Layered Structural Heme Protein Magadiite Nanocomposites with High Enzyme-like Peroxidase Activity

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Received January 28, 2004. Revised Manuscript Received April 21, 2004

Layered nanocomposites of global myoglobin (Mb)−magadiite and hemoglobin (Hb)−magadiite with the interlayer distances of 43.2 and 68.2 Å, respectively, have been successfully prepared by using tetrabutylammonium (TBA)−magadiite as the intermediate under mild conditions. The structures, details of the interaction between the host magadiite and guest proteins, and saturated bounding amounts of Mb and Hb were characterized and studied by many techniques. The enzyme activity study showed that the fresh intercalated Mb and Hb nanocomposites retained most enzyme-like peroxidase activity and about 25.0% of activities based on the formation amounts of tetramers were kept after 30 min of reaction when these nanocomposites were stored for 1 month at room temperature. The relations between the structures and the peroxidase activities were discussed based on Michaelis−Menten model analyses. Such stable and high enzyme-like peroxidase activity for immobilized Hb and Mb provide potential application in biosensors and enzyme reactors.

Introduction

Enzymes are highly efficient and specific catalysts, but their high cost and poor stability severely limit their utility in chemical reaction. Enzymes attached in or on inert matrixes can partly overcome the limitations.[1] The advantages for immobilized enzymes, which include reuse, ease of product separation from the reaction mixture, improvement of stability, and use in organic solvents, attracted much attention and have been studied extensively for several decades. The specificity of enzymes and other proteins for binding of substrates, inhibitors, or related molecules also makes them attractive candidates for molecular sensors if they can be incorporated into a matrix, which stabilizes them but does not block signal transmission.[2] One kind of successful sample is the commercial glucose electrodes, which have been developed based on immobilized glucose oxidase.[3] So far, research of the immobilization of enzymes on different matrixes, such as polymers,[4] sol−gels,[5] phospholipids,[6] mesoporous molecular sieves,[7] and self-assembled monolayers,[8] have attracted more and more attention and achieved many significant results. These hosts provide a protective environment for enzymes and improve the stability of immobilized enzymes, although the structures of most selected support matrixes and the formed composites are not well-ordered.

Research of enzymes immobilized on ordered structures such as layered inorganic materials have been reported in past decades.[9−13] Adsorption of proteins on phyllosilicate was carried out in numerous studies for the potential application in soil. Some research groups found that proteins could be introduced into the interlayers of montmorillonite in addition to the adsorption on the external surface. Lower pH value than the isoeletric point of protein and larger additional amounts of proteins to form intercalated composites were required.[9] There are several advantages to select layered inorganic materials as a support matrix. The protective environment of interlayer space can effectively inhibit the microbial degradation and their open frameworks provide convenience for substrates to access the im-

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10.1021/cm049841l CCC: $27.50
Published on Web 05/28/2004

mobilized enzymes. In addition, vast varieties of layered inorganic materials provide a diverse matrix for enzyme immobilization. Several representative groups' works are introduced as follows. Kanzaki et al.[10a] and Ding et al.[10b] reported the intercalation of proteins into layered phosphates, but these methods required extreme pH conditions (pH = 2−3 or > 8), resulting in partial denaturation of enzymes and long reaction times (as long as 17 d). For the first time, Kumar et al.[11] reported proteins of met-myoglobin (Mb), lysozyme (Lys), methemogolobin (Hb), glucose oxidase (GO), and α-chymotrypsia (CT) immobilized in the galleries of layered α-zirconium phosphate under mild conditions (pH = 7.2) and short reaction time (1 h). Results showed enhanced activity of Mb with specific substrates. They also found efficient renaturation of immobilized Hb in the galleries of α-zirconium phosphate modified by carboxymethyl after being heated to denature. Corma et al.[12] made use of delaminated zeolites as support matrixes for enzymes immobilization while preserving the enzymatic activity; the immobilized enzymes were highly stable, even in solutions with relatively high ionic strength, and the delaminated zeolites could be easily recovered and reused by simple calcinations. Self-assembly preparation of protein intercalated manganese oxide nanocomposities was reported by Gao et al.[13] and enzyme-like peroxidase activity of Mb− and Hb−TMAOL nanocomposites retained only 10% of that of free Mb and Hb.

Though several groups have focused on the protein intercalation into layered inorganic materials, there are still many problems not being solved,[14] such as the interaction between the enzyme and matrix, the effect of surfaces on the immobilized proteins, and the influence of structure and composition of layered materials' sheet on the immobilized proteins. More attention should be focused in this field. Furthermore, current research has mostly been confined to forming composites at a macroscopic level. Study of the proteins intercalation into layered inorganic materials, which would form nanocomposites, is still a new field. Nanomaterials provide a larger surface area[15] and decrease the diffuse distance for the substrate to access the immobilized enzyme, which may improve the immobilized enzyme properties.

To improve the enzyme-like peroxidase activity,[13] another kind of support matrix was chosen to increase the binding quantity and catalytic activity of the enzyme. Layered polysilicate magadiite ($Na_2Si_{14}O_{29}\cdot nH_2O$) was used as the support matrix in our study. The advantages of selecting magadiite as a support matrix are as follows. The host is cheap and has a layered structure[16] and the layers can be readily expanded to accommodate small (protons)[17] as well as large guest molecules (polymers).[18] Furthermore, layered polysilicate magadiite, a natural crystalline hydrated sodium silicate[19] which can be easily synthesized,[20] is very compatible with organisms and its surface silanol groups can react with a large number of organic compounds to form preintercalated complexes.[21] These organic preswelling complexes of magadiite can then be used as intermediates. Magadiite is thermally stable and chemically inert in acidic or basic media and also possess a high surface area.[22] The hydrophilic nature of the magadiite (due to the −OH groups) surfaces can provide an aqueous-like medium to stabilize immobilized proteins.[23] Magadiite/polymer nanocomposites have attracted a great deal of research interest recently owing to the substantial improvements over their pristine state in thermal and mechanical properties.[24] However, no research has been reported about the protein intercalated magadiite nanocomposites.

Mb and Hb are the two well-characterized proteins that we have chosen herein because they not only provide advantages for convenience of theoretical study but also are significant for practical application. These heme proteins have strong spectroscopic signatures that are suitable for monitoring protein binding or their redox activities in host environments.[25] Mb contains one heme prosthetic group, while Hb consists of four subunits similar to the Mb molecule and each subunit has one noncovalently bound heme prosthetic group. Native Mb and Hb have important biochemical functions such as in electron transport, dioxygen transport, and storage and dioxygen-related chemical transformations, for example, oxygenase, peroxidase, and catalase.[26]

In this work, we reported the global proteins, Mb and Hb, intercalated into the galleries of layered polysilicate magadiite under mild conditions. The resulting protein−magadiite nanocomposites were examined by XRD, HRTEM, SEM, FTIR, UV−vis, and activity studies. The structural models of intercalated protein nanocomposites were constructed. Activity parameters were measured for immobilized Mb and Hb proteins. Their well-ordered structures and known surface groups should be useful for further understanding the relations between the new structures and their bioactivities as well as how surface groups of the support matrix interact with proteins.

