Nanoencapsulation of Cytochrome *c* and Horseradish Peroxidase at the Galleries of α-Zirconium Phosphate

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Cytochrome *c* (Cyt *c*) and horseradish peroxidase (HRP) are encapsulated in the galleries of α-zirconium phosphate ($Zr[HPO_4]_2 \cdot nH_2O$, α-ZrP) and α-zirconium phosphonate $Zr(PO_3CH_2COOH)_2 \cdot nH_2O$ (α-ZrPAA), under mild conditions (pH 7.2, room temperature). Thermal stability, peroxidase activity, and the spectral features of the bound proteins are largely preserved. The binding constants for α-ZrP are in the range 1−100 μM^{-1}. Cyt *c* shows a much higher affinity for α-ZrP (42/μM) than HRP (1.5/μM) and α-ZrP has a much higher affinity for Cyt *c* than α-ZrPAA. The binding interactions are, thus, sensitive to the surface functional groups of the protein as well as the ZrP matrix. The α, β, and the soret absorption bands of heme protein−α-ZrP composites were essentially superimposable with those of the native protein. The FTIR spectra of the protein−phosphate composites are superimposable with those of the native proteins indicating no major changes in the secondary structures of the bound proteins. Powder diffraction data of protein/α-ZrP indicate increases in the interlayer spacings from 7.6 Å for α-ZrP to ∼33 Å for protein/α-ZrP samples, providing strong evidence for the intercalation of proteins in the galleries. Dithionite or ascorbate reduces ferric Cyt *c*/α-ZrP to the ferrous form, while hydrogen peroxide rapidly reacts with HRP/α-ZrP to form the green intermediate. Ferricyanide readily oxidizes the ferrous Cyt *c*/α-ZrP to ferric Cyt *c*/α-ZrP, and α-ZrP bound Cyt *c* retains its peroxidase activity. The thermal stability of the bound Cyt *c* is similar to that of free protein.

Introduction

Microencapsulation of proteins in inert matrices is important for their use in biosensors.[1] The matrix should protect the proteins from degradation, aggregation, or denaturation, and it should not perturb the native properties of the protein. Entrapment of proteins at the surfaces of phospholipids,[2] self-assembled monolayers,[3] silicate sol gels,[4] and polymer matrixes[5] have been reported. Such immobilization can prolong the shelf life of the protein while preserving its activity. The protective environment of the host can inhibit microbial degradation, hydrolysis, or deamidation of the bound proteins and extend their useful lifetime.[6] Retention of the native structure and access of the encapsulated protein for cofactors, substrates, or redox reagents is necessary for their application in biosensors and biocatalysis. By choosing an appropriate host, the protein can be retained in a porous medium while allowing small molecules to diffuse in and out of the matrix.[4] The binding and immobilization of redox proteins in the layered inorganic phosphates as porous matrixes under mild conditions at room temperature are described here.[7] Layered inorganic phosphates serve as excellent host materials for the nano- or microencapsulation of proteins. The surface functions of metal phosphonates can be readily altered to study how these functions influence protein structure and function. Cytochrome *c* (Cyt *c*) and horseradish peroxidase (HRP) have been chosen for the current studies as representative examples of heme proteins.

α-Zirconium phosphate ($Zr(HPO_4)_2 \cdot nH_2O$, abbreviated as α-ZrP) is a layered ion-exchange material capable of binding a variety of metal ions and organic cations in the interlayer regions.[8] The cations can be assembled as a monolayer or as a bilayer at the galleries, depending upon their charge, size, and packing properties.[9] Self-assembly of proteins can be achieved at the galler-

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ies of α-ZrP to extend the protein stability, activity, or lifetime or to modify their redox activity and specificity. Proteins that have a number of positively charged residues are expected to show a high affinity for the anionic surfaces of α-ZrP. Such proteins can be assembled as monolayers at the galleries of layered materials, under favorable conditions.[10] For example, two reports of intercalation of proteins into layered phosphates has been reported but these methods require extreme pHs (2−3 or >8) and long reaction times (as long as 17 days).[10a,b] Acid phosphatase and amylase immobilized on hydrated tricalcium phosphate matrix retained their activities with increased thermal stability.[10c] The water molecules bound to the surfaces and galleries of the phosphate can provide an aqueous-like medium. Surface-bound water and the polar surfaces of tricalcium phosphate were suggested to provide a favorable environment for the proteins. Immobilization at such polar surfaces can improve protein stabilities.[10] The α-zirconium phosphonates, where the OH groups of α-ZrP are replaced by functional groups such as 2-aminoethyl, 2-carboxyethyl, 4-aminophenyl, etc., provide different surface characteristics for interaction with proteins. Proteins can be covalently immobilized with these phosphonate functionalities without surface modifications.[11] Surface modification is essential to immobilize proteins on nylon, silica, controlled-pore glass, and cellulose. α-ZrP and phosphonates, thus, are suitable for noncovalent/covalent immobilization of proteins and to evaluate the effect of specific surface groups on the protein structure and function. Immobilization of proteins with α-ZrP will be useful in exploring the utility of these layered materials for biosensors.

Efforts to immobilize enzymes were prompted by their high cost, instability, and the difficulty in recovering active enzyme for reuse. By immobilizing enzymes on a solid phase, they can be reused, the reactions can be carried out in a continuous process, the active enzyme can be readily separated from the reaction mixture, and the enzyme properties may be improved.[10c] This could be due to increased conformational rigidity, inhibition of chemical degradation of sensitive side chains, and prevention of protein aggregation.

