Catalytic Activity of Myoglobin Immobilized on Zirconium Phosphonates

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The adsorption and catalytic activity of myoglobin (Mb) on zirconium phosphonates (α-zirconium benzenephosphonate (α-ZrBP), α-zirconium carboxyethanephosphonate (α-ZrCEP), and a novel layered zirconium fluoride aminooctyl-*N*,*N*-bis(methylphosphonate) (ZrC8)) were investigated. The maximum adsorption was reached after 16 h of contact and was greater on hydrophobic supports such as α-ZrBP and ZrC8 compared to hydrophilic supports such as α-ZrCEP. The equilibrium adsorption isotherms fitted the Langmuir equation, suggesting the presence of a monolayer of protein molecules on the support surfaces. The catalytic activities of free Mb and of the obtained biocomposites were studied in terms of the oxidation of two aromatic substrates, *o*-phenylenediamine and 2-methoxyphenol (guaiacol), by hydrogen peroxide. The oxidation catalyzed by immobilized myoglobin followed the Michaelis−Menten kinetics, similar to oxidation by free Mb. The kinetic parameters, k_{cat} and K_M, were significantly affected by the adsorption process. Mb/α-ZrCEP was the most efficient biocatalyst obtained, probably because of the hydrophilic nature of the support. The effect of immobilization on the stability of Mb toward inactivation by hydrogen peroxide was also investigated, and an increased resistance was found. The biocomposites obtained can be stored at 4 °C for months without a significant loss of catalytic activity.

Introduction

Protein adsorption on a solid surface is an important topic of biological, medical, and technological research. Although the adsorption of proteins and enzymes at the surfaces of polymers,[1] sol−gels,[2] phospholipids,[3] silica microparticles,[4] and mesoporous silicate[5] has been studied for decades, the driving forces leading to adsorption are still not well understood. However, given that the protein surface has charged groups and polar and hydrophobic regions, the most important intermolecular forces involved in the adsorption should be essentially electrostatic, with contributions from hydrogen bonding and van der Waals interactions. In this context, support materials, which play an important role in the utilization of immobilized proteins, should not only be insoluble and inert toward different substrates but also possess functional groups on the surface that interact properly with the protein surface.

In addition to the various above-mentioned supports, zirconium phosphates and phosphonates have also attracted much attention with respect to protein binding because of their good chemical and mechanical resistance as well as their ability to anchor to the surface functional groups having different hydrophilic/hydrophobic characteristics and/or ionogenic properties.[6−8]

α-Zirconium phosphate ($Zr(HPO_4)_2$, hereafter indicated as α-ZrP), is a layered inorganic material with acidic hydroxyl functions present on its lamellar surface. The hydrophilic nature of α-ZrP may play an important role in stabilizing the surface-bonded protein. α-Zirconium phosphonates ($Zr(RPO_4)_2$), in which the OH groups of α-ZrP are replaced by several R functional groups such as phenyl, 2-carboxyethyl, or sulfophenyl,[9,10] provide different surface characteristics for the interaction with proteins.

The adsorption of *Candida rugosa* lipase (CRL) on these supports has been investigated.[7,8] The different nature of the functional groups on the surface, combined with the appropriate experimental conditions, afforded selective immobilization of several CRL isoenzymes and allowed the preparation of some biocomposites having different catalytic properties.[8]

Continuing the research on the immobilization of enzymes and proteins onto supports based on zirconium phosphonates, myoglobin (Mb) has been considered. Mb is a protein that has been naturally selected for oxygen storage; it contains a single, noncovalently bonded iron protoporphyrin IX as the prosthetic group in a hydrophobic

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(1) Piletsky, S.; Piletska, E.; Bossi, A.; Turner, N.; Turner, A. *Biotechnol. Bioeng.* **2003**, *82*, 86.
(2) Smith, K.; Silvernail, N. J.; Rodgers, K. R.; Elgren, T. E.; Castro, M.; Parker, R. M. *J. Am. Chem. Soc.* **2002**, *124*, 4247.
(3) Li, J.; Rosilio, V.; Boissonnade, M. M.; Baszkin, A. *Colloids Surf., B* **2003**, *29*, 13.
(4) He, F.; Wang, Y.; Feng, J.; Zhuo, R.; Wang, X. *Polymer* **2003**, *44*, 3215.
(5) Han, Y. J.; Watson, J. T.; Stucky, G. D.; Butler, A. *J. Mol. Catal. B: Enzym.* **2002**, *17*, 1.
(6) Kumar, C. V.; Chaudhari, A. *J. Am. Chem. Soc.* **2000**, *122*, 830.
(7) Bellezza, F.; Cipiciani, A.; Costantino, U.; Negozio, M. E. *Langmuir* **2002**, *18*, 8737.
(8) Bellezza, F.; Cipiciani, A.; Costantino, U. *J. Mol. Catal. B: Enzym.* **2003**, *26*, 47.
(9) Alberti, G.; Costantino, U.; Casciola, M.; Vivani, R. *Adv. Mater.* **1996**, *8*, 291.
(10) Clearfield, A. Metal Phosphonate Chemistry. In *Progress in Inorganic Chemistry*; Karlin, K. D., Ed.; John Wiley & Sons: New York, 1998; Vol. 47.