Experimental Section

Preparation of Na−Magadiite and H−Magadiite. Na−magadiite was synthesized following the procedure described by Fletcher et al.[20c] as follows. A mixture of silica and hydrous sodium hydroxide with a molar ratio of SiO_2:NaOH:H_2O = 9.0:3.0:162 has been heated at 150 °C for 42 h under hydrothermal

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conditions. H−magadiite was obtained by ion exchange of Na−magadiite in 0.1 M HCl solution using the method of Beneke and Lagaly.[17c] The final product was filtered and dried in air. The FTIR spectra, powder X-ray diffraction patterns (XRD), and Raman spectra of Na−magadiite and H−magadiite matched very well with those of the reported data.

Preparation of TBA−Magadiite and Intercalation of Proteins Mb and Hb. TBA−magadiite suspensions were prepared by mixing H−magadiite (0.1 g) in distilled water (10.0 mL) with tetrabutylammonium hydroxide (0.2 mM). The resulting suspensions were stirred for 1 h at room temperature. Mb− or Hb−magadiite composites were achieved by dripping slowly TBA−magadiite suspensions (2.0 mM magadiite) into Mb (60.0 μM) or Hb (15.0 μM) solutions (pH = 7.2 by 10.0 mM K_2HPO_4), which were stirred for 1 h at room temperature.

Binding Constant Measurements. The binding constants are measured by the centrifugation method. Mb− and Hb−magadiite composites contained TBA−magadiite (2.0 mM magadiite) and varying concentrations of Mb (5.0, 7.5, 10.0, 12.5, 15.0, and 17.5 μM) and Hb (0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 μM). After being stirring for 1 h, the samples were centrifuged at 10000 rpm for 20 min to separate the free protein from the bound protein. Concentration of the free Mb and Hb in the supernatant was calculated from the characteristic absorbance at 407 nm for Mb and Hb. The binding data were plotted according to the Scatchard equation.[27]

$$1/C_f = (K_b \cdot n) \times 1/r - K_b \quad (1)$$

In eq 1, C_f, K_b, n, and r are the free protein concentration, the binding constant, the binding site size, and the binding density, respectively. Binding density is the ratio of the concentration of the bound protein to that of the binding sites. Binding constant (K_b) reflects the affinity of protein to magadiite matrix. The greater the K_b value is, the stronger the combination between Mb or Hb and magadiite will be.

Binding Stoichiometry Studies. Mb− and Hb−magadiite samples were prepared with increasing concentrations of Mb (30.0, 35.0, 40.0, 45.0, 50.0, 55.0, and 60.0 μM) and Hb (10.0, 15.0, 20.0, 25.0, 30.0, and 35.0 μM). After reaction for 1 h, the samples were centrifuged at 10000 rpm for 20 min. The absorbance of the supernatant at 407 nm was recorded and the bound concentrations of Mb and Hb could be obtained by total protein concentration subtracting free protein concentration. Bound protein concentration was plotted against the total protein concentration to estimate the binding stoichiometries.

Mb and Hb Activity Assay. The catalyzed reaction plots of Mb and Hb were measured using the reported method with some modifications.[11b] Free or immobilized Mb (5.0 μM) and *o*-methoxyphenol (0.5 mM) were mixed with hydrogen peroxide (0.5 mM), and blank runs contained no peroxide. Colored tetrameric products formation was monitored by the absorbance at 470 nm as a function of time by means of UV−vis spectroscopy. A similar procedure was used to determine the activity of free and immobilized Hb. The catalyzed reaction plots of immobilized Mb and Hb aging at different times were also measured while storing under room temperature. Enzyme activity of free and bound Mb and Hb obtained from the catalyzed reaction plots were compared.

Kinetic behavior of the immobilized Mb and Hb was also studied. Keeping the concentration of immobilized Mb and Hb (5.0 μM) unchanged, the initial rate of the reaction at varying *o*-methoxyphenol concentrations was determined at constant H_2O_2 concentration (0.1 mM). Kinetic constants V_{max}, K_m, K_{cat}, and K_{cat}/K_m were determined from the Lineweaver−Burk plots. Lineweaver−Burke plots were constructed using the following equation (2),[27]

$$1/V = K_m/(V_{max}[S]) + 1/V_{max} \quad (2)$$

where K_m is the Michaelis constant (substrate concentration when the reaction rate is half of V_{max}), V_{max} is the maximum reaction velocity, and [S] is the substrate concentration.

Characterization. Powder X-ray diffraction patterns were obtained with a Rigaku D/MAX-2200 diffraction using Ni-filtered Cu Kα radiation. Mb or Hb/magadiite suspensions (2.0 mL) were spread onto glass slides and air-dried. Scan rates for these runs were 0.6°/min. Infrared spectra between 4000 and 400 cm^{-1} were carried out with 4 cm^{-1} resolution on a Thermo Niocolet Fourier Transform Spectrum, using a KBr disk method. Mb/ or Hb/magadiite suspensions were centrifuged and the pellets were dried at 4 °C. UV−vis absorption spectra were measured on a Shimadzu UV-3101PC spectrophotometer. The scanning electron micrographs (SEM) were recorded on a JEOL JSM-6700F field emission electron microscope. Prior to the observation, the samples were coated with Au for 10 s by E-1030 ion sputtering. The high-resolution transmission electron microscope (HRTEM) images were performed on a JEOL JSM-200CX transmission electron microscope with an acceleration voltage at 200 kV.

Results and Discussion

Preparation, Morphologies, and Structures of Mb/Hb Magadiite Composites. As illustrated in Figure 1, Mb and Hb were not able to be intercalated directly into the galleries of Na− or H−magadiite due to the strong combination between Na^+ or H^+ ions and the inorganic layers and small interlayer distances of 1.56 nm for Na−magadiite and 1.16 nm for H−magadiite, respectively. The introduction of larger organic TBA^+ ions into the galleries of magadiite was the key for preparing the Mb/Hb intercalated magadiite composites because the intercalation of TBA^+ ion into the galleries could not only increase the distance between inorganic layers to provide larger space (for the intercalation of larger Mb/Hb proteins into the inorganic layers) but also weaken the combination between the guest organic ions and host inorganic layers.

