the Tb^{3+}-binding ability of the entrapped protein at different Ca^{2+} levels, Tb^{3+}-binding curves were generated for both proteins in the presence and absence of 50-fold excess Ca^{2+}. The response curves are shown in Figure 4. In all cases, the amount of Tb^{3+} required to reach a binding plateau was substantially higher than that required in solution. In solution, a sharp break is generally observed at a 2:1 molar ratio of Tb^{3+}:protein for both CDOM33 and Y57W.[10,11] However, the entrapped proteins required between 4:1 and

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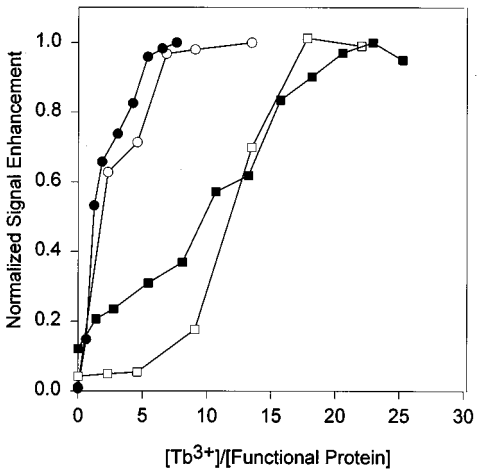

Figure 4. Binding curves obtained during the addition of Tb^{3+} to entrapped proteins: (●) CDOM33 with 50-fold excess Ca^{2+}, (■) Y57W with 50-fold excess Ca^{2+}, (○) CDOM33 with no excess Ca^{2+}, (□) Y57W with no excess Ca^{2+}.

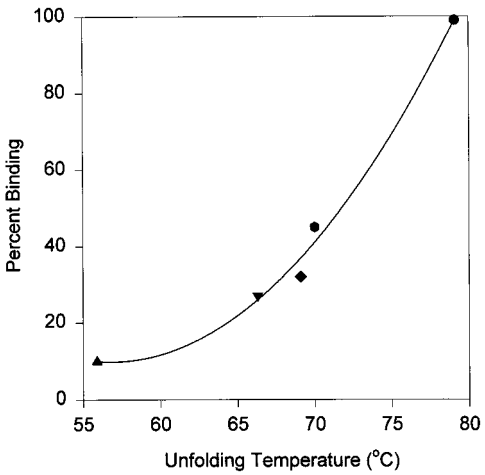

Figure 5. Percent of binding competent protein remaining after entrapment into a sol−gel-derived glass slide versus unfolding temperature of the protein in solution: (▲) Y57W in solution with no Ca^{2+} (apo), (▼) CDOM33 in solution with no Ca^{2+} (apo), (◆) CDOM33 in solution with a 2-fold ratio of Ca^{2+}:protein, (●) Y57W in solution with 50-fold excess Ca^{2+}, (●) CDOM33 in solution with 50-fold excess Ca^{2+}.

20:1 ratios of Tb^{3+}:functional protein, providing strong evidence that the equilibrium binding constants for Tb^{3+} binding were reduced on entrapment. The binding curves indicate that the binding affinity of CDOM33 is higher than that of Y57W, in agreement with the trend observed for the proteins in solution.[13] These results suggest that the presence of Ca^{2+} during entrapment affects only the fraction of binding-competent protein and not the *K* value of the entrapped protein.