Cyt *c* and HRP are redox-active heme proteins, and they possess strong spectroscopic signatures to monitor their binding or redox activity in the host environment.[12,13] Cyt *c* is an important electron-transfer protein present in the mitochondrial membranes. Cyt *c* is a small protein of molecular weight 12 500, and the heme moiety in Cyt *c* is covalently linked to the backbone through two thioether linkages. Cyt *c* contains one Fe atom bound to the heme and His23, Met80, are coordinated to the iron at the fifth and sixth coordination sites. The heme provides strong absorption bands in the visible region that are sensitive to the iron oxidation state. One of the edges of the heme in Cyt *c* is exposed from its binding site, and several lysine residues are positioned near or around the heme pocket that result in a positively charged region. This charge field around the heme pocket has been implicated in the interaction of Cyt *c* with its redox partners in the biological electron transfer.[14]

HRP is a large heme protein with a molecular weight of 44 000, and its biological function is not yet known. HRP structure was suggested to resemble that of myoglobin,[15] and HRP is capable of reacting with a variety of substrates in the presence of hydrogen peroxide. HRP contains one iron atom bound to the heme, and the fifth coordination site of iron is linked to His170 while the sixth position is vacant for reaction with a variety of ligands. HRP reacts rapidly with hydrogen peroxide to form an intermediate (compound I) which oxidizes amines, phenols, and other organic compounds.[16] Encapsulation of HRP in a rigid, inert matrix which does not alter the activity of the protein, therefore, will be attractive for catalytic transformations.

The amino acid side chains of proteins vary from hydrophobic to hydrophilic, polar to nonpolar, charged to neutral, small to large, and reactive to inert. The protein surfaces, thus, are capable of interacting with a variety of environments. The binding of proteins to charged matrices is mainly due to electrostatic interactions between the protein side chains and the host. Ionic binding, therefore, can be stronger than physical adsorption. Mild conditions required for this process may result in a high retention of enzyme structure and activity after immobilization. Binding of penicillin G acylase to anionic methacrylate polymers increased its stability at 55 °C over extended periods of time, when compared to the free enzyme.[10c] Immobilization of pullulanase on amberlite IRC-50, on the other hand, resulted in loss of stability,[10e] indicating the important role of surface functions of the host on protein stability. Investigation of the affinity, organization, and activity of redox proteins with layered phosphates and phosphonates as potential host materials will be useful in biosensors, biocatalysis, and bioanalytical applications. High-affinity binding of Cyt *c* and HRP with α-ZrP is indicated in our experiments, and the bound proteins retain their redox activity, and thermal stability.

A schematic representation of the structure of α-ZrP is outlined in Chart 1.[8,17] Two layers of phosphate anions sandwich a layer of Zr(IV) ions to form two-

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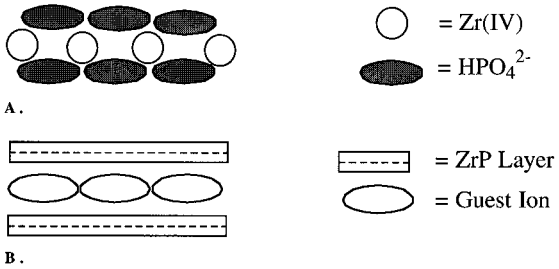
Chart 1. Schematic Representation of a Single Layer of α-Zirconium Phosphate (A) and the Intercalation of Guest Ions between Two Layers of α-ZrP (the Galleries, B) (Binding of the Guests on the Outer Surfaces Is Also Possible (Not Shown))



dimensional α-ZrP platelets with interlayer spacings of 7.6 Å. Each phosphate in the layer is bound to three Zr(IV) ions, and six phosphate oxygens are coordinated to each Zr ion in an octahedral geometry. Each phosphate anion bridges three Zr ions in a two-dimensional lattice and carries one ionizable hydroxyl group. Therefore, α-ZrP is used as an ion-exchange material for a variety of metal ions and organic cations.[18] The α-ZrP platelets are stacked with counterions intercalated in the galleries (Chart 1B), and the interlayer separation depends upon the size of the counterion present in the galleries. Adsorption may also occur on the edges and outer surfaces. The layers of α-ZrP can be exfoliated to form individual platelets, and the guests can be adsorbed at these surfaces.[20] These platelets can be reassembled under appropriate conditions to form stacks of α-ZrP platelets trapping the intercalated guests in the galleries. Small organic cations undergo spontaneous self-assembly in the galleries of α-ZrP, and these assemblies provide new materials for photochemical energy or electron-transfer reactions.[8−9,21] The surface hydroxyl groups of α-ZrP can be replaced by a variety of functional groups as in the case of zirconium phosphonates or phosphites.[22] Proteins can be covalently linked with these functional groups of immobilization. For example, polymerization of phosphonoacetic acid with zirconyl chloride leads to the formation of the corresponding acetic acid derivative of α-ZrP, $Zr(PO_3CH_2COOH)_2$, abbreviated as α-ZrPAA. The carboxylic acid functions present on the surface of α-ZrPAA provide a polar surface different from that of α-ZrP for interaction. Cyt *c* shows a much higher affinity for α-ZrP than for α-ZrPAA, as demonstrated here. Replacement of the OH groups of α-ZrP by $-CH_2COOH$, therefore, decreases its affinity for Cyt *c*. Intercalation of heme proteins at the galleries of α-ZrP, and the effect of the matrix on their properties are described below.

