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Enhanced Catalytic Activity of Hemoglobin in Organic Solvents by Layered Titanate Immobilization

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Biocatalysts have been extensively investigated in organic media over the past two decades.[1] The organic solvents have been selected to optimize the application of enzymes in biotransformation of xenobiotic pollutants due to the increased solubility of these hydrophobic substrates.[1,2] However, their industrial applications have been limited by the low activity and stability of the commonly used freeze-dried enzymes in nonaqueous environments. Immobilization of enzymes into solid supports appears to be an effective approach to enhance activity in organic media.[3−6] The treatment not only preserves the facile recovery advantages of the freeze-dried enzymes but also avoids the deleterious protein aggregation by spreading the enzymes on the support surface. The previous immobilization efforts for nonaqueous enzymatic catalysis have been made mainly by using polymers and hydrogels with cross-linked polymeric structure to mildly entrap enzymes and absorb large quantities of water.[3,4] However, the corrosion of organic solvents cannot be resisted by the hosts due to their facile dissolutions in the solvents.

In general, inorganic materials have intrinsic advantages such as stronger mechanical strength and higher resistance to organic solvents. Several groups reported that improved catalytic activities were shown for enzymes absorbed by mesoporous molecular sieves with 2−10 nm pore diameters in organic solvents.[5,6] Recently, an alternative route to bind bulky enzymes was carried out by reassembly of the exfoliated inorganic layers with guest molecules under mild conditions. A variety of enzymes were combined with α-zirconium phosphates, octahedral manganese oxides, layered silica, and so forth.[7−9] There is no report on the effect of layered inorganic hosts on the nonaqueous enzymatic activities.

Here, we report the immobilization approach by using layered titanate as a host to improve the hemoglobin biocatalytic behavior in organic solvents. As a simulated peroxidase, hemoglobin can be used to catalyze the oxidation of organic pollutants, such as polycyclic aromatic hydrocarbons and phenols. The titanate elementary sheets were obtained by the delamination of the protonic lepidocrocite-type titanate. Their detailed synthetic mechanism and assembly behavior have been reported by Sasaki et al.[10] The total procedure only required minor modification for the effective immobilization of Hb.[11] Four times the mass of Hb can be tightly combined into the titanate sheets. Negligible amounts of Hb (<1 mass %) may be desorbed from the titanate host even in 1 M NaCl solution. Prior to the reactant addition, the key step is to rinse the composite with the chosen solvent three times to remove the residue surface water.

XRD patterns (Figure 1) indicate that the *d* spacing increases from 0.93 nm for the protonic-type titanate to 7.12 nm for the composite. The observed *d* spacing is slightly larger than that of the monolayer Hb intercalated titanate, 6.45 nm (sum of the Hb average size 5.70 nm and the titanate slab thickness 0.75 nm).[7b,10] The slightly larger interlayer distance of the Hb composite can be ascribed to the swollen effect of the water molecules on the titanate. The results indicate that the immobilization process can entrap Hb and absorb large quantities of water into the interlayers.

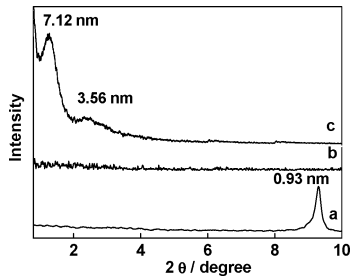

Figure 1. XRD profiles at various stages of the synthetic process: (a) the protonic lepidocrocite-type titanate; (b) the colloid of the exfoliated titanate; (c) the composite obtained by immobilizing Hb into titanate interlayers.

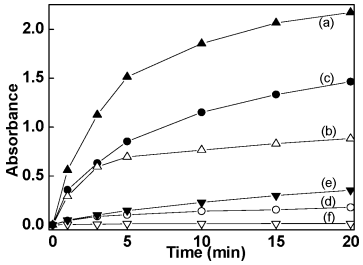

Figure 2. Catalytic reactions of immobilized and native Hb in three kinds of organic solvents, respectively (water content < 0.3%), with different hydrophobicities: (a) immobilized Hb in toluene; (b) native Hb in toluene; (c) immobilized Hb in acetonitrile; (d) native Hb in acetonitrile; (e) immobilized Hb in dioxane; (f) native Hb in dioxane.

The catalytic activity of Hb in organic solvents was studied using H_2O_2 oxidation of *o*-phenylenediamine (OPD) to phenazine as a model reaction. The reaction process of 25.0 mM H_2O_2 with 10.0 mM OPD catalyzed by Hb was measured by monitoring the absorbance at 450 nm along with the increasing time. The same concentration of 3.0 μM was chosen for immobilized and lyophilized native Hb, respectively. The absorbance increase in the first minute was defined as the initial rate. Their kinetic constants were estimated with Lineweaver−Burk plots, which were constructed with the initial rates at different *o*-methoxyphenol concentrations in the reaction.