pocket.[11] Nonenzymatic heme proteins, such as myoglobin, hemoglobin, and cytochrome *c*, can also catalyze peroxidase-like reactions. Oxidative dehydrogenation is the classical reaction catalyzed by peroxidases; these proteins also catalyze a variety of related reactions, including oxygen transfer, hydrogen peroxide cleavage, and peroxidative halogenations.[12] In the oxidative dehydrogenation, hydrogen peroxide reacts rapidly with Mb to generate an intermediate (known as compound I) which is capable of oxidizing amines, phenols, and other organic compounds.[13] The mechanism of these reactions generally proceeds through one-electron steps. In fact, one-electron reduction of compound I produces a second protein intermediate, compound II, which can also monoelectronically oxidize a molecule of substrate, albeit at a slower rate. Mb was chosen as a representative example for investigating the peroxidase-like activity of a supported protein because of its availability, well-known structure, small size, and strong spectroscopic signatures that allow protein binding and redox activity to be monitored. In addition, detailed data on the peroxidase activity of several Mb derivatives have recently been reported in the literature.[14−17]

The specific objectives of this study were to immobilize Mb on zirconium phosphonates and study the catalytic activity of the immobilized protein in the oxidation of two representative substrates, *o*-phenylenediamine and 2-methoxyphenol (guaiacol), also in comparison with the behavior of free Mb. The supports chosen were α-zirconium benzenephosphonate (α-ZrBP), α-zirconium carboxyethanephosphonate (α-ZrCEP), and a novel layered zirconium fluoride aminooctyl-*N,N*-bis(methylphosphonate) (ZrC8), which are characterized by different hydrophobic/hydrophilic groups on the surface. The effects of the immobilization process on the activity, kinetic parameters, and stability of Mb were also investigated.

Experimental Section

1. Materials. Myoglobin (from horse heart, 90% pure) was obtained from Sigma and used without further purification. Hydrogen peroxide (30 volumes in water), 2-methoxyphenol, and *o*-phenylenediamine were obtained from Aldrich.

2. Preparation and Characterization of Supports. Layered zirconium phosphonates α-ZrBP and α-ZrCEP were prepared according to the method of slow decomposition of zirconium fluoro complexes in the presence of phosphonic acids.[18] Details of the synthesis, as well as the physicochemical properties, are reported elsewhere.[7]

Zirconium fluoride aminooctyl-*N,N*-bis(methylphosphonate) (ZrC8) was prepared by allowing the zirconium fluoro complexes to decompose in a water−methanol solution containing 0.1 mol/dm^3 of $(H_2O_3PCH_2)_2NC_8H_{17}$ diphosphonic acid and maintained at 80 °C in a closed Teflon vessel for 4 days. Details about the synthesis procedures and information about the structure and properties of the compound have been previously described.[19]

(11) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in their Reactions with Ligands*; North-Holland: Amsterdam, 1971.

(12) Colonna, S.; Gaggero, N.; Richelmi, C.; Pasta, P. *Trends Biotechnol.* **1999**, *17*, 163.

(13) Dawson, J. H. *Science* **1988**, *240*, 433.

(14) Matsui, T.; Ozaki, S.; Liong, E.; Phillips, G. N., Jr., Watanabe, Y. *J. Biol. Chem.* **1999**, *274*, 2838.

(15) Monzani, E.; Alzuet, G.; Casella, L.; Redaelli, C.; Bassani, C.; Sanangelantoni, A. M.; Gullotti, M.; De Gioia, L.; Santagostini, L.; Chillemi, F. *Biochemistry* **2000**, *39*, 9571.

(16) Ozaki, S.; Roach, M. P.; Matsui, T.; Watanabe, Y. *Acc. Chem. Res.* **2001**, *34*, 818.

(17) Redaelli, C.; Monzani, E.; Santagostini, L.; Casella, L.; Sanangelantoni, A. M.; Pierattelli, R.; Banci, L. *ChemBioChem* **2002**, *3*, 226.

(18) Alberti, G.; Costantino, U.; Kornyei, J.; Luciani, M. L. *React. Polym.* **1985**, *4*, 1.

Scanning electron microscopy (SEM) micrographs were obtained using a Philips XL30 scanning electron microscope and high-resolution transmission electron microscopy (HRTEM) micrographs using a JEOL, JEM-2010 high-resolution transmission electron microscope with EDS detector.

3. Myoglobin Immobilization. Samples of immobilized Mb were prepared by treating 60 mg of support with 4 mL of protein solution at different concentrations. In the case of α-ZrBP and ZrC8, the solids were first prewetted with 0.2 mL of ethanol to facilitate contact between the hydrophobic support and the aqueous solution. The adsorption experiments were carried out in unbuffered systems because inorganic buffers could interfere with the adsorption of protein. The mixture was stirred for 16 h at room temperature. The solid containing immobilized protein was recovered by centrifuging at 3000 rpm for 15 min. The supernatant was assayed for protein content by UV spectra recorded at 410 nm ($\epsilon = 160\,000$ M^{-1} cm^{-1}). The amount of protein bound to the support was calculated by subtracting the absorbance of the supernatant solution from the initial absorbance of the protein solution. The adsorption data were fitted to the Langmuir (1) and Freundlich (2) equations[20,21]

$$Q_e = \frac{Q_{MAX}C_e}{(1/a_L) + C_e} \tag{1}$$

$$Q_e = K_F C_e^{1/n} \tag{2}$$

where C_e is the concentration of Mb in solution at equilibrium (mg/mL), Q_e is the amount of Mb adsorbed onto the support (mg/g of support), Q_{MAX} is the maximum adsorption capacity of the support (mg/g of support), a_L is the Langmuir constant (mL/mg), K_F is the Freundlich adsorption coefficient ((mg/g of support)/(mg/mL)$^{-1/n}$) and $1/n$ is the heterogeneity factor (*a*-dimensional).