Experimental Section

Spectral Measurements. The absorption spectra were recorded on a Perkin-Elmer Lambda 6 spectrophotometer, in 1 cm cuvettes. α-ZrP suspensions of appropriate concentrations (0−0.01% by weight) were used in the reference beam to compensate for the light absorption or scatter (<5% at 410 nm) by α-ZrP present in the samples. The intensity of the scattered light from α-ZrP decreased dramatically after exfoliation with tetrabutylammonium chloride.[20] The FTIR spectra of the samples in KBr pellets were recorded on a MIDAC 2000 infrared spectrometer. The powder diffraction patterns of the α-ZrP/protein complexes were recorded on a Scintag Model 2000 diffractometer, using nickel-filtered Cu Kα radiation. The interlayer separations of the materials were measured from the 00*l* reflections ($l = 1, 2, 3$, etc.) using Bragg's law. Sin(θ) was plotted as a function of l (1, 2, 3, ...), and the layer spacing was calculated from the slope of a linear fit to the data.[8,17]

ZrP and ZrPAA Syntheses. The layered phosphates were prepared according to the published procedures, with minor modifications.[8,23] Stoichiometric amounts of zirconyl chloride and phosphoric acid (8 M) or phosphonoacetic acid in water were mixed at room temperature to form a gel. Use of phosphonoacetic acid resulted in the corresponding acetic acid derivative of the α-ZrP, abbreviated as α-ZrPAA. The mixture was heated to 75 °C for 24 h, and the product was filtered, washed, and allowed to dry. The samples were exfoliated by adding stoichiometric amounts of tetrabutylammonium hydroxide (40% by weight in water) to a suspension of the layered phosphate (1 g) in water (100 mL). The exfoliated suspensions were diluted with buffer as needed for the protein binding studies.[20] Dry samples of the protein/α-ZrP mixtures were prepared for FTIR by lyophilizing the samples in a speedvac.

Preparation of the α-ZrP-Protein Samples. Cytochrome *c*/α-ZrP intercalates were prepared by mixing Cyt *c* solution (115 μM, 1.5 mL, in 12 mM K_2HPO_4 buffer, pH 7.2) with the exfoliated zirconium phosphate (1 mL, 1% by weight) suspension. The mixture was equilibrated for 12 h and centrifuged to collect a reddish pink solid. The solid was dried and then powdered for XRD and FTIR. For optical studies, the solid was resuspended in phosphate buffer (0.01% by weight), and it scattered <5% of incident light at 410 nm. A similar procedure was followed for the binding of HRP. In this case, the protein−α-ZrP mixture was allowed to dry under reduced pressure, and the resulting solid was resuspended in phosphate buffer when needed.

Binding Constant Measurements. The binding constants of the proteins with the α-ZrP matrix were estimated by the centrifugation method. Protein−α-ZrP suspensions (α-ZrP, 0.01% by weight, and the protein, 125 nM to 5 μM) were equilibrated for 12 h at room temperature. The samples were centrifuged at 12 000 rpm in a Fisher Scientific minicentrifuge (Marathon 16KM) for 5 min to separate the free protein from

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the bound protein. Concentration of the free protein in the supernatant was estimated from the absorbance of the samples at the soret band. Absorbances and their corresponding extinction coefficients at 418 nm for Cyt *c* and at 410 nm for HRP were used to estimate the free protein concentrations.[23,26] The binding data were plotted according to the Scatchard equation, and the binding constants have been estimated from these plots.[27] The binding plots are also used to determine the stoichiometry, the number of phosphate groups occupied on the α-ZrP surface per protein molecule.

Protein Sources. Horse heart cytochrome *c* (>96%) and horseradish peroxidase (HRP, 99%, type IV) were obtained from Sigma Chemical Co. have been dissolved in potassium phosphate buffer (12 mM, pH 7.2). The absorption spectra, and the extinction coefficients at the soret band of the proteins are in good agreement with the published values.[23,26]

Results and Discussion

Cytochrome *c* and horseradish peroxidase show a high affinity for the layered α-ZrP ($K_b > 1/\mu M$). Interaction of the proteins in the galleries of α-ZrP is clearly established from the following observations. The spectral properties and the chemical reactivity of the intercalated proteins are characteristic of the native proteins with some modifications.

Binding Studies. Immobilization and strong binding of Cyt *c* to the α-ZrP matrix were indicated in centrifugation studies. α-ZrP suspensions (0.072%, 12 mM K_2HPO_4, pH 7.2) containing the protein were centrifuged, and the bound protein was separated from the free protein. The solid at the bottom of the centrifuge tubes was pink, indicating the entrapment of cytochrome *c* by α-ZrP. Absorption spectra of the supernatant obtained after centrifuging a mixture of cytochrome *c* (2.7, 5.4, 8.1, 10.9, 13.6, 16.3, 19.0, and 21.7 μM) and 0.072% α-ZrP (2.3 mM) are shown in Figure 1A. At low concentrations of the protein (<8 μM), there was no free protein in the supernatant, indicating complete binding of the protein to the matrix. At higher concentrations (>8 μM), the peak absorbances were much lower than expected from the total concentration of the protein in the sample, indicating strong association of the protein to the matrix.

The binding stoichiometry can be estimated from these titration data by plotting the ratio of the concentrations of the bound protein (C_b) to that of the total protein concentration (C_t) as a function of C_t (Figure 1B, at 2.3 mM ZrP). At low concentrations of the protein (<6 μM), this ratio is close to 1, indicating complete binding of Cyt *c* to α-ZrP. At protein concentrations >6 μM, the ratio decreases with a clear breakpoint corresponding to the binding stoichiometry. A plot of free protein concentration (C_f) vs C_t, when $C_t \geq 8$ μM, was linear (inset, Figure 1B). The *y* intercept of this plot corresponds to a stoichiometry of 5.3 μM of Cyt *c* bound to 2.3 mM of α-ZrP. This translates to the occupation of 434 phosphates on the surface of α-ZrP per protein molecule. These observations clearly establish the binding of cytochrome c to α-ZrP.

Binding Constant Measurements. At low ionic strengths (12 mM K_3PO_4, pH 7.2), protein affinities (K_b) for α-ZrP were found to be >100 μM. This high affinity made it difficult to measure the binding constants using absorption spectroscopy. Therefore, the binding constants were measured in the presence of an electrolyte such as tetrabutylammonium bromide (TBA, 6−20 mM) so that the binding constant is lowered and readily measurable by absorption methods.

