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Model Systems for Flavoenzyme Activity. Specific Hydrogen Bond Recognition of Flavin in a Silicate Sol-Gel

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Abstract: Replication of the functionality and isolation provided by enzyme active sites is an important goal in the creation of effective models. To provide both of these attributes, we have covalently incorporated flavin mononucleotide (FMN) into a silicate matrix using the sol-gel process. In these sol-gels, the isolation provided by the cybotactic region of the silicate replicates the sequestered nature of an enzyme active site. Specific hydrogen bonding recognition of the flavin within the sol-gel matrix was then established via doping with diacyl diaminopyridine **3**. The presence, specificity, and magnitude of this host−guest interaction was established via quenching of the flavin fluorophore by receptor **3**.

The peptide architecture found in enzymes serves two primary functions. First, it serves to provide a scaffold for the proper geometric and dynamic presentation of functionality required for recognition and catalysis. Second, the protein superstructure allows the isolation of this active site from unwanted interactions with solvent and other reactive or disruptive entities. While researchers have been able to use model systems to explore both presentation[1] and isolation,[2] the creation of models that effectively replicate both functions remains a daunting prospect.

Flavoenzymes are proteins that use the $FADH_2$-FAD redox cycle to catalyze a variety of biological redox transformations. In these proteins, the flavin cofactor is strongly bound to the apoenzyme through noncovalent interactions. In addition to providing recognition, these interactions serve to modulate the behavior of the flavin cofactor. In the course of our research into flavoenzyme activity, we have developed model systems based on diaminopyridine receptors that replicate structural and functional aspects of flavoprotein behavior.[3,4] These solution-phase models use synthetic receptors to mimic flavoenzyme−cofactor interactions. While these models have replicated a number of aspects of flavoenzyme activity, they and other previous synthetic flavoenzyme model systems[5] all share a major limitation: they are unable to isolate the flavin from unwanted interactions with solvents and other species.[6] As a result the behavior of these models has differed significantly from that of their flavoenzyme prototypes, in terms of both efficiency and mechanism utilized. This inability to replicate a fundamental feature of protein behavior is a significant constraint in our ability to effectively model flavoenzyme behavior.

To provide a system where both flavoenzyme−cofactor

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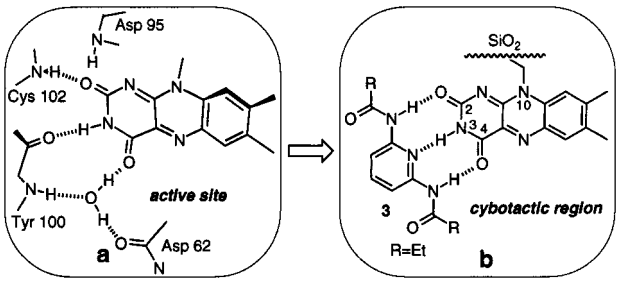

Figure 1. (a) Flavin binding site of the flavodoxin isolated from *Desulfovibrio vulgaris*. (b) Sol-gel based model system for binding of **3** to flavin.

interactions and sequestering of the flavin by the protein scaffold can be modeled, we have examined recognition and catalysis within silicate sol-gels.[7] Silicate sol-gels are readily formed through acid-catalyzed condensation of commercially available orthosilicates. Through proper choice of processing and polymerization conditions optically transparent films,[8] fibers,[9] and monoliths can be fabricated,[10] allowing direct spectroscopic analysis of incorporated chromophores. These glasses are mesoporous, allowing transport of species in and out of the gel.

The gentle conditions that can be used for the formation of sol-gels have allowed the incorporation of both inorganic[11] and organic dopants[12] into silica gels. Biomolecules such as enzymes, proteins, and antibodies have also been incorporated into silicate matrices, while still retaining their activity.[13] The examination of specific noncovalent interactions in sol-gels, as well as the application of these systems to the creation of enzyme models, is, however, an area that remains essentially unexplored.

We report here the incorporation of a flavin into a sol-gel matrix to mimic the isolation afforded by the flavoenzyme active site. Further, we demonstrate the specific recognition of the immobilized flavin by a hydrogen bonding host, mimicking the ubiquitous flavoenzyme hydrogen bonds between the apoenzyme and the O(2), N(3), and O(4) positions of the flavin nucleus (Figure 1).[14]

Incorporation of flavin into the silicate matrix was accomplished by addition of aqueous flavin mononucleotide

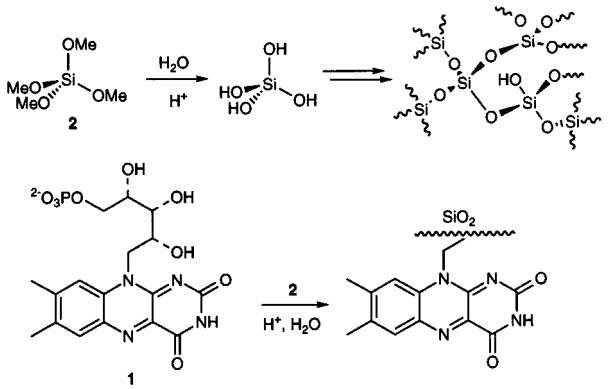

Figure 2. Formation of amorphous silicates via the sol-gel process, and incorporation of flavin into the silicate matrix.