4. Enzyme Kinetics for Free Myoglobin. The peroxidase activity assays for both free and immobilized Mb were performed by measuring the initial oxidation rates of guaiacol and *o*-phenylenediamine with H_2O_2. The kinetic parameters were determined using the Michaelis−Menten approach, through the equation

$$\frac{V}{[E_0]} = \frac{k_{cat}[S]}{K_M + [S]} \tag{3}$$

where *V* is the initial reaction rate, [S] the substrate concentration, and [E_0] the total enzyme concentration. The kinetic parameters k_{cat} and K_M were determined from a plot of $V/[E_0]$ vs [S] using a nonlinear least-squares fit of the experimental data. To measure the enzyme activity, the enzyme concentration [E_0] used in all the experiments was at least 1000-fold lower than the substrate concentration [S], to satisfy the quasi-steady-state assumption in Michaelis−Menten kinetics.

The reactions were followed by measuring the increase in absorbance at the wavelength corresponding to the product of each oxidative reaction. The initial reaction rate was obtained by considering the absorbance changes within a few seconds after the oxidant was added. During the oxidation of guaiacol, the reaction led to the formation of a colored product which had an absorption maximum at 470 nm ($\epsilon = 26\,600$ M^{-1} cm^{-1}).[22] The product of oxidation of *o*-phenylenediamine was 2,3-diaminophenazine,[23] which has an absorption maximum at 426 nm and $\epsilon = 29\,000$ M^{-1} cm^{-1}. To reduce the effect of noise in the absorbance reading, it has been found useful to monitor the difference between the absorbance of the reaction product and the absorbance at a wavelength where the absorption remains negligible during the assay ($\lambda = 700$ nm).

(19) Costantino, U.; Nocchetti, M.; Vivani, R. *J. Am. Chem. Soc.* **2002**, *124*, 8428.

(20) Al-Duri, B.; Yong, Y. P. *J. Mol. Catal. B: Enzym.* **1997**, *3*, 177.

(21) Iozzi, I.; Calamai, L.; Fusi, P.; Borsetto, M.; Stotzky, G. *Soil Biol. Biochem.* **2001**, *33*, 1021.

(22) Mahely, A. C.; Chance, B. *Methods Biochem. Anal.* **1954**, *1*, 357.

(23) Tarchia, P. J.; Chu, V. P.; Whittern, D. *Anal. Biochem.* **1987**, *165*, 230.

All UV−vis experiments were performed with a Hewlett-Packard 8452A diode array spectrophotometer.

The kinetic measurements were started by adding fixed amounts of hydrogen peroxide in a 1-cm path length quartz cell containing enzyme and substrate to a constant final volume of solution (3 mL) in 0.2 M phosphate buffer (pH = 6.0) at 25 °C.

In the first set of experiments, hydrogen peroxide was the species considered and its concentration was varied, while the substrate concentration was held constant. The following concentrations were used: for o-phenylenediamine, [Mb] = 1.0 μM, [substrate] = 20 mM, [H$_2$O$_2$] = 0−450 mM; for guaiacol, [Mb] = 1.0 μM, [substrate] = 30 mM, [H$_2$O$_2$] = 0−450 mM.

The conditions of hydrogen peroxide saturation were determined for the two substrates and, in a new series of experiments, the hydrogen peroxide concentration was kept constant while the substrate concentrations were varied. The following concentrations were used: for o-phenylenediamine, [Mb] = 1.0 μM, [H$_2$O$_2$] = 330 mM, [substrate] = 0−20 mM; for guaiacol, [Mb] = 1.0 μM, [H$_2$O$_2$] = 67 mM, [substrate] = 0−60 mM.

5. Enzyme Kinetics for Immobilized Myoglobin. The experimental procedure was similar to that described in part 4. In all the experiments, a fixed amount of supported Mb was added in a cuvette containing the substrate dissolved to a final volume of 3 mL of phosphate buffer (0.2 M, pH = 6.0). The mixture was then vigorously shaken, and the reaction was started by adding hydrogen peroxide. The enzyme matrix was kept outside the light path with stirring and the increase in absorbance per second was monitored.

6. Determination of Mb Activity as a Function of Enzyme Loading. A sample of 10 mg of biocomposite, containing different amounts of protein, was added to 3 mL of guaiacol solution (20 mM in sodium phosphate buffer 0.2 M, pH = 6.0, 25 °C) contained in a cuvette. The oxidation product formed upon the addition of 70 μL of H$_2$O$_2$ (10 M) was monitored at 470 nm. The enzymatic activity was determined using the following formula

$$U = \frac{(\text{absorbance/s})_{470\text{nm}}}{\epsilon c} \tag{4}$$

where $\epsilon = 26.6 \times 10^{-3} \ \mu\text{M}^{-1} \ \text{cm}^{-1}$ and c = micromoles of immobilized Mb/ volume of reaction mixture (l).