The XRD patterns of Na−, H−, TBA−, Mb−, and Hb−magadiite composites are presented in Figure 2. The expanded interlayer distances from 2.56 nm to 4.32 and 6.82 nm indicated the introduction of Mb and Hb into the galleries of the TBA−magadiite matrix, respectively, based on the XRD analyses. The layer thickness of inorganic magadiite[19] is about 1.12 nm and the dimensions of TBA^+ ions,[28] Mb, and Hb are about 0.95−1.05, 3.0 × 4.0 × 4.0, and 5.3 × 5.4 × 6.5 nm,[29] respectively. The expansions of 3.20 and 5.70 nm for intercalated Mb− and Hb−composites were in good agreement with the short axis dimensions of Mb and Hb with a little larger distances of 0.20 and 0.30−0.40 nm, possibly due to the contribution of H_3O^+ ions (0.28 nm) and TBA^+ between the inorganic layers, or a certain degree of inclination of the long axis dimensions of Mb and Hb proteins. The narrow XRD peaks for Hb−magadiite and broad peaks for Mb−magadiite indicated a distribution of orientations. Similar results were also found in protein intercalated zirconium phosphate systems reported by Kumar et al.[11] Such distribution was important for the substrates to access the active sites of the bound proteins.[11b]

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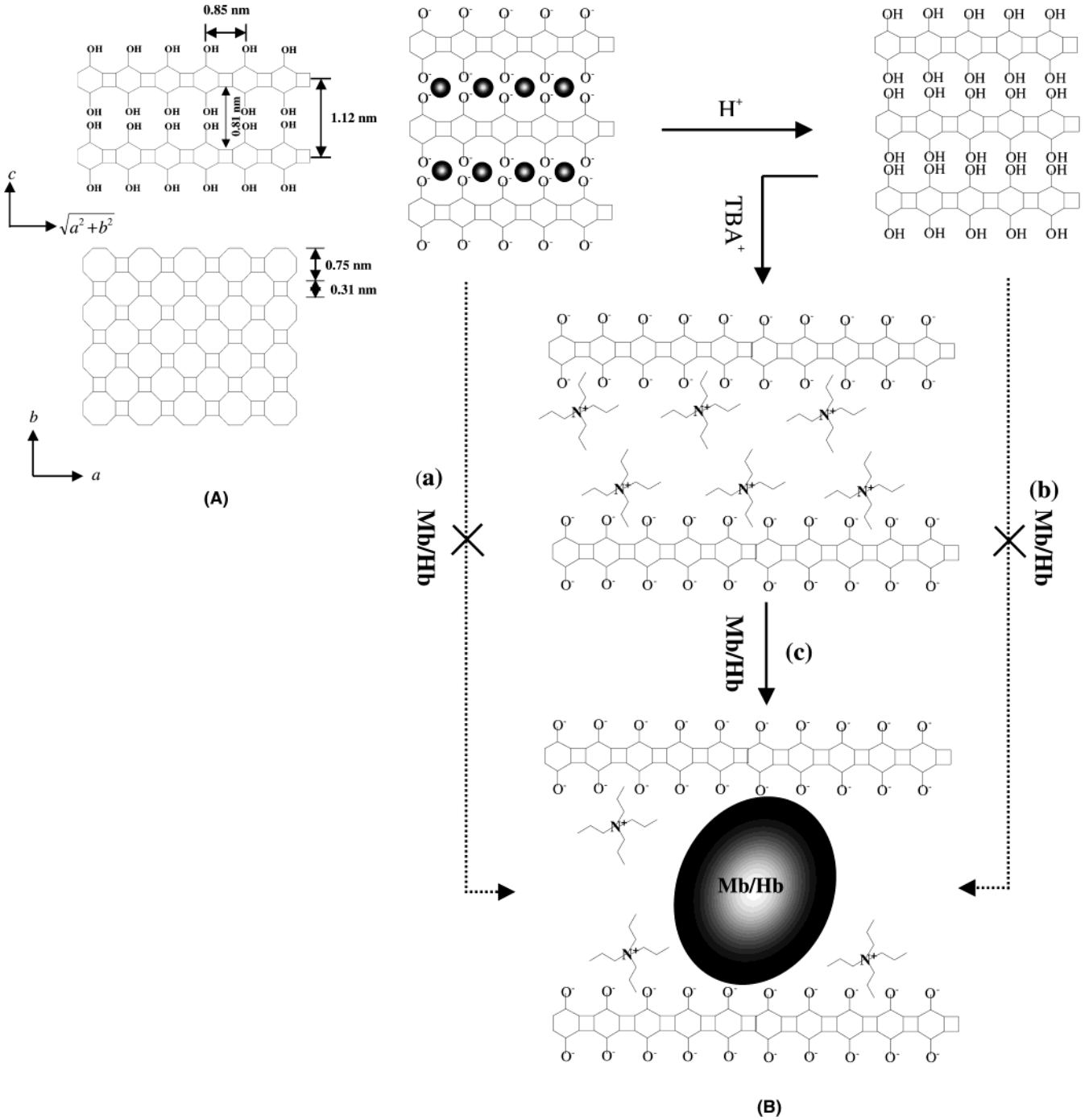

Figure 1. (A) Schematic representation of the sheet structure of magadiite. (B) The syntheses of Mb and Hb intercalated magadiite composities: (a) Mb and Hb directly intercalated into Na−magadiite; (b) Mb and Hb directly intercalated into H−magadiite; and (c) Mb and Hb directly intercalated into TBA−magadiite.

Visual evidence for the cross section of sheets of TBA−magadiite and Mb−magadiite are provided by HRTEM (Figure 3) images. It appeared to feature well-aligned planes with a periodic arrangement of dark lines and bright ones, where the discrete bright lines represented intercalated guest layers and dark lines represented the structure of the matrix. HRTEM images show that the layer thickness of about 1.2 nm is close to the inorganic layer of magadiite, the volume of 2.6 nm corresponds to the basal spacing of TBA−magadiite, and that of 4.4 nm corresponds to the interlayer distance of Mb−magadiite, which were close to those from XRD analyses, further conforming the encapsulation of molecular Mb in the galleries of magadiite. SEM images of Na−, H−, TBA−, and Mb−magadiite are shown in Figure 4. A platelike morphology was observed for all of them. The sizes for Na−magadiite and H−magadiite particles were about 1−2 μm. The particle sizes of TBA−magadiite decreased to about 50−150 nm and they were well-dispersed, while the sizes of Mb−magadiite particles further decreased to about 30 nm with some aggregations.

It could be seen clearly that large particles of Na− and H−magadiite have changed into nanosized Mb/Hb−magadiite particles during the ion-exchange process.[30] A similar process was also reported in manganese oxide systems.[28] The process could be described as follows. In the beginning of TBA reaction with

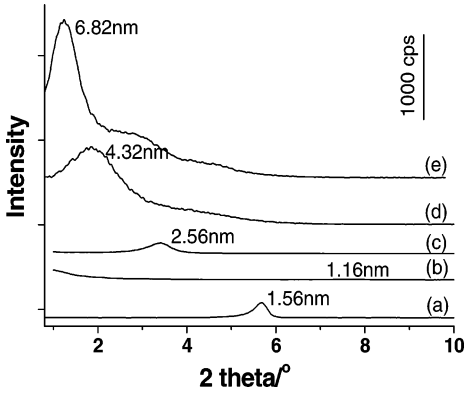

Figure 2. Powder X-ray diffraction patterns for Na−magadiite (a), H−magadiite (b), TBA−magadiite (c), Mb−magadiite (d), and Hb−magadiite (e).

