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High temperature peroxidase activities of HRP and hemoglobin in the galleries of layered Zr(IV)phosphate†

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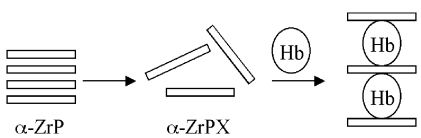
Horseradish peroxidase, and met hemoglobin, when intercalated in the galleries of α-Zr(IV) phosphate, show peroxidase activities at elevated temperatures (86–90 °C) and the rates increased to 2–3.6 times the rates observed at room temperature.

Enzymes are efficient and specific biocatalysts, but their use in chemical reactions is limited due to their fragility, expense, and sensitivity to temperature, pH or organic solvents.[1] Enzymes bound in the galleries of α-zirconium phosphate (α-$Zr(HPO_4)_2{\cdot}nH_2O$, α-ZrP) or other solids can overcome some of these limitations, and the solid support improves the enzyme properties in specific cases.[2] Enzyme binding to solids often results in some loss of activity, and this is due to changes in enzyme structure or conformation. Improved stability of the bound enzyme, however, can more than compensate for this loss.[3] Improved stability of enzymes also facilitates enzyme activities at high temperatures, and high temperature activity of enzymes is attractive for rate acceleration, and for driving endothermic reactions. Here, we present the first report of the high temperature activities of horseradish peroxidase (HRP) and met hemoglobin (Hb) intercalated in the galleries of α-ZrP.

While HRP is important as a catalyst for the oxidation of organic substrates, Hb is an inexpensive model system for HRP peroxidase activity. Hb is an oxygen transport protein[4] and Hb is extensively studied for oxygen storage and extraction. Although Hb does not function as an enzyme in biological systems, its peroxidase activity is well documented.[5] Hb can oxidize phenols, amines, and aromatic hydrocarbons[6] in the presence of hydrogen peroxide, *via* a high valent Fe(IV)oxo intermediate, in a manner similar to HRP.[7] Peroxidase-like activity of Hb was also observed *in vivo*,[8] and this may have significant implications in tissue damage subsequent to injury, stroke, or heart attacks.

Facile intercalation of a number of proteins in the galleries of α-ZrP has been reported from this laboratory,[9] and the thermal stabilities of some of the intercalated proteins are improved.[10] Enzyme binding was accomplished by the delamination of the α-ZrP layers with tetrabutylammonium hydroxide (TBA), followed by the exposure of the exfoliated platelets to the enzyme (Scheme 1). Intercalation of the enzymes in α-ZrP is indicated by the expanded interlayer distances, as monitored in the powder X-ray diffraction studies, and the immobilized enzymes retain structure/activity to a significant extent.[10]



Scheme 1 Exfoliation of the stacks of α-ZrP plates, binding of the protein to the exposed plates, and the reassembly of protein/ZrP matrix.

† Electronic supplementary information (ESI) available: a plot of the amide vibrational frequencies of Hb/α-ZrP as a function of temperature. See http://www.rsc.org/suppdata/cc/b2/b206988a/

Intercalation of HRP and Hb into the α-ZrP galleries was accomplished as reported earlier.[11] Intercalation of Hb into the galleries, for example, increased the *d*-spacings of α-ZrP from 7.6 Å to 65 Å for Hb/α-ZrP, and the layer spacing is consistent with the binding of Hb (Hb diameter 54 Å + the thickness of α-ZrP, 7.6 Å) in the galleries.[11] The UV-Vis, circular dichroism, and attenuated total reflectance FTIR spectra of Hb/α-ZrP agreed with the previously reported data.[1,11] The various spectral data indicate retention of the bound protein structure to a significant extent. Thermal stabilities of the intercalated proteins were examined by following the amide I and amide II vibrational band positions as a function of temperature. These bands are sensitive to protein structural changes, and they provide an important handle to monitor protein denaturation.[12] A plot of the amide vibrational frequencies as a function of temperature indicated improved thermal stabilities of the bound protein (see ESI†) when compared to that of the free protein. The denaturation temperature increased from 73 °C for Hb to 98 °C for Hb/α-ZrP. The improved thermal stability prompted us to monitor enzyme/α-ZrP activities at elevated temperatures.

The high temperature activity of HRP/α-ZrP (2 μM HRP/1 mM α-ZrP, 100 μM guaiacol, 0.5 mM H_2O_2) at 86 °C, and the peroxidase-like activity of Hb/α-ZrP (1 μM Hb/1 mM α-ZrP, 100 μM guaiacol, 0.5 mM H_2O_2) at 90 °C are demonstrated in Fig. 1. The corresponding free HRP or Hb did not show any activity at these temperatures. Peroxidase activities are determined at room temperature as well as at elevated temperatures by following the reported procedure.[13] Guaiacol was used as the substrate and the product formation was monitored as a function of time by following product absorbance at 470 nm. The product absorption spectra recorded at elevated temperatures are superimposable with that observed at room temperature, and both proteins gave the same product spectrum at both temperatures. Furthermore, no product was observed in the absence of H_2O_2, or the protein/enzyme or the α-ZrP matrix at elevated temperatures, confirming the activities of the HRP/Hb bound to α-ZrP at these temperatures.