7. Stability Assay for Free and Immobilized Myoglobin. The inactivation of Mb by hydrogen peroxide was measured by following the oxidation of guaiacol after having incubated the protein (free or immobilized) in a fixed volume of phosphate buffer solution (0.2 M, pH = 6.0, 25 °C) containing different concentrations of hydrogen peroxide for different times. The reaction was started by adding a fixed volume of 1 M guaiacol in ethanolic solution. The residual activity was expressed in terms of the percentage of the initial activity.

8. Desorption of Mb from the Supports. The possible desorption of protein from the support was tested in the presence of water and phosphate buffer (0.1 M, pH 6.0), and in both cases no significant desorption was observed. The same experiment was carried out with the aromatic substrates used to evaluate the protein activity. The biocomposites were stirred in the presence of guaiacol and o-phenylenediamine (60 mM and 20 mM in phosphate buffer 0.1 M, pH 6.0, respectively) for 3−5 min, and then the mixture was centrifuged. The supernatant was assayed for activity by adding hydrogen peroxide and following the formation of oxidation product with UV−vis spectroscopy.

In the case of guaiacol, no significant traces of activity were observed in any of the biocomposites obtained, showing no desorption of protein from the supports.

In the case of o-phenylenediamine, a partial desorption of protein from the α-ZrCEP support was observed. This effect made it impossible to evaluate the Mb/α-ZrCEP biocomposite activity for this substrate.

Results and Discussion

1. Effect of the Surface Characteristics of Supports on Immobilization. For the sake of clarity, Figure 1 shows a pictorial representation of the layered structures of the supports employed. It may be noted that, in ideal
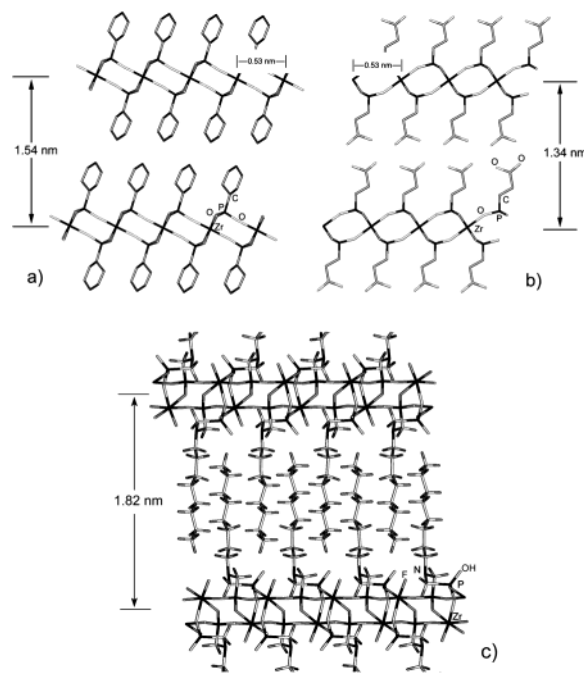

Figure 1. Schematic representation of the sequence of two layers of (a) α-ZrBP, (b) α-ZrCEP, and (c) ZrC8.

conditions, the functional groups anchored on the surface of α-ZrBP and α-ZrCEP form a well-packed monofilm of regularly spaced units of about 5.3 Å each (Figure 1a,b). In the case of ZrC8, the different linkage between ZrO$_6$ octahedra and PO$_3$R tetrahedra creates a thicker layer and the presence of only one organic group for each zirconium atom generates a less compact monofilm of C$_8$H$_{17}$ moiety spaced about 11 Å from each other (Figure 1c).

An attempt was made to immobilize Mb in these zirconium phosphonates. The differences in the chemical structures of the surfaces of the supports should lead to differences in the adsorption of protein. Hydrophobic supports will bind Mb via hydrophobic interactions. In contrast, hydrophilic supports will bind Mb via electrostatic interactions between the protein (which is positively charged at pH < 7.0) and the negative groups of the supports.

Preliminary experiments carried out to determine the conditions for maximum adsorption of Mb at equilibrium (data not shown) showed that these conditions involve 16 h of contact between protein and support. Therefore, the supports were routinely equilibrated with the Mb solutions for 16 h to obtain the equilibrium adsorption isotherms. The protein uptake vs the amount of protein in solution is reported in Figure 2. α-ZrBP took up almost all of the protein present in solution up to 38 mg/g. In the other two cases, the affinity was up to at least 20 mg of protein added per gram, after which the uptake isotherms reached a saturation plateau.

The protein seems to be adsorbed essentially on the external surface of the zirconium phosphonates. X-ray powder diffraction (XRPD) patterns of the biocomposites are identical to those of the original supports; in particular, there is no detectable variation in the interlayer distance.