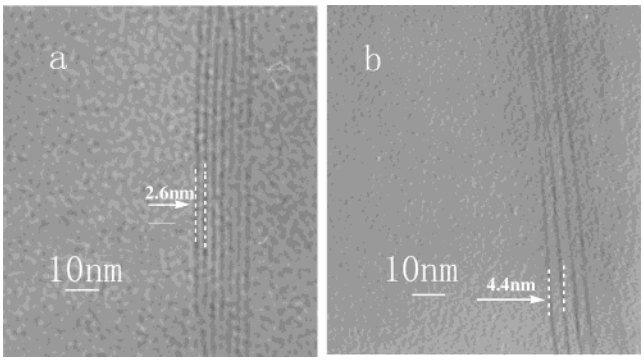

Figure 3. High-resolution transmission electron microscope images for TBA−magadiite (a) and Mb−magadiite (b).

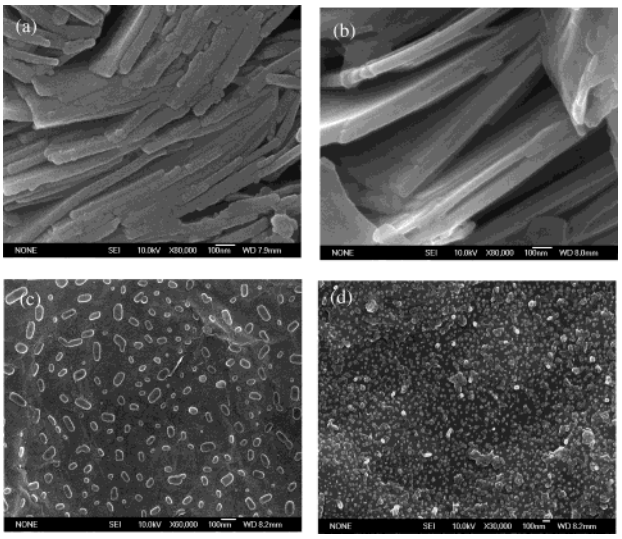

Figure 4. Scanning electron micrographs for Na−magadiite (a), H−magadiite (b), TBA−magadiite (c), and Mb−magadiite (d).

magadiite, H−magadiite kept its normal particle size of about 1−2 μm based on SEM analyses. Succedent intercalation of TBA^+ ions led to larger distances between the inorganic layers. Static repulsion between TBA cations and weak van der Waals forces between bilayer arrangements of intercalated TBA cations led to a smaller number of stacks of layers. Because the

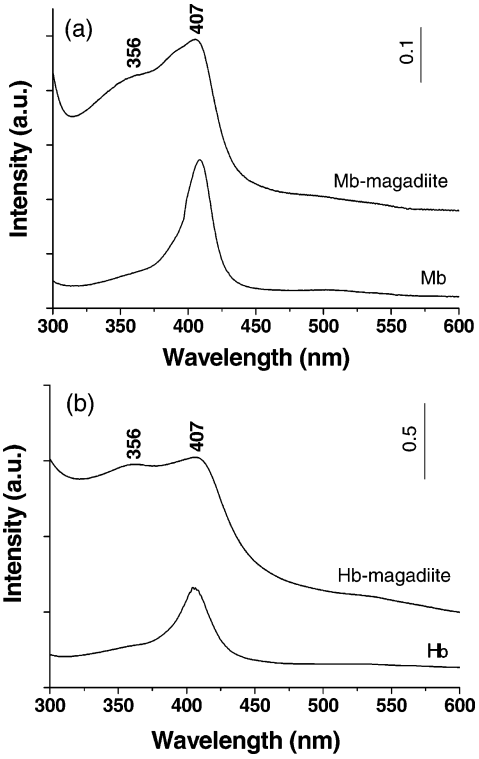

Figure 5. Absorption spectra of free and immobilized proteins for Mb (a) and Hb (b).

interactions between inorganic layers and TBA ions are weaker[31] than those between inorganic layers and Na^+ and H_3O^+, the layers were then easy to break. The colloids with 50−150-nm particles formed after stirring for 1 h at room temperature. When Mb/Hb was introduced into the galleries of magadiite, the number of stacks of layers became much smaller. Smaller particles of Mb/Hb−magadiite could be easily obtained after 1 h of stirring at room temperature.

The combination between inorganic magadiite layers and Mb/Hb proteins was investigated by spectroscopic measurements. UV−visible absorption bands were sensitive to a transitional metal coordinated microenvironment,[32] structure,[33] and oxidation state.[34] The changes observed in the absorption spectra may be indicative of the perturbation of the protein bound to the magadiite matrix. The absorption spectra of Mb− and Hb−magadiite composites are shown in Figure 5a,b. The soret bands at 407 nm for Mb and Hb were unaffected by the matrix after immobilization. At the same time, a new band appeared at 356 nm for Mb− and Hb−magadiite, possibly due to the combination between intercalated proteins and magadiite matrix, further proving the interaction between intercalated proteins and magadiite matrix.

Immobilization of proteins at solid surfaces can result in the loss of native conformation to a significant extent or denaturation in some cases.[35] Infrared absorption provided a sensitive measure for Mb− and Hb−maga-

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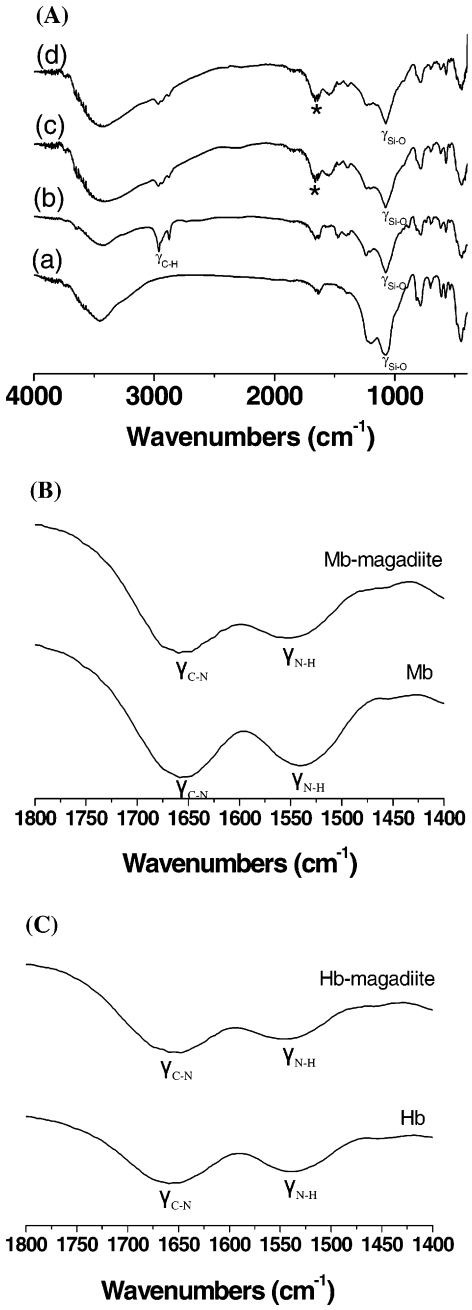