To further examine the binding constants for the entrapped proteins, Scatchard analysis was attempted for each binding curve. On the basis of the results of the DPA titrations, it is reasonable to assume that reliable *K* values could be obtained. However, given that a small amount of Tb^{3+} binding to the silicate matrix was observed, the equilibrium binding constants given below should be treated as *apparent K* values that are specific to the proteins entrapped under the conditions described above. In cases where incomplete binding was obtained (i.e., total signal level was less than 100% that obtained in solution), it was not possible to accurately determine binding constants since nonlinear Scatchard plots were obtained. However, it was possible to obtain a *K* value for CDOM33 entrapped with 50-fold excess Ca^{2+}. Scatchard analysis of the binding curve provided a *K* value of $(3.4 \pm 1.1) \times 10^4\ M^{-1}$. For comparison, the *K* value for Tb^{3+} binding to CDOM33 in solution is estimated to be $\sim 10^7-10^8\ M^{-1}$,[23,24] confirming that the Tb^{3+}-binding constant is lowered by more than 1000-fold on entrapment. These results are in qualitative agreement with results obtained for entrapped antibodies, where a drop of 100-fold in the affinity constant was observed on entrapment,[1c] and with previous results obtained for entrapped parvalbumin.[25] The basis of the lower *K* values is not fully understood, but is likely due to electrostatic interactions between the negatively charged silicate matrix and the negatively charged groups in the binding loops of the protein.

Thermal Stability Studies for Entrapped CDOM33 and Y57W. Figure 5 shows the correlation between the thermal unfolding temperature of the protein in solution and the fraction of binding competent protein remaining after entrapment. The thermodynamic data for the proteins in solution was obtained from ref 13 and was used since it reflects the stability of the proteins *before* entrapment. The thermodynamic data are given in Table 1. The correlation between these parameters is good, although not linear. A plot of the free energy of unfolding against the binding capacity gave a similar correlation (see Table 1 for free energy values). These results indicate that the thermodynamic stability of the protein before entrapment affects the fraction of protein that survives the entrapment conditions and hence the overall performance of the entrapped protein. The results indicate that, whenever possible, the thermodynamic stability of proteins should be maximized before entrapment into sol−gel-derived matrixes.

To further explore the relationship between binding capacity and protein stability, we examined the thermodynamic stability of the entrapped proteins at different Ca^{2+} levels. The thermal denaturation experiments were performed using the following samples: encapsulated protein with no extra Ca^{2+} in either the gelation buffer or the surrounding buffer, protein with a 50-fold excess of calcium in the gelation buffer but no extra Ca^{2+} in the surrounding buffer, and protein with a 50-fold excess of calcium in both the gelation buffer and the surrounding buffer. The unfolding curves for entrapped CDOM33 are shown in Figure 6 (closed symbols). The thermophysical data for the entrapped CDOM33 mutant, calculated using eqs 3 and 4, are shown in Table 2.

The thermal unfolding curves and thermodynamic data clearly show that the thermodynamic stability of the entrapped protein increases as the level of Ca^{2+} present during entrapment and in the surrounding solution increases. These results are in agreement with the finding that excess levels of Ca^{2+} generally cause improvements in the stability of Ca^{2+}-binding pro-

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Table 1. Thermodynamic Parameters for Thermally Induced Denaturation of Y57W OM and CDOM33 in Solution with Different Levels of Ca^{2+} Present

sample matrix	$\Delta H°_{un}$ (kJ mol^{-1})	$\Delta S°_{un}$ (J K^{-1} mol^{-1})	T_{un} (°C)	$\Delta G°_{un}$ (kJ mol^{-1})[a]
Y57W in solution with no Ca^{2+} present (apo)[b]	117.6 ± 18.5	357.0 ± 25.2	55.9 ± 2.5	10.1 ± 1.3
Y57W in solution with a 50:1 ratio of Ca^{2+}:protein[c]	119.3 ± 13.0	347.8 ± 36.6	70.0 ± 3.7	12.6 ± 1.7
CDOM33 in solution with no Ca^{2+} present (apo)[d]	121.0 ± 25.0	356.4 ± 36.6	66.4 ± 2.8	12.6 ± 2.0
CDOM33 in solution with a 2:1 ratio of Ca^{2+}:protein[e]	256.1 ± 32.2	753.0 ± 73.9	69.1 ± 3.1	35.0 ± 5.3
CDOM33 in solution with a 50:1 ratio of Ca^{2+}:protein[f]	321.7 ± 32.2	913.6 ± 73.9	79.1 ± 3.1	54.0 ± 5.3

