Tuning the Properties of Hb Intercalated in the Galleries of α-ZrP with Ionic Strength: Improved Structure Retention and Enhanced Activity

Akhilesh Bhambhani and Challa V. Kumar*

Department of Chemistry, U-3060, University of Connecticut, Storrs, Connecticut 06269-3060

Received September 19, 2005. Revised Manuscript Received December 8, 2005

The influence of ionic strength on the behavior of met-hemoglobin (Hb) bound to layered α-zirconium phosphate (α-ZrP) was investigated. Small increases in the ionic strength (5 mM to 80 mM K_2HPO_4, pH 7.2) indicated large improvements in the bound protein structure, activity, and stability. The number of ZrP units occupied per bound Hb (stoichiometry) increased from 773 to 2000 units. The Soret absorption band and the corresponding circular dichroism (CD) spectra (Soret) indicated that there are considerable improvements in the protein structure around the heme pocket with increase in ionic strength. Monitoring the UV−CD in the 190−250 nm range indicated only minor changes with ionic strength, and some formation of α-helical coiled coils was indicated. The peroxidase-like activity of the bound Hb increased approximately threefold when the ionic strength was increased from 10 mM to 40 mM K_2HPO_4 (pH 7.2). Free Hb did not indicate similar improvements in structure or activity. Differential scanning calorimetric studies showed that bound Hb was denatured over a wide range of temperature and a significant portion of the protein was stabilized by the solid. This study provides a simple method to improve the bound protein properties, and such approaches aid in engineering more effective synthetic materials to maximize bound enzyme function.

1. Introduction

Enzymes and specific biocatalysts with superb chemo-, regio-, stereo-, and chiral selectivities are highly efficient, but their use in chemical transformations is severely limited.[1] This is because enzymes are often expensive, sensitive to pH/temperature, and unstable in organic media.[2] Binding of proteins/enzymes on solid supports can partly overcome these limitations,[3] and in specific cases, such binding improved the enzyme properties to a significant extent.[4] Some advantages of solid-bound enzymes are that they can be readily separated from the reaction mixture by filtration, they can be recycled to lower the cost, or the catalyst can be used in a continuous process, and the catalysis may be carried out in organic solvents. However, binding of enzymes or proteins on solids often results in some loss of activity. The extent of activity loss depends on the solid used, the nature of the protein/enzyme, and the method used for the binding. Therefore, developing methods to improve the bound enzyme activity or protein structure are quite valuable. Such improvements in bound enzyme activity or protein structure is of direct interest in biosensors, protein arrays, and biomedical applications.[5]

The current report focuses on improving the properties of proteins bound in the galleries of inorganic solids. Intercalation of several enzymes and proteins in the galleries of layered α-zirconium phosphate ($Zr(HPO_4)_2 \cdot nH_2O$,[6] abbreviated as α-ZrP, R═OH, Chart 1), was reported earlier.[7] Intercalation resulted in significant retention of enzyme activity and protein structure.[7,8] Binding to α-ZrP did not result in aggregation or spontaneous denaturation of the enzyme/protein. Furthermore, the support matrix permitted access to the bound enzymes by substrates or redox reagents.[9,10] Narrow galleries of α-ZrP/protein materials (47−116 Å,[8] depending on the intercalated protein) restrict access to microbes, and this in turn can inhibit microbial degradation of the bound protein. α-ZrP and its derivatives are chemically

* To whom correspondence should be addressed. E-mail: challa.kumar@uconn.edu.

(1) Scholz, C., Gross, R. A., Eds. *ACS Symposium Series*; American Chemical Society: Washington, DC, 2000; Vol. 764, p 356. *Applied Biocatalysis*, 2nd ed.; Straathof, A. J. J., Adlercreutz, P., Ed.; Harwood: Amsterdam, The Netherlands, 2000. *Biocatalysis*; Abramowicz, D. A., Ed.; Van Nostrand Reinhold: New York, 1990. *Biocatalysis and biomimetics*; Burrington, J. D., Clark, D. S., Eds.; American Chemical Society: Washington, DC, 1989.

(2) Fessner, W.-D. Biocatalysis − From Discovery to Application. *Top. Curr. Chem.* **1999**, 200. Cowan, D. A. *Comp. Biochem. Physiol., A* **1997**, *118*, 429−438.