Figure 6. FTIR spectra of (A): (a) H−magadiite, (b) TBA−magadiite, (c) Mb−magadiite, and (d) Hb−magadiite with the stretching vibration of C−H absorption bands at 2960 cm^{-1} (γ_{C-H}), the characteristic Si−O vibration at 1078 cm^{-1} (γ_{Si-O}), and the amide I and II bands located at 1658 and 1546 cm^{-1} (*), respectively. FTIR spectra of (B) and (C): the amide I (γ_{C-N}) and II (γ_{N-H}) bands of free and immobilized proteins for Mb and Hb, respectively.

diite composites. The vibrational bands of the amide group present in proteins provided sensitive signatures of the protein secondary structure.[36] For the amide vibrational frequencies of proteins were sensitive to hydrogen-bonding interactions of the amide function with the solvent, and with the neighboring functionalities, protein denaturation could modify these interactions to a significant extent and resulted in the amide vibrational frequency shifting. Accordingly, the amide I (assigned to the stretching of the carbonyl coupled to the C−N) and amide II (assigned to the stretching of N−H) vibrational bands were usually chosen as the sensitive markers of protein structural changes. Upon denaturation of α-helix, for example, the amide I band shifts from 1650 to 1640 cm^{-1}, while the amide II band shifts to higher frequencies from 1550 cm^{-1}.[11] The FTIR spectra of H−, TBA−, and Mb/Hb−magadiite are shown in Figure 6A. Seen from curve b in Figure 6A, the sharp absorption band at 2960 cm^{-1} was assigned to the stretching vibration of C−H, indicating the introduction of TBA^+. The characteristic Si−O vibration at 1078 cm^{-1} and the amide I and II bands located at 1658 and 1546 cm^{-1}, respectively, were observed in Mb− and Hb−magadiite as shown in curves c and d in Figure 6A, demonstrating the combination of heme proteins with magadiite layers. Furthermore, the amide I and II bands were essentially the same as those of the native proteins as shown in Figure 6B,C. These spectra indicated the undisturbed secondary structure for the bound proteins. The tertiary structure is important for understanding the bioactivity of the combinated heme proteins and fluorescence spectroscopy is a very useful method and will be carried out in the future to study the composites.

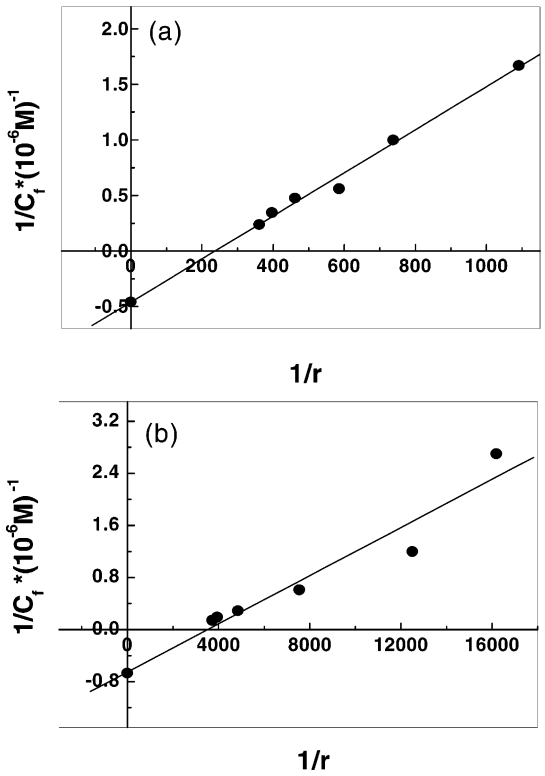

Figure 7. Scatchard plots for the binding of Mb (a) and Hb (b).

Binding Studies. Equilibrium mixtures of magadiite with increasing concentrations of protein were centrifuged; the Mb− and Hb−magadiites settled to the bottom and were separated from the free proteins. The concentrations of the bound protein could be estimated. These data were analyzed using the Scatchard equation (eq 1). The binding plot for Mb and Hb are shown in Figure 7. The binding constant K_b for Mb calculated from this plot was 4.6×10^5 M^{-1}, higher than that of

(35) Torii, H.; Tasumi, M. In *Infrared Spectroscopy of Biomoleculaes*; Mantsh, H. H., Chapman, D., Eds.; John Wiley & Sons: New York, 1996; pp 1−18.

(36) (a) Kimm, S.; Bandekar, J. *Adv. Protein Chem.* **1986**, *38*, 181. (b) Susi, H.; Byler, D. M. *Methods Enzymol.* **1986**, *130*, 290. (c) Pancoska, P.; Wang, L.; Keiderling, T. A. *Protein Sci.* **1993**, *2*, 411.

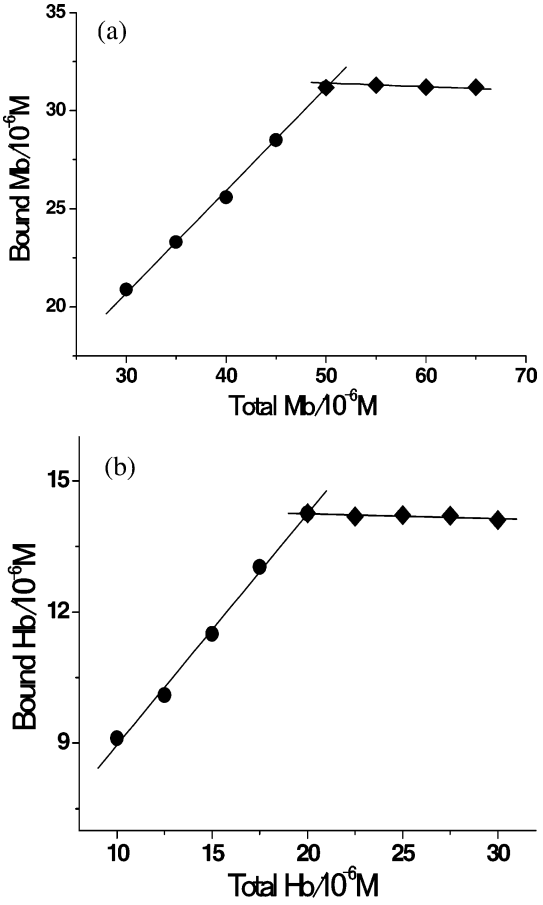

Figure 8. Stoichiometry plots for Mb (a) and Hb (b). The concentration of magadiite was kept constant at 1.8 mM.