SEM micrographs show that the shape and size of the microcrystals were almost unchanged after the immobilization procedure of the protein. As an example, parts a and b of Figure 3 show micrographs of α-ZrBP samples as such and of its Mb composite, respectively. In both cases the lamellar shape of the aggregated microcrystal
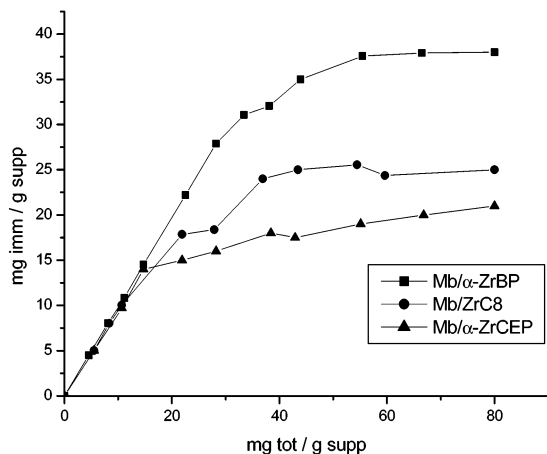
Figure 2. Adsorption isotherms of myoglobin on the indicated supports as a function of the amount of protein added in solution (60 mg of support, 4 mL of protein solution, concentration range 0.1–10 mg/mL, 16 h, room temperature).
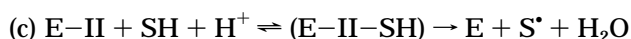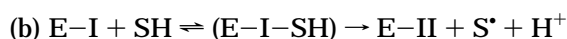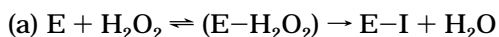
is evident and the immobilization procedure only seems to cause a small fragmentation of the aggregates.

The biocomposites were also examined with HRTEM, in an attempt to detect protein particles in the interlayer region and/or on the surface of the layered microcrystals.

The micrographs show a regular stacking of the layers without the faults that could have arisen if protein particles had effectively inserted in the interlayer region. Moreover, the repeating distance measured on the HR-TEM micrographs is very near to the interlayer distance of the lamellar supports, measured by XRPD. As an example, parts a and b of Figure 4 show the hexagonal morphology of the Mb/ZrC8 composite and the section orthogonal to the sheet, respectively. The measured spacing between the layers (Figure 4b) corresponds to about 2 nm, a value very near 1.82 nm, the interlayer distance of the compound. Attempts to detect protein particles on the surface of the microcrystals failed because the samples rapidly become amorphous under the electronic beam and it is practically impossible to detect an amorphous particle (the protein) on a surface with faults and imperfections that have the same size and shape as the protein itself.

The adsorption isotherms can be described by the Langmuir equation (see eq 1 and Table 1), which suggests the presence of a monolayer of protein molecules on the regular and homogeneous surface of the supports. The amount of protein adsorbed at equilibrium onto α-ZrCEP is lower than that adsorbed onto α-ZrBP and ZrC8, even if the specific surface areas of the supports follow a reverse order (5.2, 8.0, 12.7 m^2/g, respectively). This fact could be explained considering the different degrees of hydrophobicity of the surfaces.

2. Kinetic Studies on Free and Immobilized Myoglobin. The behavior of the immobilized Mb compared with that of free enzyme was studied in terms of redox activity. The oxidation of substrates catalyzed by peroxidases usually follows the scheme[24]

$$\text{(a)} \quad E + H_2O_2 \rightleftharpoons (E-H_2O_2) \rightarrow E-I + H_2O$$

$$\text{(b)} \quad E-I + SH \rightleftharpoons (E-I-SH) \rightarrow E-II + S^\bullet + H^+$$

$$\text{(c)} \quad E-II + SH + H^+ \rightleftharpoons (E-II-SH) \rightarrow E + S^\bullet + H_2O$$

Generally, step c is the rate-limiting step. When the rate of step b is much higher than that of step c, the scheme

can be reduced to a simple bimolecular ping-pong mechanism. The resulting rate equation is[17]

$$V = \frac{k_{cat}[E_0]}{1 + \dfrac{K_M(H_2O_2)}{[H_2O_2]} + \dfrac{K_{M(S)}}{[S]}}$$

where $K_{M(S)}$ contains terms which refer only to step c.

To analyze the primary kinetic data with a Michaelis–Menten equation, the term $K_{M(H_2O_2)}/[H_2O_2]$ must be negligible. This condition is fulfilled when the hydrogen peroxide concentration is high enough to make step a faster than step c. Once the conditions for hydrogen peroxide saturation were set up for the various substrates, the kinetic data were analyzed with the Michaelis–Menten scheme; the data obtained are reported in Tables 2 and 3.

The rates of substrate oxidation by the immobilized Mb showed Michaelis–Menten saturation kinetics (Figure 6), demonstrating that the immobilized protein reacts similarly to Mb in solution (Figure 5). As expected, the K_M and k_{cat} values were significantly affected by the adsorption of Mb onto zirconium phosphonates. The kinetic data, and in particular, the lower k_{cat} value, show that the reaction rate of the immobilized protein is slower than that observed for the same amount of Mb in solution. The loss of activity upon immobilization is the result of a combination of several factors, such as hindrance of accessibility of substrate to the active site, multiple point binding, or partial denaturation of the enzyme. If the immobilization process does not control the orientation of the enzyme to the surface, only a fraction of the immobilized enzyme is properly oriented with its active sites exposed for the reaction, and this results in a lower reactivity of the immobilized protein.