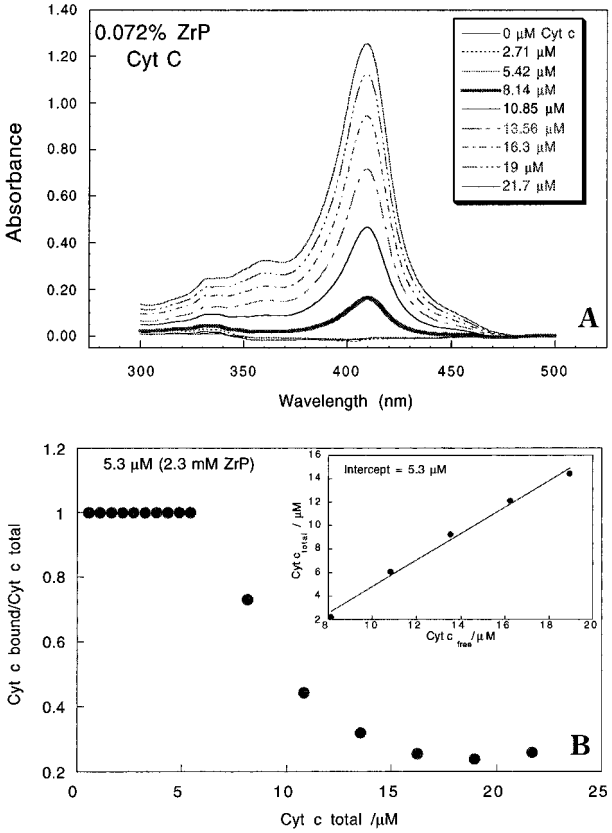

Figure 1. (A) Absorption spectra of the supernatant, after centrifugation of a mixture of Cyt *c* and α-ZrP (230 μM phosphate). The concentrations of the protein were 2.7, 5.4 (close to baseline absorption), 8.1, 10.9, 13.6, 16.3, 19.0, and 21.7 μM, with increasing absorbances. (B) Plot of the ratio of bound cytochrome *c* to that of the total protein as a function of the total protein concentration (2.3 mM α-ZrP). The inset shows the plot of C_f vs C_t for $C_t \geq 8$ μM. The *y* intercept of a linear fit to the data (solid line) equals the maximum amount of protein (5.3 μM) bound to 2.3 mM of α-ZrP.

Equilibrium mixtures of zirconium phosphate (320 μM α-ZrP, 20 mM TBA) with increasing concentrations of Cyt *c* were centrifuged, and the absorption spectra of the supernatant have been recorded. The absorbances at the soret band (418 nm) were used to estimate the concentrations of the free protein and binding isotherms were constructed from this data, using the Scatchard equation:[27]

$$r/C_f = k_b(n - r) \qquad (1)$$

In eq 1, *r*, C_f, K_b, and *n* are the binding density, free protein concentration, the binding constant, and the binding site size, respectively. Binding density is the ratio of the concentrations of the bound protein to that of the binding sites and the binding plot of Cyt *c* is shown in Figure 2A. The simple model fits the data well, and the data do not suggest any binding cooperativity.

The corresponding binding plot for α-ZrPAA (6 mM TBA) and Cyt *c* is shown in Figure 2B. The slight curvature observed here may be due to cooperativity.

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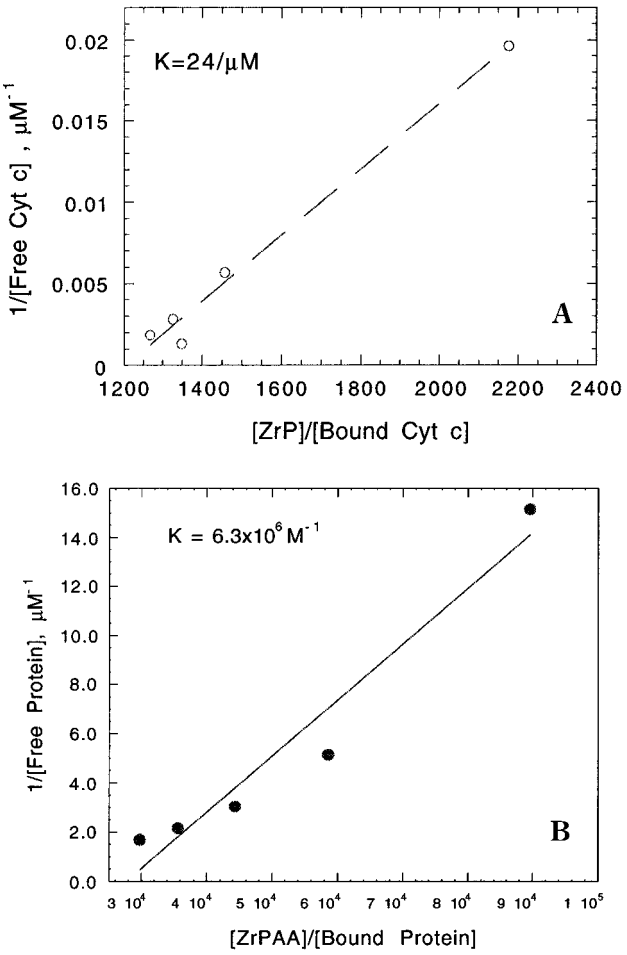

Figure 2. (A) Scatchard plot for the binding of cytochrome *c* to α-ZrP at 20 mM tetrabutylammonium bromide (12 mM potassium phosphate buffer, pH 7.2). (B) Scatchard plot for the binding of cytochrome *c* to α-ZrPCH_2COOH at 6 mM tetrabutylammonium bromide (12 mM potassium phosphate buffer, pH 7.2).