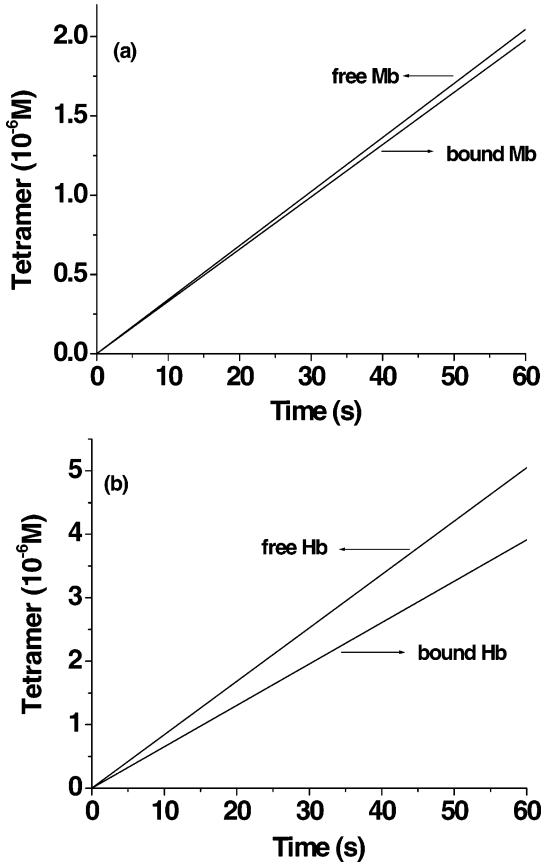

Figure 9. Activities comparison between free and immobilized Mb (a) and Hb (b).

Mb bound to α-zirconium phosphate.[11b] In the case of Hb, the binding constant K_b was 1.6×10^6 M^{-1}, higher than that of Mb. These protein affinities to magadiite clearly demonstrated the ability of the magadiite matrix to bind Mb and Hb. The combinations between Hb and magadiite inorganic layers were stronger than those of Mb composites, which were consistent with the XRD results. The narrow XRD peaks for Hb−magadiite also showed that the combinations between the guest Hb and host layers were stronger and a very narrow distribution of the orientations appears. The weak combinations between Mb and inorganic layers led to the broad peaks for Mb−magadiite, indicating broad distribution of orientations.

To investigate the binding amount of Mb and Hb in magadiite, binding stoichiometries were also studied. When equilibrium mixtures of Mb/Hb−magadiite suspensions were centrifuged, the free protein concentrations in the supernatant were determined by means of spectroscopic measurements. The corresponding binding stoichiometry plots are shown in Figure 8. It could be found that an increase in the amount of the immobilized proteins occurs along with the concentration of proteins increasing. Upon a certain concentration of proteins being reached, no more proteins would be immobilized. Then we could estimate the binding stoichiometries by means of the point intersection of the two lines. The C_b values (1.8 mM magadiite) estimated for Mb and Hb were 31.4 and 14.4 μM, respectively. These studies clearly established the binding of the proteins to the magadiite matrix.

On the basis of the above data, we could construct the structure models for intercalated TBA^+ and immobilized Mb and Hb. The inorganic magadiite layer thickness is about 1.12 nm. The expansion for TBA−magadiite was about 1.44 nm, far larger than the monolayer of TBA^+ between the layers of magadiite. The combination structure characteristic of the sheet (Figure 1A) and size of TBA^+ and the bilayer stack of TBA^+ between layers was constructed, as shown in Figure 1B. The monolayer of Mb/Hb was intercalated in the interlayers with the interlayer expansions by 3.20 and 5.70 nm. The short axis dimensions were perpendicular to the inorganic layers with a layer of H_3O^+ ions or TBA^+ between the inorganic layers or the long axis dimensions with a certain degree of inclination also existed possibly between the layers (Figure 1B). The interaction between proteins and matrix was generally thought to be electrostatic interactions. However, the immobilized Mb/Hb was highly stable, even in the solutions with a relativity high ionic strength. More than 90% of immobilized proteins were retained in the magadiite support in 0.8 mol/L NaCl solutions based on the UV−vis analyses, indicating that stronger interactions existed between magadiite and intercalated proteins, such as the formation of hydrogen bonding between residual nitrogen on the surface of proteins and the oxygen atom of the hydroxyl of magadiite.

On the basis of the structure reported by Brindley et al.,[37] the unit parameters of magadiite were $a = b =$ 0.73 nm, $c = 1.57$ nm, and $\beta = 96.8°$. The available unit

(37) Brindley, G. W. *Am. Miner.* **1969**, *54*, 1583.

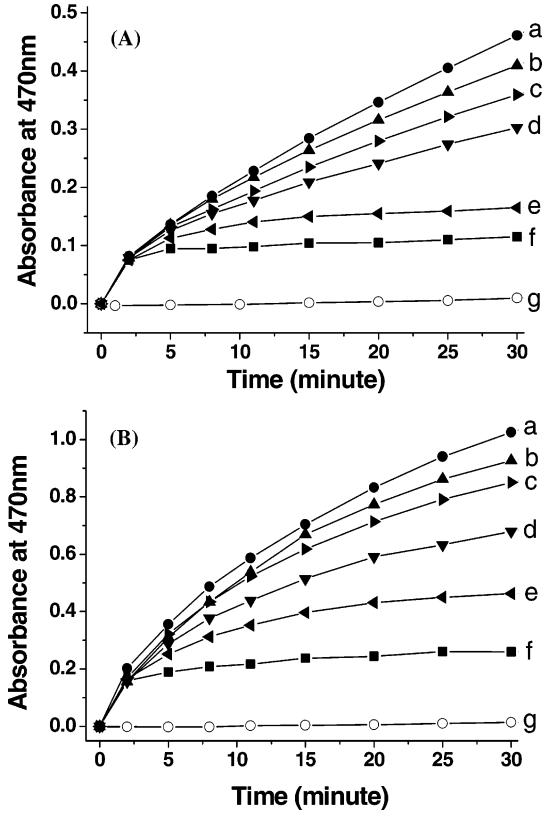

Figure 10. Hydrogen peroxide activated peroxidase activity for oxidation of *o*-methoxyphenol to tetramer product with the absorbance at 470 nm at different interval times for free and immobilized Mb (A) and Hb (B) with (a) free Mb and Hb, (b) aging for 1 h, (c) aging for 5 d, (d) aging for 10 d, (e) aging for 20 d, (f) aging for 30 d, and (g) TBA−magadiite. (Aging process was developed at room tempreture. $[H_2O_2] = 0.5$ mM, [*o*-methoxyphenol] = 0.5 mM, [Mb] = [Hb] = 5.0 μM).