(FMN) (**1**) and dilute acid to tetramethyl orthosilicate (TMOS, **2**) (Figure 2). The onset of gelation was observed at 25 h, with complete gelation observed at 50 h. Throughout the course of processing, the solution and gel remained transparent, with no evidence of light scattering. After gelation was complete, the flavin moieties were completely immobilized: repetitive washing of crushed FMN gels with water and CH_2Cl_2 did not result in any appreciable extraction of FMN from the gels.[15]

With immobilization of the flavin established, we next explored the creation of specific hydrogen bonding within the porous sol-gel matrix. To investigate this process, we coincorporated diaminopyridine receptor **3** (Figure 1b) into the sol-gel monolith. Receptor **3** has been previously shown to recognize flavins in solution[16] via a three-point hydrogen bond network to O(2), N(3), and O(4) of the isoalloxazine nucleus.

The optical transparency of the sol-gel monoliths allows us to directly observe the recognition of incorporated flavins by host molecules via fluorescence spectroscopy. Quenching of the characteristic fluorescence of flavins is generally observed in flavoenzymes,[17] and is attributed to the formation of strong hydrogen bonding interactions.[17b] Fluorescence quenching has also been observed upon addition of hydrogen bonding solvents to solutions of flavins in noncompetitive media.[18] In directly relevant studies, the quenching of the flavin fluorophore in

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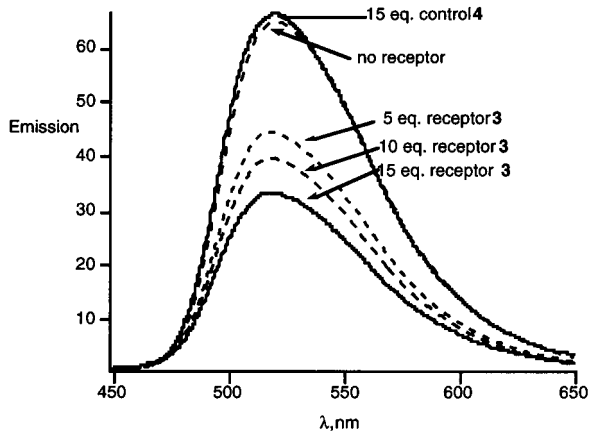

Figure 3. Fluorescence emission spectra of FMN-containing gels in the presence of receptor **3** and control **4**. Emission units are arbitrary; the excitation wavelength for these studies was 445 nm. Uncertainty of the fluorescence values was ±5%. Quantity of FMN (**1**) 0.1965 mmol/dm^3; quantity of receptor **3** 0.982 mmol/dm^3 (5 equiv), 1.965 mmol/dm^3 (10 equiv), and 2.947 mmol/dm^3 (15 equiv); quantity of control **4** 2.947 mmol/dm^3 (15 equiv).

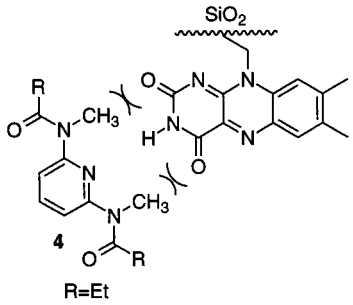

Figure 4. Non-hydrogen-bonding control **4**.

aprotic media by diaminopyridine-based receptors[19] including host **3**[3a] has also been demonstrated.

Sol-gels containing FMN and varying quantities of receptor **3** were formed by addition of the receptor prior to hydrolysis of the orthosilicate. In these gels, neither the flavin nor the receptor could be extracted, indicating complete immobilization of these species. As shown in Figure 3, addition of increasing quantities of receptor **3** to the gel increased the observed quenching of flavin fluorescence. To demonstrate that quenching of flavin fluorescence occurred via specific hydrogen bond formation, the methylated receptor analog **4** (Figure 4) was incorporated into gels as a non-hydrogen-bonding control. As shown in Figure 3, control **4** does not quench fluorescence.[20] This demonstrates that specific hydrogen bond interactions are responsible for the quenching observed with receptor **3**.