Table 1. Binding Constants of Cytochrome *c* and Horseradish Peroxidase with α-ZrP and α-ZrPAA (12 mM Potassium Phosphate Buffer, pH 7.2, Room Temperature)

protein/matrix	[TBA] (mM)	K_b
Cyt *c*, α-ZrP	0	>100/μM
Cyt *c*, α-ZrP	20	42/μM
Cyt *c*, α-ZrPCH_2COOH	6	8/μM
HRP, α-ZrP	20	1.5/μM

The binding constants calculated from these data using the above binding model are 24/μM and 6/μM for α-ZrP and α-ZrPP, respectively. Thus, the binding affinity was much lower with α-ZrPAA when compared to α-ZrP, even though the latter was measured at a much lower ionic strength (6 mM TBA). The binding constants for HRP with α-ZrP were also measured in a similar manner and the data are collected in Table 1. Cyt *c* shows a much higher affinity for α-ZrP than does HRP, and the binding constants are in the 10^6-10^8 M^{-1} range depending on the ionic strength and the surface functions.

Absorption Spectra of Intercalated Proteins. The effect of binding environment on the protein structure was investigated in spectroscopic measurements. For example, the UV−visible absorption bands of Cyt *c* are sensitive to its mocroenvironment,[24a] structure,[24b] and oxidation state.[4c] Any changes observed in the absorption spectra will be indicative of the perturbation of the protein bound to the α-ZrP matrix. For example, denaturation of Cyt *c* has been known to cause a blue-shift in the soret absorption band.[24b] The absorption spectra of Cyt *c*, and HRP bound to α-ZrP are shown in Figure 3A,B. The position of the soret band of Cyt *c* is unaffected by the matrix, and the extinction coefficient is increased by ~7% upon binding to α-ZrP. Similarly, no changes were observed in the 500−600 nm region of the absorption spectrum. These results are to be compared with the red-shifts observed when Cyt *c* was immobilized in sol gels, polymer matrixes, and colloidal silica.[25] The weak absorption band of Cyt *c* at 695 nm, attributed to the coordination of the Met80 to the Fe center,[24d] was shifted to 650 nm. This shift in the band is significant and suggests an altered coordination environment of the heme.[24b,c] Minor changes were observed with HRP/α-ZrP intercalates, and these observations establish that α-ZrP is an attractive medium for the immobilization of heme proteins.

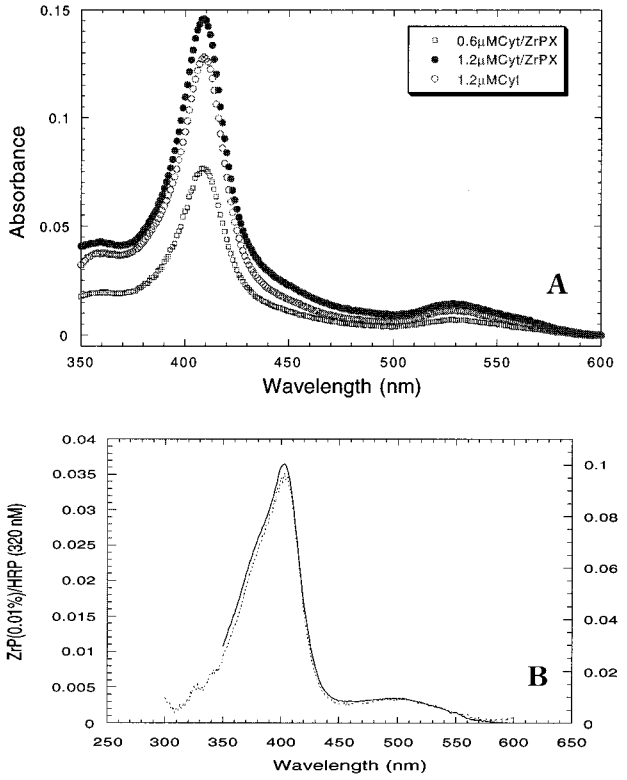

Figure 3. (A) Absorption spectra of cytochrome *c* (0.6 μM, small squares; 1.2 μM, filled circles) intercalated in α-ZrP (0.02%), and in the absence of α-ZrP (1.2 μM, open circles). (B) Absorption spectra of horseradish peroxidase (320 nM); dots represent the free protein, and the solid line represents the protein intercalated in α-ZrP (0.02%). The absorption values on the right and left *y* axes are in the presence and absence of α-ZrP, respectively.

Vibrational Spectra of the Intercalated Proteins. The vibrational bands of the amide group present in proteins provide sensitive signatures of the protein secondary structure.[28] The hydrogen-bonding interactions of the amide group with the solvent and with the neighboring residues are sensitive to the

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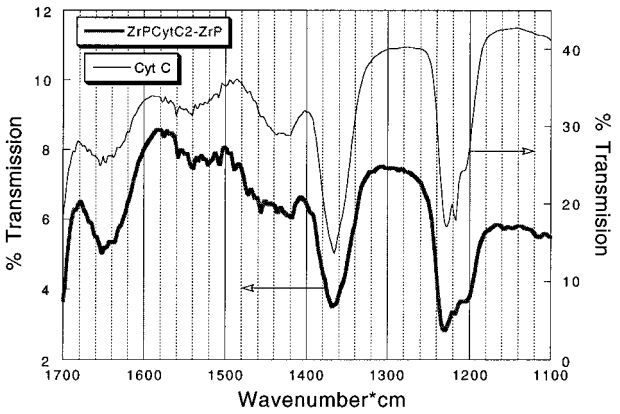

Figure 4. FTIR spectrum of cytochrome *c* (23 μM) bound to α-ZrP (0.01%) and that of the free Cyt *c* in KBr matrix.

protein structural changes. The amide I, II, and III vibrational bands observed in the 1700−1000 cm^{-1} region respond to the changes in the hydrogen bonding. For example, the amide I band assigned to the stretching of the carbonyl coupled to the C−N is sensitive to its hydrogen-bonding environment. Similarly, the amide II band is assigned to the NH stretching, and it is used as a sensitive measure of the protein structural changes. The FTIR spectrum of Cyt *c* bound to α-ZrP is shown in Figure 4 after subtracting the contributions from α-ZrP. The amide I and III bands of the bound protein are located at 1655 and 1230 cm^{-1}, respectively. These bands are essentially the same as those of the native protein (1657 and 1225 cm^{-1}),[29] and the two spectra are superimposable. These spectra indicate the undisturbed secondary structure for the bound protein. Binding to α-ZrP does not appear to denature the protein, and no detectable changes occur in the protein vibrational bands.