(3) (a) Gubitz, G.; Kunnsberg, E.; van Zoonen, P.; Jansen, H.; Gooijer, C.; Velthorst, N. H.; Fei, R. W. In *Chemically Modified Surfaces*; Leyden, D. E., Collins, W. T., Eds; Gordon and Breach: London, 1988; Vol. 2, p 129. (b) Gorton, L.; Marko-Varga, G.; Dominguez, E.; Emneus, J. In *Analytical Application of Immobilized Enzyme Reactors*; Lam, S., Malikin, G., Eds.; Blackie Academic & Professional: New York, 1994; p 1.

(4) (a) Monsan, P.; Combes, D. *Methods Enzymol.* **1988**, *137*, 584. (b) Mozhaev, V. V.; Berezin, I. V.; Martinek, K. *Crit. Rev. Biochem.* **1988**, *23*, 235.

(5) Markovic, N.; Ginic-Markovic, M.; Dutta, N. K. *Polym. Int.* **2003**, *52*, 1095.

(6) (a) Clearfield, A.; Stynes, J. A. *J. Inorg. Nucl. Chem.* **1964**, *26*, 117. (b) Alberti, G.; Casciola; Costantino, U. *J. Colloid Interface Sci.* **1985**, *107*, 256.

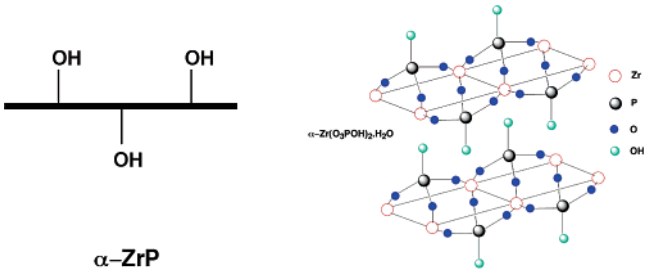
(7) Kumar, C. V.; Bhambhani, A.; Hnatiuk, N. In *Handbook of Layered Materials*; Carrado, K. A., Dutta, P. K., Auerbach, S. M., Eds.; Marcel Dekker: New York, 2004; Vol. II.

(8) Kumar, C. V.; Chaudhari, A. *J. Am. Chem. Soc.* **2000**, *122*, 830.

(9) Rosevear, A.; Kennedy, J. F.; Cabral, J. M. S. *Immobilized Enzyme and Cells*; Adam Hilger: Philadelphia, 1987.

10.1021/cm0521065 CCC: $33.50
Published on Web 01/12/2006

Chart 1. Schematic Representation of α-ZrP[a]



[a] The OH groups are oriented perpendicular to the plane of metal ions.

inert at or below pH 7, and these solids are suitable for enzyme/protein intercalation.[7]

Intercalation of met-hemoglobin (Hb) and horseradish peroxidase (HRP) in the galleries of α-ZrP and its derivatives improved the thermal stabilities of the proteins. For example, Hb/α-ZrP and HRP/α-ZrP catalyzed the oxidation of *o*-methoxyphenol by H_2O_2 (peroxidase activity) at elevated temperatures (>90 °C), while the free enzyme/protein showed no activity at these elevated temperatures.[11,12] It is also noteworthy that the intercalated Hb or HRP underwent reversible thermal denaturation in these solids.[12,13] These interesting properties of the intercalated Hb/HRP samples prompted us to find methods to further improve the retention of structure and/or activity of these biomolecules after intercalation.

In the current studies, the effect of ionic strength on the bound protein behavior is investigated. Binding studies were carried out with phosphate buffer where the phosphate concentration has been increased from 5 to 80 mM K_2HPO_4 (pH 7.2). Increased ionic strength is expected to weaken electrostatic interactions and attenuate protein−protein as well as protein−solid interactions. Current results show that the peroxidase activity of the intercalated Hb is improved severalfold by simply raising the ionic strength. Consistent with this observation, the secondary structure of the protein also showed detectable improvements. Such improvements may form a strong basis for engineering more effective synthetic materials to maximize enzyme function.

2. Experimental Section

Protein Source. Hb (bovine) was purchased from Sigma Chemical Co. and used without further purification. Hb solutions were prepared in potassium phosphate buffer (K_2HPO_4, 5 mM to 80 mM, pH adjusted to 7.2) over a small range of ionic strengths ([*I*] = 10 mM to 195 mM by varying K_2HPO_4 concentration from 5 mM to 80 mM).