[a] Free energy values were calculated using eq 4 with $\Delta C_{p,un} = 1100$ cal mol^{-1} K^{-1} (from ref 13), with a reference temperature of 293K. [b] Fitting parameters were as follows: $F_{0N} = 0.997$, $F_{0U} = 0.821$, $s_N = -0.010$, $s_U = -0.008$. [c] Fitting parameters were as follows: $F_{0N} = 1.001$, $F_{0U} = 0.922$, $s_N = -0.013$, $s_U = -0.007$. [d] Fitting parameters were as follows: $F_{0N} = 1.001$, $F_{0U} = 0.724$, $s_N = -0.009$, $s_U = -0.006$. [e] Fitting parameters were as follows: $F_{0N} = 1.195$, $F_{0U} = 0.173$, $s_N = -0.009$, $s_U = -0.001$. [f] Fitting parameters were as follows: $F_{0N} = 1.034$, $F_{0U} = 0.484$, $s_N = -0.009$, $s_U = -0.003$.

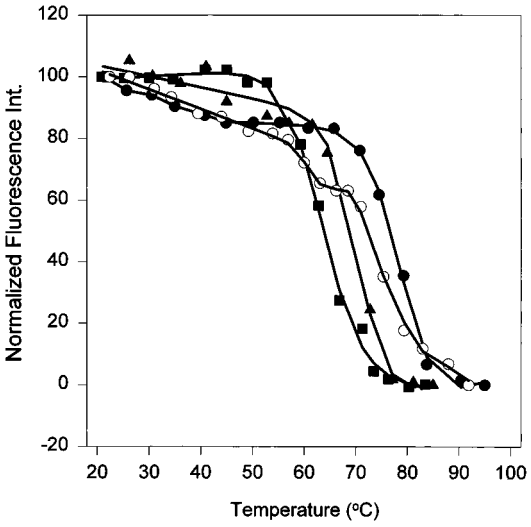

Figure 6. Changes in fluorescence intensity during thermal denaturation of entrapped proteins: (■) CDOM33 entrapped with no extra Ca^{2+}, (▲) CDOM33 entrapped with 50-fold excess Ca^{2+} in the gelation buffer, (●) CDOM33 entrapped with 50-fold excess Ca^{2+} in the gelation buffer and tested with 50-fold excess Ca^{2+} in the surrounding buffer, (○) Y57W entrapped with 50-fold excess Ca^{2+} in the gelation buffer and tested with 50-fold excess Ca^{2+} in the surrounding buffer. The symbols are the experimentally derived data points. The solid lines are the lines-of-best-fit as determined by fitting to eq 3. The data were normalized by setting the initial intensity to a value of 100 and the final intensity to a value of 0 to provide overlap of each unfolding curve.

teins.[13,26] The relatively poor stability for the holoprotein which was entrapped with no extra Ca^{2+} in the gelation buffer was expected, since the final calcium:protein ratio was ca. 0.05:1, after accounting for dilution of entrapped Ca^{2+} by the surrounding Ca^{2+}-free buffer. Thermal denaturation of CDOM33 with a 50-fold excess of calcium in the gelation buffer, but no additional calcium in the surrounding buffer, resulted in a small improvement in $\Delta H°_{un}$, with more substantial improvements in $\Delta G°_{un}$ and T_{un}. Thus, entrapment with excess Ca^{2+} in the gelation buffer did result in improvements in the stability of the entrapped protein, in agreement with the trend observed from the Tb^{3+}-binding data.

Addition of a 50-fold excess of Ca^{2+} to both the gelation buffer and the surrounding buffer caused $\Delta S°_{un}$, $\Delta H°_{un}$, $\Delta G°_{un}$, and T_{un} to increase substantially compared to the values obtained for CDOM33 without Ca^{2+} in the surrounding buffer. Interestingly, the stability of the entrapped protein was higher than that obtained for free CDOM33 with a similar amount of Ca^{2+} in solution.[13] This result suggests that the entrapped protein in the presence of excess Ca^{2+} was at least as stable as the protein in solution. Hence, entrapment with a 50-fold excess of Ca^{2+} in the surrounding buffer resulted in full retention of the thermodynamic stability of the protein, correlating well with the ability of the protein to retain 100% binding activity using these entrapment conditions.