Expanded Interlayer Spacings. The powder diffraction patterns of the Cyt *c*−α-ZrP indicate increased interlayer separations from 7.6 Å for α-ZrP to 32 Å for the protein samples. The XRD patterns of α-ZrP, α-ZrP/Cyt *c*, and α-ZrP/HRP are presented as Supporting Information (as 1−3, respectively). The peaks due to α-ZrP are completely absent in the XRD patterns of the protein intercalates. New bands appeared at lower 2θ values indicating increased interlayer spacings. This increased interlayer separation indicates the intercalation of Cyt *c* (or HRP) in the galleries of α-ZrP.[30] The peaks are broad indicating a distribution of interlayer distances. This could be due to several possible orientations of the bound proteins in the interlayer regions. These various spectral and XRD data provide strong evidence for the binding and intercalation of the proteins in the galleries of α-ZrP.

Effect of Ionic Strength on the Binding Affinity. The contribution of electrostatic interactions to the overall binding affinity was illustrated by the reduced binding of the protein in the presence of an electrolyte. Addition of NaCl, $MgCl_2$, or TBA to the α-ZrP suspension shifts the binding equilibrium toward the free

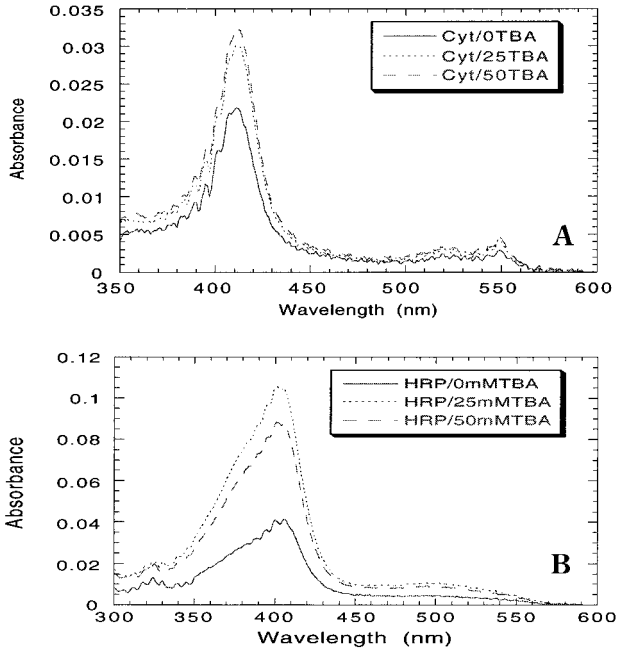

Figure 5. (A) Effect of ionic strength on the binding of cytochrome *c* (360 nM) to α-ZrP (0.01% at 0, 25 and 50 mM TBA). (B) Effect of ionic strength on the binding of horseradish peroxidase (440 nM) to α-ZrP (0.01%, 0, 25, and 50 mM TBA).

protein. The absorption spectra of the supernatant after centrifugation of a mixture of α-ZrP and Cyt *c* with increasing concentrations of TBA are shown in Figure 5A. The spectra indicate that the binding affinity decreases with increasing concentrations of TBA, at a given concentration of α-ZrP. Similar results were also observed for HRP, and the corresponding absorption spectra of the supernatant are shown in Figure 5B. These results clearly establish the contribution of electrostatic interactions to the binding of Cyt *c* and HRP, as expected. Furthermore, addition of NaCl (2 M) to the protein/α-ZrP mixtures releases the free protein, and this result clearly establishes the reversible binding of the proteins to the matrix. The absorption spectra of the released protein were superimposable with that of the free protein.

Redox and Peroxidase Activities of the Bound Proteins. The encapsulated proteins should retain their chemical reactivity and the immobilized proteins should also be readily accessible for external reagents. This is essential for the application of these materials in the construction of biosensors or chemical sensors.[29] The redox nature of Cyt *c* provides a convenient probe of both the chemical reactivity and access to the bound protein. Ascorbate or dithionite reduce ferri Cyt *c*, and the reduced protein can be identified from the soret band or the low-energy bands around 550 nm. Addition of ascorbate to Cyt *c* bound to α-ZrP resulted in the facile reduction of the metal center, and the sharp absorption at 550 nm due to the reduced form grows with time (Figure 6). The reduction was complete in 20 min, despite the fact that ascorbate is negatively charged, and the negative charge field of α-ZrP is expected to repel the reagent from entering into the galleries of α-ZrP. Oxidation of the reduced Cyt *c* bound to α-ZrP matrix by ferricyanide also proceeds to completion, restoring the oxidized from (data not shown). Oxidation and reduction of the bound protein can be readily achieved due to the large interlayer distances.