During the hydrolysis and gelation, there is negligible change (<2%) in total volume for the systems studied during the sol-gel process. Therefore, the approximate pore volume for the final solvated monolith can be estimated by calculating the amount of methanol released during the hydrolysis of the orthosilicate.

Assuming the complete hydrolysis of TMOS, the final gel will be 12% silicate and 88% methanol. Given this volume,

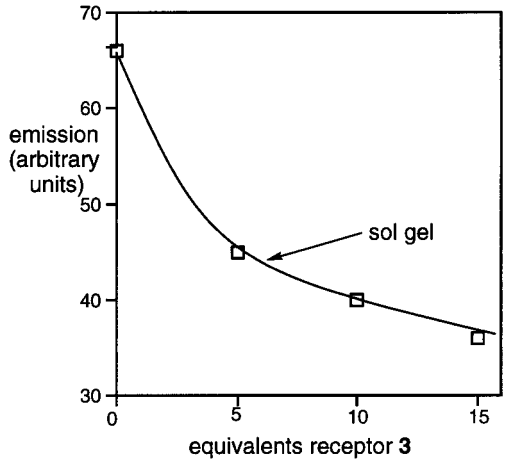

Figure 5. Plot of fluorescence emission vs concentration of receptor **3** at the emission maxima in the silicate sol-gel. Uncertainty of the fluorescence values was ±5%. Curve fit for the sol-gel is to the 1:1 isotherm calculated for the K_a = 900 M^{-1} isotherm based on overall pore volume.

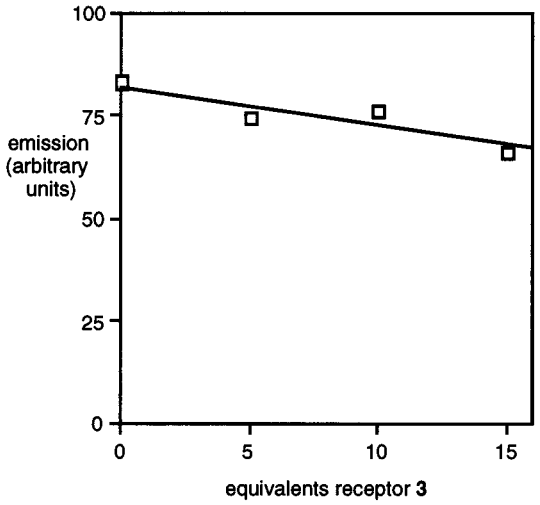

Figure 6. Plot of fluorescence emission vs concentration of receptor **3** at the emission maxima in methanol solution. Concentrations for the solution titration: 0.2232 mM flavin, the calculated concentration of FMN in the pore of the sol-gel; receptor **3** 1.12 mM (5 equiv), 2.23 mM (10 equiv), and 3.35 mmol/dm^3 (15 equiv).

the association constant (K_a) for the flavin−receptor **3** interaction can be estimated by fitting the fluorescence emission data to a 1:1 binding isotherm, providing a value of 900 ± 350 M^{-1} (Figure 5).[21] This is an unexpectedly favorable association constant, considerably larger than that found for *N*(10)-isobutylflavin[22] −receptor **3** complex in methanol (K_a < 1 M^{-1}) (Figure 6),[23] and comparable to that of the *N*(10)-isobutylflavin (K_a 537 M^{-1}) with receptor **3** in chloroform.[3d]

From the enhanced binding observed between flavin **1** and receptor **3** in the sol-gel, it is apparent that the silicate matrix fosters hydrogen-bonding interactions. To better understand the relation of recognition to the dynamic process of gelation, fluorescence quenching of FMN-functionalized gels and FMN gels doped with receptor **3** and control **4** were monitored over the course of gelation. Fluorescence emission at the initiation

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(20) Addition of higher quantities (>30 equiv) of both receptor **3** and control **4** resulted in a very gradual decrease in fluorescence intensity. This presumably arises from other interactions possible between receptor and flavin, such as π-stacking or nonspecific aggregation. Due to these nonspecific interactions, complete saturation of flavin with receptor **3** could not be achieved.

(21) The uncertainty presented is the asymptotic standard error (A.S.E.). The value presented is for comparison purposes, as this system is obviously not in the standard state.

(22) Niemz, A.; Rotello, V. *J. Am. Chem. Soc.* **1997**, *119*, 887−892.

(23) N(10) isobutyl flavin was used for these studies due to the low solubility of FMN in methanol. Since the only difference between this flavin and FMN is in the spatially remote side chain, recognition at the O(2), N(3), and O(4) surface of this flavin is expected to be comparable.