The differences in $\Delta H°_{un}$, $\Delta S°_{un}$, and $\Delta G°_{un}$ for the proteins entrapped under different conditions indicate that the initial and final conformational states of the entrapped protein change, on the basis of the entrapment conditions, since each of these values depends only on the nature of the initial and final conformations.[27] The similarity in the thermophysical data for free and entrapped CDOM33 with a 50-fold excess of Ca^{2+} in solution indicates that the entrapped protein is able to fully unfold, even when entrapped. This assertion is supported by the observation that the emission maximum for the entrapped protein red-shifted to 345 nm upon denaturation (identical to the result obtained in solution). However, the enthalpy and entropy values dropped considerably for entrapped CDOM33 that was denatured with no excess Ca^{2+} in the surrounding solution. The lower values for the thermophysical parameters, compared to the entrapped protein with excess Ca^{2+} present, indicate that the initial and/or final states of the entrapped protein in the silicate matrix were not the same in all cases. On the basis of the known sensitivity of the protein to the level of excess Ca^{2+},[13] it can be concluded that the protein was entrapped in a fully folded state in the presence of excess Ca^{2+} but was entrapped in a partially unfolded state in the absence of excess Ca^{2+}, confirming that Ca^{2+} can be used to maintain the structure and stability of the protein during entrapment.

Thermal denaturation of entrapped Y57W was also attempted using the conditions listed above. However, unfolding curves for the holoprotein entrapped with no Ca^{2+} in the gelation buffer and for the protein entrapped with Ca^{2+} in the gelation buffer, but not in the surrounding buffer, showed immediate unfolding transitions and could not be adequately fit to eq 3 (unfolding

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Table 2. Thermodynamic Parameters for Thermally Induced Denaturation of Entrapped CDOM33

sample matrix	$\Delta H°_{un}$ (kJ mol^{-1})	$\Delta S°_{un}$ (J K^{-1} mol^{-1})	T_{un} (°C)	$\Delta G°_{un}$ (kJ mol^{-1})[a]
entrapped protein with no Ca^{2+} added[b]	225.3 ± 21.4	668.0 ± 50.7	64.1 ± 3.5	29.5 ± 2.4
entrapped protein with 50-fold excess Ca^{2+} in the gelation buffer[c]	239.1 ± 20.9	690.9 ± 42.5	69.1 ± 3.7	34.3 ± 2.9
entrapped protein with 50-fold excess Ca^{2+} in the gelation and surrounding buffer[d]	383.7 ± 25.0	1090.6 ± 89.6	78.8 ± 2.8	64.4 ± 6.0

[a] Free energy values were calculated using eq 4 with $\Delta C_{p,un} = 1100$ cal.mol^{-1}.K^{-1} (from ref 13), with a reference temperature of 293K. [b] Fitting parameters were as follows: $F_{0N} = 1.006$, $F_{0U} = 0.173$, $s_N = -0.008$, $s_U = -0.002$. [c] Fitting parameters were as follows: $F_{0N} = 1.014$, $F_{0U} = 0.175$, $s_N = -0.014$, $s_U = -0.001$. [d] Fitting parameters were as follows: $F_{0N} = 0.990$, $F_{0U} = 0.089$, $s_N = -0.013$, $s_U = -0.001$.

curves not shown). Thermal stability studies of entrapped Y57W obtained with a 50-fold excess of calcium in both the gelation buffer and the surrounding buffer solution resulted in the unfolding curve shown in Figure 6 (open symbols). Fitting of the curve to eq 3 was unsuccessful due to the two-step unfolding profile. However, qualitative comparison of the unfolding curve with that of CDOM33 entrapped and tested under similar conditions shows that entrapped Y57W has a lower T_{un} value than is obtained for CDOM33, as well as a broader unfolding profile. This result clearly indicates that (1) Y57W is less stable than CDOM33 after entrapment and aging and (2) Y57W exists in a distribution of different environments and/or conformations. The lower overall stability of Y57W compared to CDOM33 is consistent with the observed loss of binding capacity on entrapment being due to a fraction of the protein that is unable to survive the sol−gel encapsulation process, even with excess calcium present.