The possibility of multipoint attachment of the enzyme could render it less flexible allowing only a fraction of the immobilized enzyme to be attached to the support in such a way that would allow its active sites to face away from the surface and consequently be accessible to substrate binding.

A higher K_M value was generally observed for the supported proteins, and this indicates a significant reduction in the affinity of the protein for the substrates.[25] The reduction in affinity could be caused by structural changes in the protein conformation induced by the immobilization procedure and by a lower accessibility of the substrate to the active site (as discussed before). Of all the heterogeneous biocatalysts obtained, Mb/α-ZrCEP is the most efficient, probably because the support is the most hydrophilic, having polar groups on the surface.

It has been reported that the enzymes may be attached to the hydrophilic supports at the ε-amino functionality of lysine residues on the proteins.[26] The presence of various lysine residues distributed over the surface of horse Mb[11] could lead to different orientations of the protein with respect to the support. In particular, three residues (Lys-62, Lys-63, and Lys-96) are located near the carboxylic groups of the heme, on the surface area where the substrates approach the active site.

The interactions of these lysines with the carboxylic groups of α-ZrCEP probably reduce the activity of the protein (k_{cat}) but increase its affinity for the substrate

(24) Dunford, H. B. *Heme Peroxidases*; Wiley-VCH: New York, 1999.
(25) Bathia, R. B.; Brinker, C. J.; Gupta, A. K.; Singh, A. K. *Chem. Mater.* **2000**, *12*, 2434.
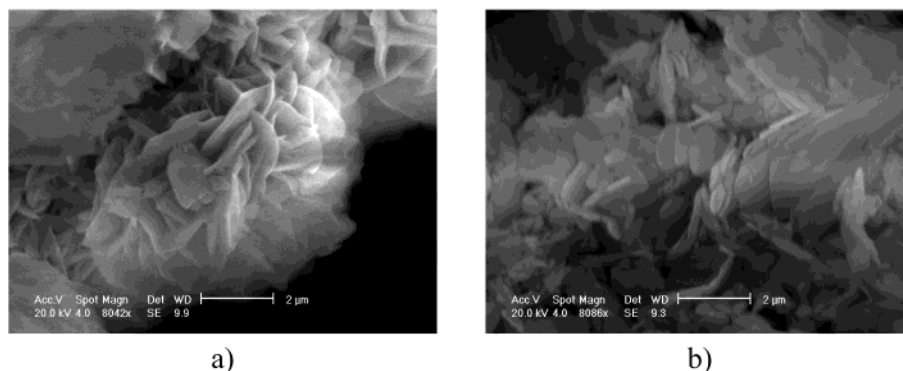(26) Tisher, W.; Wedekind, F. Immobilized Enzymes: Methods and Applications. *Top. Curr. Chem.* **1999**, *200*, 95.

Figure 3. SEM micrographs of α-ZrBP (a) and of its Mb composite (b).
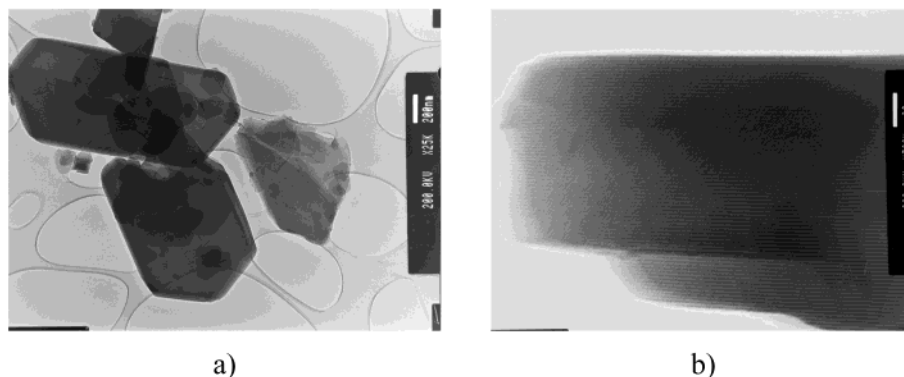

Figure 4. HRTEM micrographs of a sample of Mb/ZrC8 biocomposite showing the hexagonal shape of the microcrystals (a) and the section perpendicular to the *ab* plane that shows the regular packing of the lamellae (b).