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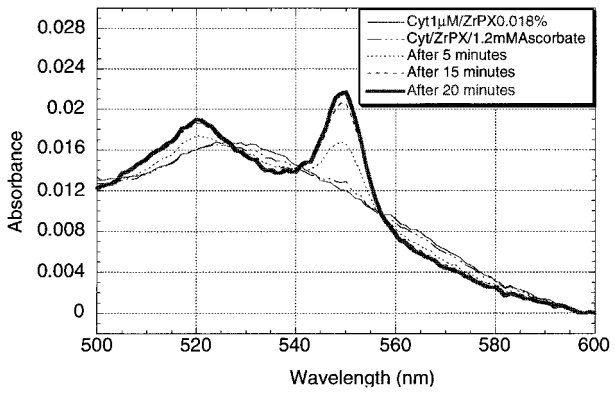

Figure 6. Facile reduction of cytochrome *c* (1.1 μM) bound to α-ZrP (0.018%) by ascorbate (1.2 mM) in potassium phosphate buffer (pH 7.2).

Similar activity was also observed for HRP (data not shown) intercalated in the galleries of α-ZrP. HRP bound to α-ZrP rapidly reacts with hydrogen peroxide to produce a green product, compound I, and this reacts with amines or phenols to produce a red product, compound II, as indicated from the absorption spectra. Cyt *c* and HRP, immobilized in α-ZrP, retain their redox activities, and they are readily accessible to external reagents.

Peroxidase activity of Cyt *c* bound to α-ZrP was also demonstrated here. Addition of hydrogen peroxide to a mixture of Cyt *c* (1 μM) and α-ZrP (0.01%) in the presence of 2-methoxyphenol (18 mM) resulted in the formation of the corresponding tetramer, as observed by its absorption spectrum and the growth of absorbance at 470 nm as a function of time (Supporting Information 4). Similar growth of absorbance at 470 nm was also observed in the absence of α-ZrP indicating the peroxidase activity of Cyt *c*. Oxidation of 2-methoxyphenol by Cyt *c* bound to phospholipids has been reported previously.[31] Cyt *c* bound to the α-ZrP matrix is, therefore, active, and the bound protein is competent.

Thermal Stability and Activity of Cyt *c*/α-ZrP. Thermal denaturation of Cyt *c* (1.2 μM) in the absence and in the presence of α-ZrP (0.03%) is shown in Figure 7. The absorption spectra were recorded at various temperatures, and the soret absorption was plotted as a function of temperature. The soret absorption of Cyt *c* in solution phase decreases gradually up to ~60 °C and then rapidly with a break around 60 °C. However, in the presence of α-ZrP, the absorbance change follows that of the native protein up to ~55 °C and then increases rather sharply before it starts to decrease with increase in temperature. This behavior is unexpected and further experiments are in progress to understand this unusual temperature dependence. The absorption spectra at these high temperatures are similar to the room temperature spectra with minor broadening of the soret band. Thermal stability of the intercalated Cyt *c* in the range 25−55 °C is similar to that of the native protein.

The utility of α-ZrP in the nanoencapsulation of cytochrome *c* and horseradish peroxidase under mild ambient conditions is demonstrated here. Encapsulated proteins retain their major structural features and spectroscopic signatures. Bound proteins are accessible to reagents and retain their activity.

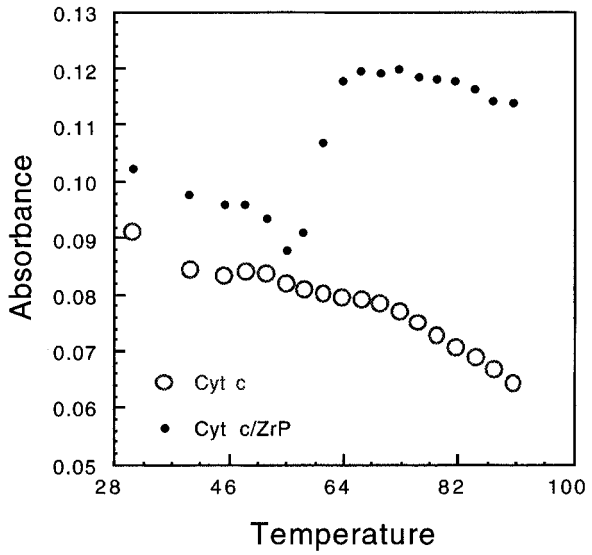

Figure 7. Changes in the soret absorption (410 nm) of Cyt *c* (1.2 μM) free (open circles) and bound to α-ZrP (0.03%, closed circles). The temperature is indicated in centrigrade on the *x* axis. Clear deviation of the encapsulated sample behavior from that of the free protein is evident.

Conclusions

Large molecules such as cytochrome *c* and HRP can be readily intercalated in the galleries of α-ZrP under mild conditions, at ambient temperature. At low ionic strengths, the binding constant for Cyt *c* with α-ZrP is in excess of 10^7/M. The binding constant of Cyt *c* is nearly 30-fold higher than that of HRP, indicating the strong role played by the large number of positively charged lysine side chains of Cyt *c* in the binding interaction. The negatively charged α-ZrP lattice should prefer binding of such positively charged proteins. The binding constant for Cyt *c* with α-ZrP was found to be much higher (42/μM at 20 mM TBA) than with α-ZrPAA (8/μM at 6 mM TBA) even though the latter was measured at a lower ionic strength, indicating the subtle role of the surface functional groups in the binding interaction. This is not obvious from the structure of the protein or the binding surface. Similar effect of surface residues on the protein binding affinity was recently reported.[3b] Such specific information on the role of the surface functionalities on protein binding is likely to be useful in understanding how biological molecules adhere to surfaces. Zirconium phosphonates, prepared from well-characterized phosphonic acids, pave the way to demonstrate the effect of specific surface functions on the bound protein structure and function. Current investigations prompt and support the feasibility of such studies. Varying the surface functionalities of sol−gels, hydrogels, polymers, and other solid matrixes for similar studies are more difficult due to the prior surface modification that is required and characterization of their modified surfaces is challenging. The contribution of the electrostatic interactions to the overall binding free energy is also demonstrated by the effect of electrolytes on the binding affinities, observed here. Addition of tetrabutylammonium bromide to the protein/α-ZrP suspensions lowered the binding affinities. Addition of NaCl or $MgCl_2$ releases the bound

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proteins from the galleries, and the absorption spectrum of the released protein is superimposable with that of the free protein. Reversible binding of the proteins to the matrix is clearly demonstrated by this observation.