Response Time for Reactions with Entrapped Proteins. The change in the Tb^{3+} luminescence signal on binding to the entrapped proteins was used to determine the response times for proteins entrapped at the different Ca^{2+} levels. Previous work has shown that the kinetics describing the interaction of charged species with entrapped proteins is complicated and depends on several factors.[2a,5] Such factors include the ability of the analyte to enter the glass matrix,[28] the rate of diffusion of analyte through the monolith (mainly determined by viscosity effects),[29] the electrostatic interactions between the analyte and the silicate matrix,[2a,5] and the ability of the entrapped protein to bind the analyte (based on distributions of protein function and accessibility[4]). As a result of these difficulties, it was not possible to accurately determine the kinetics of the protein−ligand interaction; therefore, only the response times were determined.

The response curves for the interaction of Tb^{3+} with sol−gel-entrapped CDOM33 and Y57W were monitored with different loadings of K^+ or Ca^{2+} in the gelation buffer to see if the response time was affected by the addition of secondary ions. The response curves for the interaction of terbium with CDOM33 are shown in Figure 7. The response times, t_{max} (time required to reach 100% of the total signal change) and initial slopes were determined for each protein, as shown in Table 3. The response times showed that the interaction of Tb^{3+} with entrapped proteins in the absence of extra Ca^{2+} was relatively slow and that the addition of excess K^+ had a minimal effect on the kinetic parameters (data not shown). The insensitivity to potassium ion concentration would be expected since the initial ionic strength, before addition of extra K^+, was already greater than 100 mM, due to the presence of KCl and PIPES buffer salts in the solution.

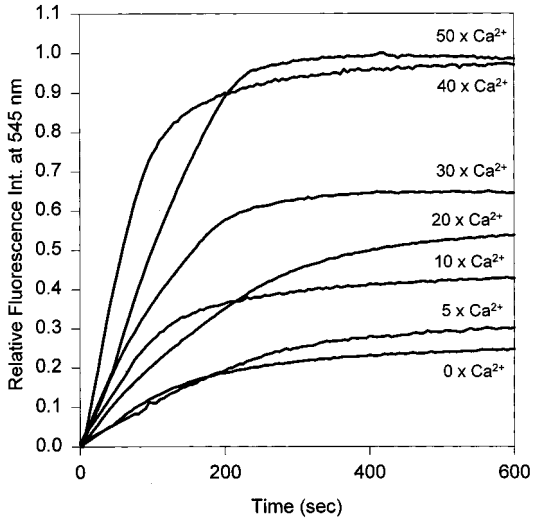

Figure 7. Kinetic response curves for interaction of Tb^{3+} with entrapped CDOM33 with different levels of calcium present in the gelation buffer used to prepare the sol−gel-derived matrixes.

Table 3. Response Times for the Interaction of Tb^{3+} with Entrapped CDOM33 and Y57W

	CDOM33		Y57W	
$[Ca^{2+}]/[pro]$	t_{max} (s)[a]	initial slope (cps/min)	t_{max} (s)[a]	initial slope (cps/min)
0	1020	600	1275	700
5	920	1200	490	2600
10	800	3200	410	4800
20	700	2500	295	6800
30	380	4500	190	7200
40	580	8700	260	5100
50	320	6200	285	6000
60	530	7600	520	4800
80	670	5700	355	4800
100	560	6200	305	5000

[a] t_{max} is the time for the response curve to reach the maximum signal intensity, determined qualitatively from inspection of the binding curve. The measurement error on t_{max} is ∼5%.