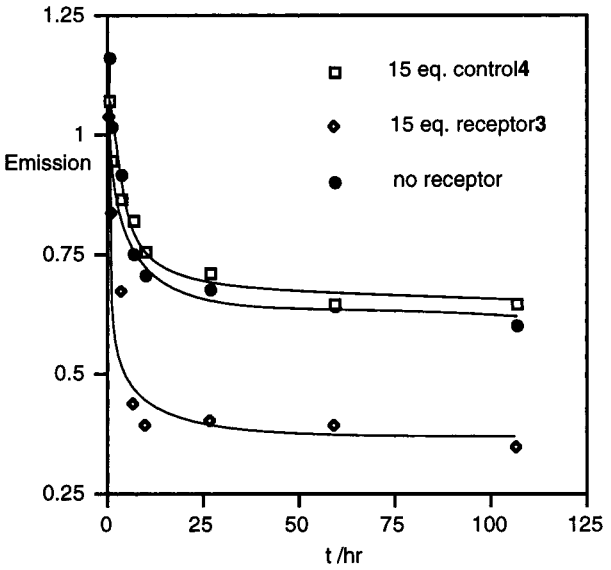

Figure 7. Time-based reduction in fluorescence emission for gels incorporating receptors **3** and **4** against undoped FMN gels. The uncertainty of the fluorescence values was ±5%.

of the hydrolysis process is essentially identical in gels containing receptor **3** and control **4** and in the gels functionalized with FMN only. A reduction in emission of equal magnitude for all three systems is observed through the hydrolysis process, where the solvent environment undergoes a transformation from primarily aqueous to methanolic. This indicates that recognition between receptor **3** and flavin **1** does not occur in the homogeneous solution. During the gelation process and subsequent formation of the silica matrix (6−110 h), however, significant quenching of flavin fluorescence in gels doped with **3** develops relative to the gels containing control **4**, or FMN (**1**) only. This demonstrates conclusively that hydrogen-bonding recognition in the receptor−flavin dyad is a consequence of the formation of the silicate matrix. The onset of recognition between receptor **3** and flavin **1** can be attributed to two seperate causes. First, polycondensation of the sol and formation of pores might increase the effective concentrations of the host and guest in localized regions. Second, the increased hydrophobicity observed in the cybotactic region during gelation will favor the hydrogen-bonding process.[24]

In summary, we have demonstrated that flavin derivatives can be immobilized via incorporation into a sol-gel silicate glass. We have also shown that specific hydrogen bond contacts to the incorporated flavin can be established through addition of a synthetic receptor to the sol-gel.[25] Taken together, these two effects mimic the isolation and specific apoenzyme−cofactor interactions found within the active sites of flavoenzymes. This offers the potential of model system fidelity that is to date inaccessible. Further studies into the enhanced recognition observed in the sol-gel matrix as well as application of silicate sol-gels incorporating flavins to the creation of biomimetic catalytic systems is currently underway, and will be reported in due course.

Experimental Section

FMN-Functionalized Sol-Gels. Sol-gels were prepared by addition of 0.690 mL of an aqueous solution containing FMN monosodium salt (**1**) (0.688 μmol) and HCl (4.0 μmol) to tetramethyl orthosilicate (2.771 mL; 18.8 mmol) to provide a solution containing 1.96×10^{-4} M FMN. The biphasic mixture was sonicated for 20 min, becoming homogenous, with an approximate pH of 6.5. The solution was then pipeted into 1-mL polystyrene cuvettes, which were then sealed and stored at room temperature in darkness. During the course of gelation, the overall volume decreased by 1.3%, providing a final FMN distribution of 1.99×10^{-4} mol/dm^3.

FMN-Receptor Functionalized Sol-Gels. Gels containing flavin with receptor **3** and control **4** were formed as above, through addition of 0.690 mL of aqueous stock solutions of FMN (0.688 μmol), HCl (4 μmol), and varying quantities of receptor/control (3.44 (5 equiv), 6.88 (10 equiv), and 10.32 μM (15 equiv) of receptor **3**, 10.32 μM (15 equiv) of control **4**).

Fluorescence Measurements. Fluorimetric studies were made on a PTI fluorimeter (QM1), using a 75-W xenon arc lamp (A1010). The excitation wavelength was 445 nm, the scan step was 0.25, and the integration time was 0.05.

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(25) While it was possible to diffuse solvents into the silicate monolith, neither receptor **3** nor flavin **1** could be added or removed from the preformed silicate. This is analogous to the flavoenzymes, where the cofactor is strongly bound by the protein.