The specific activity of HRP/α-ZrP is doubled at 86 °C, and the specific activity of Hb/α-ZrP is increased by a factor of 3.6 at 90 °C, when compared to the specific activities measured at room temperature (Table 1). In contrast, free HRP and Hb are

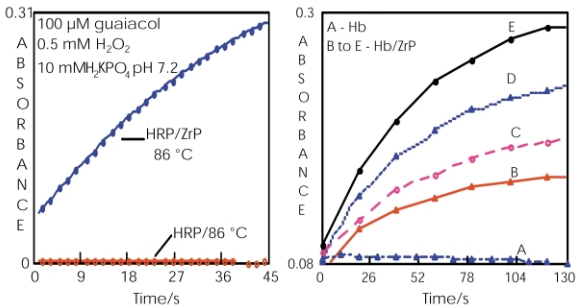


Fig. 1 A (left panel) Peroxidase activity of HRP/ZrP at 86 °C, and the lack of activity of HrP. B (right panel) Peroxidase activity of Hb/ZrP at 90 °C (curves B–E, at increasing concentrations of guaiacol) and the lack of activity of Hb (curve A), under similar conditions.

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Table 1 Catalytic properties of HRP and Hb bound to α-ZrP

Sample	Specific activity (mM s^{-1})		K_m (mol dm^{-3})		V_{max} (μmol dm^{-3} s^{-1})	
Temperature	25 °C	90 °C	25 °C	90 °C	25 °C	90 °C
Hb	0.35	0	100	0	0.03	0
Hb/α-ZrP	0.45	1.6	110	90	0.04	0.13
HRP	3.6	0	—	0	—	0
HRP/α-ZrP	1.8	3.6[a]	—	—	—	—

[a] Measured at 86 °C.

rapidly deactivated at the corresponding elevated temperatures. The enzymatic activities at elevated temperatures, therefore, are due to the protection of the proteins by the galleries of the inorganic layered material. The activity data were collected at 90 °C, at several substrate concentrations, and the data were used to construct the corresponding Lineweaver–Burk plots[14] (Fig. 2). The Michaelis constant K_m and the maximum reaction velocity V_{max} are estimated from these plots (Table 1). Thus, V_{max} is increased 3.2-fold by increasing the temperature to 90 °C, while there was a 20% decrease in the K_m, indicating rate accelerations and enhanced substrate binding.

The retention of protein structure at high temperature was also confirmed from the CD and UV-Vis spectra of the samples monitored at 90 °C. The 222 nm band in the CD spectrum and the soret absorption band at ~410 nm of heme proteins are sensitive to protein secondary/tertiary structural changes.[15] The intensities of the 222, 210 nm bands in the CD spectrum of Hb/α-ZrP, observed at 25 °C, were retained at 90 °C while that of free Hb indicated rapid loss. A plot of the absorption of Hb/α-ZrP at the Soret band (412 nm) indicated retention of the Soret band at 90 °C over an extended period of time, while that of free Hb rapidly decreased due to protein denaturation. To test if the high temperature activity observed here is due to the heme released from the protein, activities of thermally denatured Hb/α-ZrP were monitored, and no activity was detected for these samples, suggesting that the observed activity is not due to the free heme released from the protein. These observations are consistent with the extremely low activities of free heme.[16] The observed activities at elevated temperatures, therefore, are due to intercalated protein/enzyme.

The activities of HRP and Hb at elevated temperatures illustrate improved thermal stabilities of the intercalated proteins. Stabilization of the native state, and destabilization of the denatured state, by the inorganic matrix can account for enhanced thermal stability on a thermodynamic basis. Strong binding of Hb and HRP to α-ZrP have been reported,[11] and

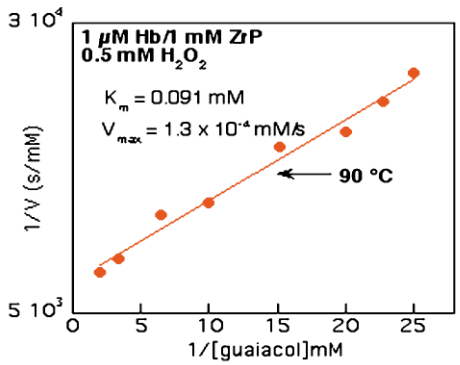

Fig. 2 Lineweaver–Burke plot for Hb/α-ZrP at 90 °C. A similar plot could not be generated for free Hb due to the loss of activity at 90 °C.

these affinities indicate the stabilization of the native state with respect to the solution conditions. The dimer–dimer (αβ) interface of Hb is hydrophobic and its exposure to the ionic matrix is expected to be unfavorable.[17] Additional possible factors contributing to the increased stabilization may include salt-bridge formation with the matrix, and such contacts improve stability in a manner similar to that of the engineered proteins.[18] The rigid galleries of the layered inorganic matrix provide a confined space, and such confinement may impose an additional barrier for protein denaturation.[19] Dehydration of the polar groups and hydration of the nonpolar groups are suggested to play a critical role in protein denaturation,[20] and reduced activity of water in the galleries is also expected to improve protein stability. High temperature activities of enzymes bound to inorganic matrixes, nevertheless, may provide alternatives to enzymes isolated from thermophiles, or specially designed mutant proteins for use in chemistry and molecular biology.

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