Addition of calcium to the gelation buffer before encapsulation generally resulted in a substantial reduction in the response time and an increase in the initial slopes for both CDOM33 and Y57W. In both cases, the response time dropped from over 1000 s to between 200 and 300 s and the initial slope of the response increased by 15-fold when the molar excess of calcium increased

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from 0 to over 30-fold. The trend is fairly consistent, with only a few anomolous results, which are likely due to slightly different aging conditions for each slide which caused the thickness of each to be a bit different. Overall, these findings demonstrate that the response time is sensitive to the calcium level before encapsulation. The reduced response time is indicative of easier passage of the terbium ions through the glass matrix when the glass is loaded with calcium ions. The studies done with extra KCl show that the improved response times are not based simply on ionic strength effects. Instead, it is likely that the change in these values is due to a combination of charge-screening effects and improved stability of the entrapped protein, which reduces the number of forms of the protein which interact with added Ca^{2+}. The possibility also exists that specific interactions between Ca^{2+} and the glass matrix may be responsible for the improved response times. We are currently investigating this possibility further.

Reversibility and Regenerability of Tb^{3+} Binding. A key issue in the development of sensors is reversibility and regenerability, since it is desirable to be able to reuse the device for several analyses. Reversibility was tested by simply rinsing the Tb^{3+}-loaded slide with Tb^{3+}-free PIPES buffer (both with and without 3.0 mM Ca^{2+}) several times to remove free and bound terbium. In both cases the Tb^{3+} signal remained constant, even after multiple rinse cycles with extended incubation times (over 24 h each). This result indicated that Tb^{3+} binding could not be reversed by dilution methods and is consistent with the binding constant for Tb^{3+} binding.

TCA was added to the sol−gel-derived slides to determine if the entrapped protein could be regenerated by reducing the pH so that the binding loops became protonated and unable to bind metal ions. Addition of TCA to slides containing the entrapped protein caused the terbium peak at 545 nm to be completely eliminated within 5 min. The TCA and free Tb^{3+} were removed by rinsing the slide with distilled water (pH ~5) several times. The slide was then rinsed with 10 mM PIPES buffer containing 25 mM Ca^{2+} several times to exchange any residual Tb^{3+} out of the matrix and adjust the pH to physiological values. The intensity of Trp signal was fully recovered after the second rinsing step with no Tb^{3+} signal present. Addition of 2.0 mL of a 1.0 mM terbium solution to the regenerated slide resulted in the recovery of 68 ± 3% of the initial terbium signal. A second cycle of regeneration followed by addition of Tb^{3+} resulted in recovery of 59 ± 2% of the original signal, while the third regeneration cycle resulted in a 58 ± 1% recovery of the initial signal. Five subsequent regeneration cycles produced no further changes in signal recovery.

The initial loss in the Tb^{3+} signal over the first two regeneration cycles indicated that a significant fraction of the protein was irreversibly denatured using this method. Addition of TCA to proteins in solution is known to result in precipitation of the protein,[10,16] and the recovery of native protein often requires passage of the protein through a size-exclusion column. For the entrapped protein, the presence of the proteins in individual pores should restrict protein−protein interactions, significantly reducing aggregation. The initial losses, combined with increases in the spectral full-width-at-half-maximum for the tryptophan emission spectrum (shown in Figure 8), provide evidence that aggregation occurs, with a resultant increase in light scattering. Hence, a large amount of protein is not entrapped as single protein molecules in individual pores but instead either must be entrapped with other protein molecules in the same pore or must have the ability to move between pores. At present we are unable to discriminate between these two possibilities. Even though some protein is denatured, it is clear that proteins which do survive the initial regeneration cycles can be regenerated multiple times with no further loss of binding capability, indicating that such proteins do not undergo irreversible aggregation. Hence, entrapment of proteins within sol−gel-processed materials can reduce protein−protein interactions that lead to aggregation, extending the useful lifetime of devices based on this technology.