Table 1. Fitting of the Adsorption Data of Myoglobin onto Zirconium Phosphonates with the Langmuir and Freundlich Models and Relative Adsorption Parameters (see text for details)

	Langmuir			Freundlich		
system	Q_{MAX} (mg/g of support)	a_L (mL/mg)	r^2	K_F (mg/g of support)/ (mg/mL)$^{-1/n}$	$1/n$	r^2
Mb/α-ZrBP	35.6	0.53	0.86	18.9	0.11	0.61
Mb/ZrC8	22.9	0.48	0.78	9.8	0.15	0.59
Mb/α-ZrCEP	23.1	0.14	0.73	9.0	0.15	0.55

Table 2. Kinetic Parameters for Myoglobin-Catalyzed Oxidation of *o*-Phenylendiamine by Hydrogen Peroxide in 0.2 M Phosphate Buffer, pH = 6.0, 25 °C

biocatalyst	[Mb] (μM)	[H$_2$O$_2$] (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} s^{-1})
free Mb	1.0	330	1.38 ± 0.05	2.2 ± 0.2	$(6.3 \pm 0.8) \times 10^{-1}$
Mb/α-ZrBP[a]	3.3	300	$(3.0 \pm 0.3) \times 10^{-1}$	18.4 ± 4.6	$(1.6 \pm 0.4) \times 10^{-2}$
Mb/ZrC8[a]	6.0	300	$(1.8 \pm 0.3) \times 10^{-1}$	50.8 ± 14.4	$(3.5 \pm 1.0) \times 10^{-3}$

[a] Constants were calculated with an assumption that all the molecules of immobilized myoglobin were active.

Table 3. Kinetic Parameters for Myoglobin-Catalyzed Oxidation of Guaiacol by Hydrogen Peroxide in 0.2 M Phosphate Buffer, pH = 6.0, 25 °C

biocatalyst	[Mb] (μM)	[H$_2$O$_2$] (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} s^{-1})
free Mb	1.0	67	5.5 ± 0.2	14.2 ± 1.6	$(3.9 \pm 0.5) \times 10^{-1}$
Mb/α-ZrBP[a]	4.0	500	$(2.2 \pm 0.3) \times 10^{-1}$	35.8 ± 8.6	$(6.1 \pm 1.6) \times 10^{-3}$
Mb/ZrC8[a]	3.0	67	$(1.4 \pm 0.1) \times 10^{-1}$	1.9 ± 0.7	$(7.2 \pm 2.5) \times 10^{-2}$
Mb/α-ZrCEP[a]	3.0	133	$(1.3 \pm 0.1) \times 10^{-1}$	0.44 ± 0.17	$(2.9 \pm 1.1) \times 10^{-1}$

[a] Constants were calculated with an assumption that all the molecules of immobilized myoglobin were active.

(K_M) so that the catalytic efficiency (k_{cat}/K_M) is similar to that of free Mb. For this reason, α-ZrCEP is the support that immobilizes Mb in the best way. Moreover, the high affinity for the substrate allows the protein to react close to its maximum efficiency even at substrate concentrations as low as 1 mM. Considering that the physiological concentration of aromatic substrates is at the millimolar level, the interest for this type of support is evident.

3. Effect of the Enzyme Loading on the Activity. The effect of enzyme loading on the activity of the immobilized enzyme was studied by recording the performance of the biocatalyst as a function of the amount of Mb adsorbed onto the surface. The activity decreased with the increased protein loading, as is evident from the plot in Figure 7. This result could be due to having very limited control of the orientation and packing of the
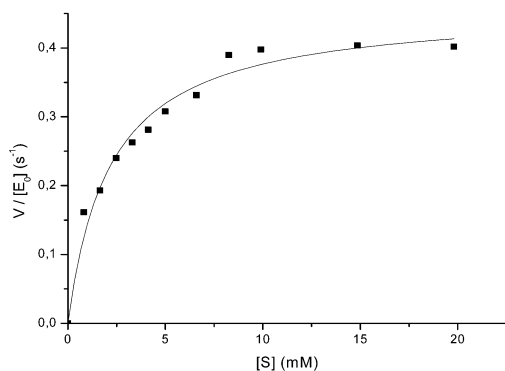
Figure 5. Initial rate of free myoglobin-catalyzed oxidation of *o*-phenylenediamine as a function of substrate concentration ([Mb] = 1.0 μM, [H$_2$O$_2$] = 330 mM).
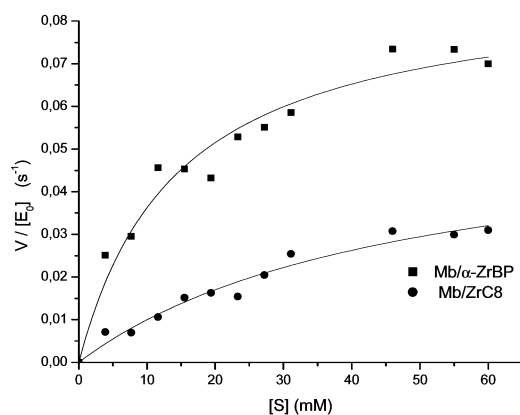

Figure 6. Initial rate of myoglobin immobilized on α-ZrBP and ZrC8 catalyzed oxidation of *o*-phenylenediamine as a function of substrate concentration.
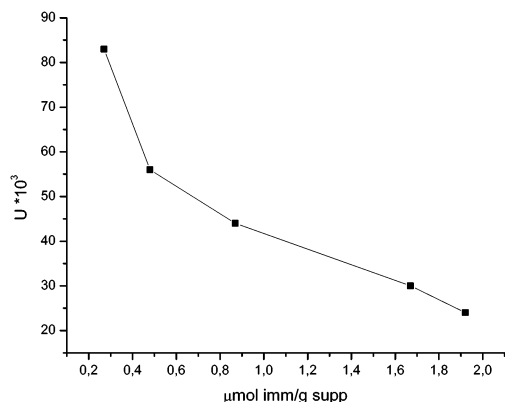

Figure 7. Effect of the protein loading on the activity of the immobilized Mb/α-ZrBP.

biomolecules onto the surface, resulting in the formation of a nonspecific monolayer, which reduces the accessibility of the substrate to the active center of the protein as the amount of adsorbed protein increases.