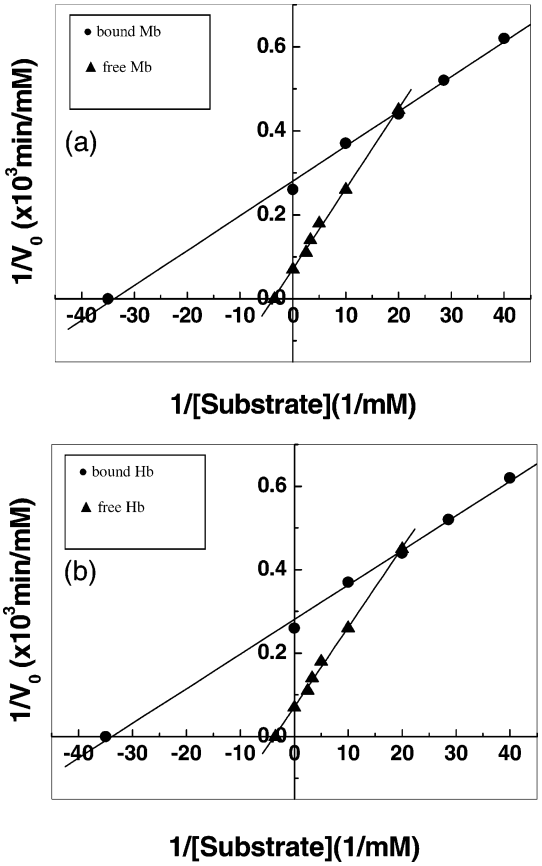

Figure 11. Lineweaver−Burke plots for the peroxidase activity of free and bound heme proteins with *o*-methoxyphenol as the substrate.

surface area in the interlayer space was estimated to be 0.53 nm^2. The cross-sectional area of an intercalated Mb and Hb molecule were about 12.56 and 33.17 nm^2, respectively, based on the longer axis distances. According to the estimated binding stoichiometries C_b, there were 1/57 Mb and 1/125 Hb molecules per units on the magadiite layers, respectively. If the unit surface was fully occupied by proteins in cubic or hexagonal aggregates, one Mb (Hb) molecule would occupy 30 (80) and 26 (69) magadiite units, respectively. In fact, the occupancy was only about 53% (64%) or 46% (55%) for Mb−magadiite (Hb−magadiite) composites related to cubic or hexagonal aggregates, respectively, according to the results of binding stoichiometries. This loose packing of the proteins was perhaps important in maintaining the access to the bound proteins by substrates and other reagents.

Activities of the Bound Proteins. Immobilized enzymes should retain their activity and be readily accessible for substrates.This is essential for the application of these materials in the construction of biosensors or chemical sensors.[38] Peroxidase activity of Mb and Hb were monitored using a spectrophotometric method. The addition of hydrogen peroxide (0.5 mM) to protein−magadiite (including 0.5 μM Mb−magdiite and Hb−magdiite in enzymes) dispersions in the presence of *o*-methoxyphenol (0.5 mM) resulted in the formation of the tetramer, as observed in its absorption spectrum and the growth of absorbance at 470 nm as a function of time.

Table 1. Kinetic Constants of Free Heme Proteins and Bound Heme Proteins

heme proteins	K_m (mM)	V_{max} (μM/s)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})
free Mb	1.50	0.14	0.028	0.019
bound Mb	0.35	0.07	0.014	0.040
free Hb	0.26	0.23	0.046	0.177
bound Hb	0.03	0.06	0.012	0.400

Enzyme activity plots of free and bound Mb and Hb are shown in Figure 9. Calculated from Figure 9a, the initial formation rate of tetramer for free Mb was 0.034 μM/s, while the initial formation rate for bound Mb was 0.032 μM/s, indicating about 94.1% of enzyme activity was kept after Mb was immobilized. In the case of Hb, the initial formation rate of tetramer for free Hb was 0.084 μM/s and 0.066 μM/s for bound Hb, indicating about 78.6% of enzyme activity was kept after Hb was immobilized. These results showed enzyme activities of Mb and Hb were kept in a large scale after they were immobilized.

To further investigate the stability of immobilized enzyme, the catalyzed reaction plots for bound Mb and Hb at different aging times storing under room temperature were measured (Figure 10). Compared with free Mb (Figure 10A), about 88.9% (the amounts of tetramer formation were 23.0 and 20.5 μM for free Mb and immobilized Mb) of the activity based on the

(38) Dong, A.; Huang, P.; Caughey, W. S. *Biochemistry* **1992**, *31*, 182.