Each Cyt *c* molecule occupies nearly 435 phosphate groups on the α-ZrP surface. Since, each phosphate occupies 24 Å^2, this corresponds to a footprint of ∼100 × 100 Å^2 for each Cyt *c* molecule. This is much larger than the area of cross section of Cyt *c*, and this fact indicates the binding of co-ions or counterions, required for charge neutralization, along with the protein molecules. Such dispersion of proteins is perhaps the reason for the facile diffusion of small molecules into and out of the α-ZrP matrix. Such access has been demonstrated, and this is important for the application of protein/α-ZrP matrices in the development of sensors.[32] Loose packing perhaps plays an important role in preventing the aggregation and denaturation of the proteins in the galleries.

The FTIR spectra of the protein−phosphate composites show essentially no changes in the positions of the amide bands, and they are superimposable with those of the native proteins. The increase in the interlayer separations from 7.6 Å for α-ZrP to ∼32 Å for protein/α-ZrP composites, determined from the powder diffraction data, provides strong evidence for the intercalation of the proteins in the galleries of α-ZrP. These distances match well with the dimensions of the bound proteins.[30] The increased interlayer separations facilitate the diffusion of reagents to the encapsulated proteins. Thus, α-ZrP isolates single layers of the protein molecules and prevents them from aggregation or denaturation.

The absorption spectra of the α-ZrP−heme protein complexes show only minor changes when compared to those of the free proteins. The α, *β*, and the soret absorption bands of bound Cyt *c* were essentially superimposable with those of the free protein, demonstrating the native like environment available for the bound proteins. The 695 nm band of free Cyt *c*, characteristic of the coordination of Met80 to iron is shifted to 630 nm, on binding to α-ZrP, suggesting a possible change in the spin state of the heme iron and its coordination environment. Perhaps, the Met80 is not coordinated to Fe(III) in the bound protein, and the absorption corresponds to a high-spin iron. The effect of immobilization in sol−gels, polymers, and silica on the 695 nm band of Cyt *c* is not reported in the literature for comparison. The absorption spectrum of metmyoglobin has a band at 631 nm assigned to the coordination of a water molecule to the heme iron, as the sixth ligand. In a similar manner, the 630 nm band observed for Cyt *c*/α-ZrP is perhaps due to the replacement of the Met80 coordinated to the iron, by a water molecule. Such a conformational change has been reported for Cyt *c* in alkaline media.[24] Subtle conformational changes produced in the protein backbone due to the negatively charged surfaces of α-ZrP may be responsible for this band. This observation is in contrast to the complete disappearance of the 695 nm band when Cyt *c* binds to negatively charged phospholipids.[2]

The heme proteins immobilized in the layers of α-ZrP undergo rapid redox reactions, indicating the accessibility of the bound proteins to external reagents, and retention of their redox activity after binding to the matrix. Dithionite or ascorbate reduces the ferric Cyt *c*/α-ZrP to the corresponding ferrous form, while hydrogen peroxide reacts with HRP/α-ZrP to form the green intermediate (compound I). Ferricyanide readily oxidizes the ferrous Cyt *c*/α-ZrP to ferric Cyt *c*/α-ZrP. Retention of the redox reactivity of the bound proteins, and their access to extraneous ligands is clearly demonstrated by these transformations. The peroxidase activity of Cyt *c* is preserved upon binding to α-ZrP and 2-methoxyphenol can be converted to the corresponding tetramer. The displacement of Met80 by a water molecule is expected to provide an opportunity for the coordination of small ligands to the heme iron needed for catalytic transformations. HRP bound to ZrP also demonstrates similar activity. The increased interlayer separations of the galleries, the large interprotein distances, and the binding of spectator ions make these composites porous. Therefore, extraneous ligands can readily diffuse in and out of the layers.

Thermal stability of a number of proteins and enzymes has been improved by immobilization.[33] Adsorption of amylase and acid phosphatase at the hydrophilic surfaces of tricalcium phosphate improved their thermal stabilities[34] when compared to those of the free enzymes. This could be due to increased conformational rigidity, inhibition of chemical degradation of sensitive side chains, and prevention of protein aggregation. Thermal stability of the bound Cyt *c* appears to be similar to that of the native protein up until 55 °C and the strong change in the soret absorption band around 60 °C is intriguing. Structural changes associated with such absorption changes and the activity of the bound protein at these temperatures are currently under investigation.

The spontaneous self-assembly of monolayers of proteins at the galleries of α-ZrP, at room temperature, and pH 7.2 in a buffer, is a characteristic feature of the current methodology. The facile diffusion of small molecules or ions in to and out of the galleries of protein−α-ZrP intercalates, demonstrated here, is necessary and attractive for the incorporation of these nanomaterials in chemical or biological sensors.

Acknowledgment. The financial support of this work was provided by NIH, and we thank Ms. A. Chaudhary for assistance in recording the XRD and FTIR spectra of the samples. We thank the reviewers for useful comments.

Supporting Information Available: XRD patterns of α-ZrP (0.008%) before protein intercalation, intercalated with Cyt c (5.3 μM), and intercalated with HRP (2.7 μM); growth of tetramer derived from the oxidation of 2-methoxyphenol (18 mM) by Cyt *c* (1 μM) and hydrogen peroxide (10 mM, 12 mM K_2HPO_4), in the absence (0−0.5 full scale) and in the presence of α-ZrP (0.01%, 0−0.1 full scale); bound protein was active although at a slower rate than the free protein (4 pages). Ordering information is given on any current masthead page.

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