Figure 2 shows OPD oxidation reaction processes catalyzed by the immobilized and lyophilized Hb in different solvents, respectively, including toluene, acetonitrile, and dioxane. The immobilized Hb shows enhanced catalytic activity with maximum activities of 2.46 and 8.13 times enhancements in the 20 min average reaction rates in toluene and acetonitrile solvents, respectively. In the most hydrophilic dioxane medium the native Hb powder hardly shows a catalytic effect, while the immobilized Hb shows a certain reaction rate. The immobilized Hb shows an about 100 times rate enhance-

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Table 1. Reaction Kinetic Constants of the Immobilized and Lyophilized Hb Powders in the Organic and Water Solvents

	solvent	V_{max} (μM/s)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})
immobilized Hb powder	toluene	1.35	3.55	0.45	0.13
	acetonitrile	0.86	3.43	0.29	0.08
	dioxane	0.22	3.23	0.07	0.02
	water	2.11	2.57	0.70	0.27
lyophilized Hb powder	toluene	0.72	9.85	0.24	0.03
	acetonitrile	0.07	*a*	0.02	*a*
	dioxane	<0.01	*a*	<0.01	*a*
	water	2.62	2.85	0.87	0.31

[a] Undetected data due to the large relative error for the initial rate measurement.

ment, compared to that of the native Hb. The results (Figure 2) also indicate that the immobilized Hb has improved stability in different organic media compared to that of the native Hb. The immobilized Hb can preserve catalytic ability after 20 min for the reaction in those solvents, while the native Hb lost most of the catalytic ability after 5 min. Additionally, the immobilized Hb in titanate has a comparative rate enhancement in organic solvents, as other enzymes do which were immobilized in polymers, hydrogels, or mesoporous sieve hosts.[3,4]

To explain the effect of the solvents on Hb, the kinetic constants of the immobilized and native Hb in various systems are listed in Table 1. A 0.81 times V_{max} value (stands for the Hb intrinsic catalytic ability) was found for the immobilized Hb in water, compared to that of the native Hb. The result indicates that the immobilization is effective. On the basis of the V_{max} values in Table 1, one can find that the activities of the immobilized Hb are related to the solvent polarity. Their catalytic activity increases along with the increase of the solvent hydrophobicity (log *P*).[12] The V_{max} values for the immobilized Hb in toluene (log $P = 2.5$), acetonitrile (log $P = -0.33$), and dioxane (log $P = -1.1$) were 0.64, 0.41, and 0.11 times that in water, respectively. For the native Hb the activities in organic solvents are evidently related to the solvent polarity too. But the native Hb catalytic ability is sharply reduced in these organic solvents. The V_{max} values in toluene, acetonitrile, and dioxane were only 0.27, 0.03, and <0.01 times that in water, respectively. The correlation is in accordance with the theoretical prediction, which demonstrates that the enzyme catalytic ability is low in polar solvents and high in apolar solvents.[12a] This result is also consistent with the ability of the organic solvents to distort the essential water layer on the Hb molecular surface.

It has become apparent that the effect of organic solvents on the enzyme is primarily due to the interaction with the essential enzyme-bound water layer rather than with the enzyme itself.[12] The higher remaining activities for the immobilized Hb in organic solvents could be due to the protection of the interlayer water molecules for the essential water layer on the Hb surface.[6,12] On the contrary, a certain amount of residue water in the native Hb powder is severely distorted by these organic solvents. In the same organic solvent environment, the interlayer water around Hb can protect its essential water layer from distorting.

Further conclusion can be obtained by analyses of other kinetic constants. The titanate immobilization results in reduction of the enzymatic K_m values (Michaelis constants) in water, which can be clearly seen from Table 1. Low K_m values are the indicators of the microenvironment changes in the immobilized Hb titanate composites, which may be attributed to an increase in the affinity of Hb to substrate as a consequence of steric restriction in layered titanate.[7,8,12] In the case of nonaqueous biocatalytic reactions, it is evident that the K_m values of the immobilized Hb increase with the increase of the solvent hydrophobicity. This observation is in accordance with that of native enzyme in organic solvents.[12] Among three types of organic solvent systems, the native Hb kinetic constants can only be measured in toluene because of the low catalytic activity. One can find that the difference is small among the K_m values for the immobilized Hb in different organic solvents, compared to that of the native Hb. The approximative K_m values indicate that the immobilized Hb should have a similar microenvironment in different organic solvents.[8,12] This result confirms that the attack from the organic solvents can be relieved by the interlayer water molecules. The catalytic turnover constant ($K_{cat} = V_{max}/[Hb]$) and the catalytic specificity constant (K_{cat}/K_m) are found to have the same trend as that of V_{max} values, which is also ascribed to the solvent polarity and the protective effect of interlayer water molecules.

In conclusion, the simple immobilization of Hb into a layered titanate results in an enhanced catalytic activity in organic media compared to that of native Hb. The enhanced nonaqueous catalytic activities of the immobilized Hb may be due to the interlayer water resistance to the attack by the organic solvents. This property suggests that the immobilization procedure would have wide application as a simple and economical way of preparing biocatalysts for reactions in organic media.

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Supporting Information Available: SEM and TEM images of the immobilized Hb composites. Lineweaver−Burk plot for the immobilized and free Hb in different solvents. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) In a typical procedure, $H_{0.7}Ti_{1.825}\square_{0.175}O_4 \cdot H_2O$ ($\square$ express vacancy) was prepared by calcining a stoichiometric mixture of Cs_2CO_3 and TiO_2 (1:5.3 molar ratio) twice at 1027 K for 20 h and sequence acid exchange by a 1.0 M hydrochloric acid solution. Then the protonic lepidocrocite-type titanate (1.0 g) was added to a 25.0 mM TBAOH aqueous solution (100 mL). The mixture was stirred vigorously at 298 K for 8 days, and the resulting suspensions were centrifuged (8000 rpm) to obtain translucent sols. Stock solutions of the Hb (1.0 mg/mL) and the exfoliated titanate (1.0 mg/mL) were mixed in a 4:1 ratio. Previous to the reaction, the sols should be adjusted from pH 12.0 to 6.0 with 0.1 M acetic acid solutions. The mixture was stirred for 1 h and then left for a further 3 h to precipitate rufous solid. The Hb concentration in the upper solution was <0.01 mg/mL by spectroscopic measurement at 410 nm.
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