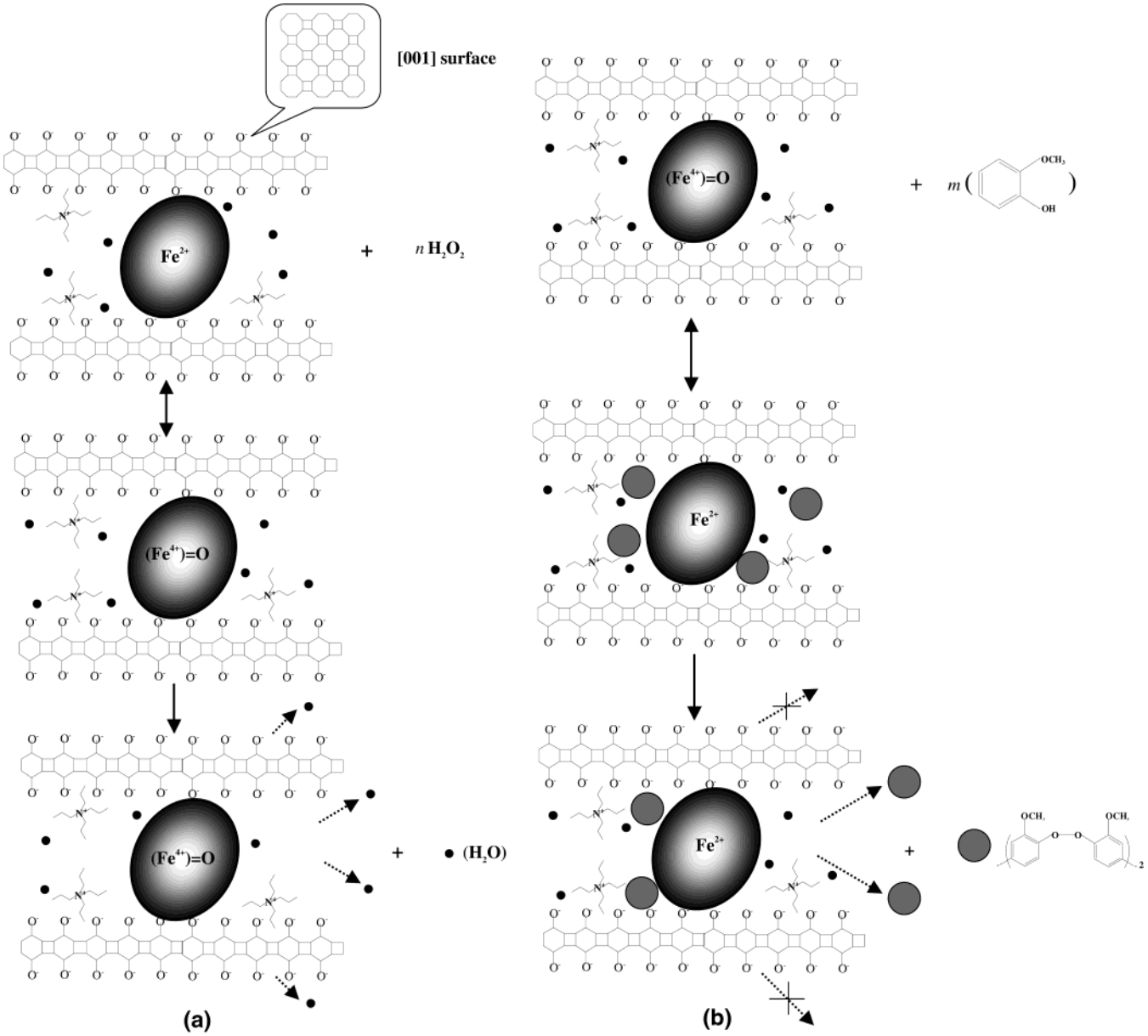

Figure 12. Schematic representation of structures and catalytic mechanisms of heme proteins immobilized in the galleries of magadiite. (a) Reacted with hydrogen peroxide. (b) Reacted with *o*-methoxyphenol. (The small closed circle = water, the larger close circle = tetramer.)

formation amount of tetramer was kept after 30 min for freshly prepared Mb−magadiite. With the increase of aging time, the activities on the formation amount of tetramer of Mb−magadiite nanocomposites began to decrease gradually. About 25.0% (the amount of tetramer formation was 5.7 μM) of the activity was kept after 30 min for Mb−magadiite composites after aging for 30 days at room temperature. But the initial formation rate of tetramer was 0.030 μM/s, indicating about 88.2% of enzyme activity (the initial formation rate of tetramer) was kept after aging for 30 days for Mb−magadiite than for free Mb. Similar to Mb−magadiite composites, the activity of bound Hb was slightly lower than that of free Hb. About 90.4% (the amounts of tetramer formation were 51.2 and 46.3 μM for free Hb and bound Hb) of activity (the formation amount of tetramer) was retained after 30 min for freshly prepared Hb−magadiite. The activity in the formation amount of tetramer of Hb−magadiite composites decreased gradually with the increase of aging time. Only 25.3% (the amount of tetramer formation was 13.0 μM) of activity for Hb−magadiite composites was kept after 30 min, while the initial formation rate of tetramer was unchanged compared with that of freshly prepared Hb−magadiite. These results demonstrated the facile diffusion of the reactants through the layered support and the facile access to the active sites of the immobilized proteins. More importantly, the intercalated enzyme properties were not attenuated evidently by the matrix. It was further proved that magadiite was an attractive matrix for heme proteins.

Lineweaver−Burk plots of enzyme activity were plotted for bound and free Mb and Hb in Figure 11. A comparison of the values of the kinetic constants for the free and the bound Mb and Hb is represented in Table 1. It is evident from the table that free Mb and Hb had the larger values of K_m and V_{max} than bound Mb and Hb. There was a 4-fold decrease and an 8-fold decrease in the K_m values for bound Mb and Hb compared with that of free Mb and Hb, respectively. The V_{max} for the

bound Mb was 0.07 μM/s, half that of the free Mb, while the V_{max} for bound Hb nearly decreased 4 times that of free Hb. The catalytic turnovers (K_{cat}) of bound Mb and Hb were also found to be lower than that of free Mb and Hb. The catalytic specific constants (K_{cat}/K_m) for bound Mb and Hb were improved greatly compared to those of free Mb and Hb.

Decreasing K_m values for the bound Mb and Hb indicated that a lower substrate concentration was required to achieve half of the maximum rate of reaction observed for the free Mb and Hb. Low K_m values for the bound Mb and Hb were exhibitive of the changes in the microenvironment of bound Mb and Hb. This might be the result of Mb and Hb being bound to the interlayer of magadiite, which might alter the interactions between the substrate and the enzyme. On the other hand, the K_m value represented the decompounded constant of ES (enzyme−substrate intermediate) according to the Michaelis−Menten model.[39] That was to say, the smaller the K_m value was, the steadier binding of ES would be. Compared with free Mb and Hb, low K_m values might indicate that the tetramer was not so easy to diffuse out of the interlayer due to the confined environments, thereby decreasing the value of K_m. Higher values of K_{cat}/K_m for bound Mb and Hb also resulted from the faster decrease in the value of K_m than V_{max}. The catalytic processes bound in the gallery of magadiite were illustrated as follows.

As shown in Figure 12, due to the confined environments, products were retained in the interlayer. Because H_2O_2 and *o*-methoxyphenol molecules were small, they could access the enzyme through layers or pores on silicate sheets without difficulty, while it was more difficult for the tetramer to diffuse out of the interlayer space. The confined environments to the tetramer made the values of K_m for bound Mb and Hb based on *o*-methoxyphenol concentration were far lower than those of the free Mb and Hb calculated from the Lineweaver−Burke plot (Figure 11). The result further proved that Mb and Hb were entrapped in the galleries of magadiite. On the basis of the above discussion, nanocomposities of hemeprotein intercalated magadiite were important for preserving the peroxides activities, which effectively increase the contact probability for substrates and decreased diffusion distance of tetramer out of the galleries. It also should be mentioned that both the substrates and product are neutral in our experiment, which avoids potential electrostatic interactions with the matrix. The use of charged analytes may lead to alterations in K_m and K_{cat} values.

(39) Wang, X. In *Biochemistry*; Tsinghua University Publishing Company, 2001; p 69.

Conclusion

Novel kinds of layered structural Mb− and Hb−magadiite nanocomposites with high enzyme-like peroxidase activity have been successfully synthesized by means of TBA−magadiite as an intermediate. HRTEM and XRD indicated that Mb and Hb were entrapped in the galleries of magadiite. SEM images showed that the Mb and Hb intercalated nanocomposities formed. FTIR and UV−vis spectra indicated no detectable changes in immobilized Mb and Hb structures. On the basis of the above results, structure models for intercalated Mb and Hb were discussed in detail. In light of the binding stoichiometry study, occupied amounts of intercalated heme proteins on the magadiite units were estimated. The activity studies indicated that Mb− and Hb−magadiite preserved their most activity and obtained facile diffusion of substrate access to the active site of bound Mb and Hb. Kinetic parameters calculated from Lineweaver−Burk plots for bound Mb and Hb were obtained. The lower values of K_m for bound Mb and Hb were discussed and illustrated based on the Michaelis−Menten model. Nanocomposities of protein intercalated layered inorganic material provide substantial applications in the field of chemical or biological nanosensors.

Acknowledgment. This work was financially supported by Chinese National Science Funding (No. 20201013) and "Plan of Outstanding Talents" of Chinese Academy of Science.

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