4. Resistance of Free and Immobilized Myoglobin to Hydrogen Peroxide Inactivation. As in the case of peroxidases, Mb is inactivated by an excess of hydrogen peroxide or by an oxidant in the absence of an oxidizable organic substrate.[27-29] To date, the inactivation mechanism has not been clearly elucidated but it seems to be
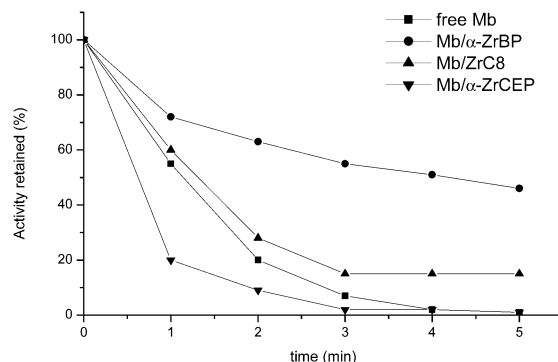
(27) Valderrama, B.; Ayala, M.; Vazquez-Duhalt, R. *Chem. Biol.* **2002**, *9*, 555.

(28) Arnao, M. B.; Acosta, M.; del Rio, J. A.; Varón, R.; Garcío-Cánovas, F. *Biochim. Biophys. Acta* **1990**, *1041*, 43.

(29) Arnao, M. B.; Acosta, M.; del Rio, J. A.; Varón, R.; Garcío-Cánovas, F. *Biochim. Biophys. Acta* **1990**, *1038*, 85.

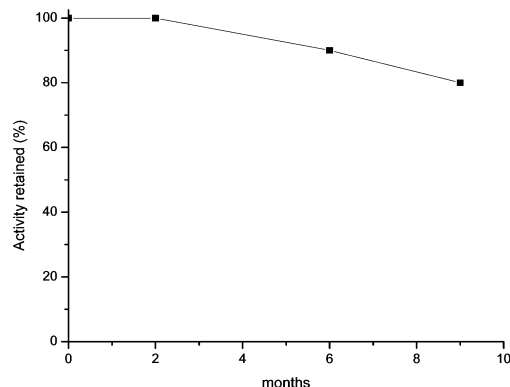**Figure 8.** Inactivation of free and immobilized Mb in the presence of hydrogen peroxide.

Figure 9. Activity of the immobilized Mb/α-ZrBP as a function of time.

correlated with the accessibility of hydrogen peroxide to the active site. This could be determined by both the exposed surface of the heme group and the hydrophobic interactions in the heme pocket.

In general, an increased rate of substrate oxidation is accompanied by a decreased resistance of the protein to hydrogen peroxide inactivation. This correlation suggests that the access of hydrogen peroxide to the active site regulates the oxidation rate and stability toward inactivation, which is probably due to further reactions by the oxidant.[30,31]

Immobilization of Mb on zirconium phosphonates may cause structural alterations of the protein, which could explain the changes of catalytic properties. Therefore, we decided to investigate the effect of immobilization on the stability of Mb toward inactivation by hydrogen peroxide. The results obtained are illustrated in Figure 8. Two supports (α-ZrBP and ZrC8) immobilized Mb in such a way that the protein stability toward hydrogen peroxide increased compared to that of free Mb. Since this probably depends on the reduced accessibility of active sites, Mb/α-ZrBP and Mb/ZrC8 were the least efficient biocatalysts (Table 3).

5. Storage Stability. Stability of the immobilized Mb, stored at 4 °C, was investigated over several months (Figure 9). Only a small drop in the activity occurred over a 9-month period.

Conclusion

Myoglobin adsorption onto zirconium phosphonates has allowed heterogeneous biocatalysts to be obtained

(30) Villegas, J. A.; Mauk, A. G.; Vazquez-Duhalt, R. *Chem. Biol.* **2000**, *7*, 237.

(31) Torres, E.; Sandoval, J. V.; Rosell, F. I.; Mauk, A. G.; Vazquez-Duhalt, R. *Enzyme Microb. Technol.* **1995**, *17*, 1014.

that are active in the oxidation of aromatic substrates by hydrogen peroxide. The immobilization process slightly increases the protein resistance toward inactivation by hydrogen peroxide. Although the driving forces responsible for adsorption are still not well understood, it is possible to assume that for Mb/α-ZrCEP the main contribution is given by electrostatic interactions between lysine residues on the protein surface and the negative charges on the functional groups of the support. In the case of Mb/α-ZrBP and Mb/ZrC8 the main contribution is made by the hydrophobic interactions between the functional groups on the protein surface and the supports. With a better knowledge of the enzyme−solid surface interactions it should be possible to prepare new support materials that are suitable for immobilizing the protein on a specific portion of the surface, such that both the stability of the immobilized protein and the catalytic activity toward exogenous substrates could be increased. Our future work exploring the activity of supported Mb biocatalysts will be developed along these lines.