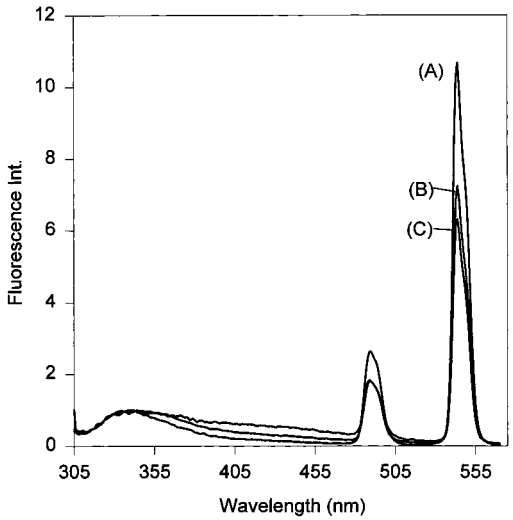

Figure 8. Emission spectra showing Trp fluorescence and Tb^{3+} luminescence signals from entrapped CDOM33 as a function of regeneration cycle for entrapped CDOM33: (A) initial response, (B) response after first regeneration cycle, (C) response after second and subsequent regeneration cycles.

Long-Term Stability and Leaching of CDOM33. Other issues important to sensor development are long-term stability and protein leaching. Both the emission of the intrinsic Trp residue and the ability of the proteins to bind Tb^{3+} were examined for samples which were aged at 4 °C over a period of 4 months. All samples tested were fresh (i.e., not regenerated after addition of Tb^{3+}) and were prepared with a 50-fold molar excess of Ca^{2+} in the gelation buffer. Tb^{3+}-binding studies indicated that CDOM33 was able to maintain over 90% of its binding ability for a period of over 50 days when a 50-fold excess of Ca^{2+} was added in the gelation buffer and the slide was stored in air in sealed cuvettes under 100% humidity conditions. However, only 30% of the initial binding ability remained after 3.5 months of storage under similar conditions. Y57W retained only 10% of its initial binding activity after 2 months and <5% after 3 months of storage, consistent with the lower thermodynamic stability of this protein.

Storage of the slides in a buffer containing a 50-fold excess of Ca^{2+} was able to completely eliminate denaturation of the entrapped proteins over a period of at

least 4 months, with only a 5% loss of signal after 6 months (this study is still ongoing). This is consistent with the improved thermodynamic stability of the entrapped protein when present in buffer solutions containing excess Ca^{2+} and shows that an understanding of the factors which affect protein stability can be used to improve both the initial stability and the long-term stability of the entrapped protein.

The intensity of the Trp signal from both the aged slide and the storage solution were tested at regular intervals and referenced to the intensity of a standard solution of tryptophan to examine protein leaching. No changes in either monolith or solution fluorescence were observed over a period of several months, indicating that no leaching of entrapped protein was occurring. This observation is in agreement with previous results obtained from entrapped monellin present in similarly aged monoliths, where no leaching was observed.[2a]

Conclusions

The ability to maximize the fraction of binding compentent protein which survives entrapment into sol−gel-processed glasses by tuning the reaction conditions to maximize protein thermodynamic stability before encapsulation is a significant finding. The addition of Ca^{2+} has the effect of stabilizing the protein and thus protects the protein from ethanol which is present initially in the sol−gel solution. While reductions in the ethanol level within the sol should, in principle, have a similar beneficial effect,[8a] this modification is often not practical since large amounts of ethanol are required, for example, to produce thin films from dipcasting of sol−gel solutions.[30] It is important to note, however, that the ability of the Ca^{2+}-binding proteins to withstand the sol−gel process depends not only on the relative thermodynamic stability (as determined by the level of Ca^{2+} present) but also on the maximum stability of the protein obtained with optimal levels of Ca^{2+} present. Thus, the more stable CDOM33 mutant was better able to withstand entrapment compared to the less stable Y57W mutant. This result correlates well with the results of Edmiston et al., which suggest that the sol−gel entrapment process is more disruptive to the structure of "soft" (i.e., unstable) proteins than "hard" (i.e., stable) proteins.[2b] However, this is the first time that a quantitative correlation has been made between the thermodynamic stability of a protein in solution and the ability of the protein to withstand entrapment into sol−gel-derived matrixes. The results clearly show the need to tailor the sol−gel entrapment protocol to optimize the stability of the biomolecule prior to entrapment.

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