α-ZrP Synthesis. This material was prepared according to published procedures,[6] with minor modification. Phosphoric acid (17 g, 172 mmol, 9 M) was added to $ZrOCl_2$ (10 g, 31 mmol) in excess. The reaction mixture was heated at 90 °C for 24 h. The resulting white product was washed twice with 50 mL of water and once with 20 mL of acetone. The solid was dried overnight at 60 °C. The Fourier tranform infrared spectra and the powder X-ray diffraction (XRD) pattern of the sample matched those reported (Table 1).[14]

Table 1. Observed *d* Spacings from the Powder XRD Patterns and Amide I Peak Position (Errors ±0.1%) of Hb/α-ZrP

	d spacing (Å)	protein diameter[14] (Å)	amide I frequency[12] (cm^{-1})
Hb		57	1654
α-ZrP	7.6		
Hb/α-ZrP	63	55.4	1653

XRD Studies. Hb/α-ZrP suspensions (50−100 μL) were spotted on glass slides and air dried overnight. A Scintag model 2000 diffractometer using nickel filtered Cu Kα radiation was used to record the XRD patterns, at a scan rate of 2°/min. The interlayer separations were measured from the 00*l* reflections (l = 1, 2, etc.) using Bragg's law. XRD patterns matched those published earlier (Table 1).[8]

Exfoliation of the α-ZrP and Intercalation of Hb in the Galleries. Exfoliated α-ZrP (2 wt %) suspensions were prepared by adding 0.1 g of α-ZrP suspended in 5 mL of distilled water to stoichiometric amounts of tetrabutylammonium hydroxide (40 wt % in water). The Hb/α-ZrP samples were prepared as reported previously,[8,15,16] but a brief description follows. Exfoliated α-ZrP (2 wt %) suspensions were mixed with stock solutions of Hb (100 μM, 6.45 mg/mL, 5−80 mM K_2HPO_4, pH 7.2) to the desired final concentrations. The mixture was equilibrated overnight and centrifuged to collect the intercalated protein. For spectral and calorimetric studies, the solid was re-suspended in phosphate buffer of desired ionic strength. The XRD pattern of Hb/α-ZrP (Table 1) is in agreement with previous work.[8,11]

Spectral Measurements. The absorption spectra were recorded on a Milton Roy 3000 ARRAY spectrophotometer using 1 cm path length cuvettes. Suspensions of α-ZrP of appropriate concentrations were used in the reference beam to compensate for light scattered by α-ZrP (scatter was <5% above 400 nm). Upon exfoliation with tetrabutylammonium hydroxide, the α-ZrP suspensions became translucent, and these scattered much less light than nonexfoliated suspensions.

Circular Dichroism (CD) Studies. A JASCO model 710 spectropolarimeter was used to record the CD spectra from 190 to 250 nm. Scan rates were 20 nm/minute with step resolution of 0.2 nm/data point. Bandwidth and sensitivity were 1 nm and 20 millideg, respectively. Several scans (8−16) were accumulated for each sample using a optical path length of 0.2 cm. Hb/α-ZrP suspensions were prepared in phosphate buffer (pH 7.2) of different ionic strengths, as described earlier. All Hb/α-ZrP suspensions were diluted as needed for the CD measurements and corrected for light scatter by α-ZrP. This was done by placing an appropriate concentration of α-ZrP, in a separate cuvette, in front of the reference cuvette. This accounted for any minor attenuation of the CD signal due to light scatter by the α-ZrP particles, and flat baselines have been obtained.

(10) (a) Miksa, B.; Slomkowski, S. *Colloid Polym. Sci*. **1995**, *273*, 47. (b) Yoshinaga, K.; Kondo, K.; Kondo, A. *Polym. J*. **1995**, *27*, 98. (c) Tiberg, F.; Brink, C.; Hellsten, M. *Colloid Polym. Sci*. **1992**, *270*, 1188. (d) Yoishinaga, K.; Kito, T.; Yamaye, M. *J. Appl. Polym. Sci*. **1990**, *41*, 1443 (e) Shabat, D.; Grynszpan, F.; Saphier, S.; Turniansky, A.; Avnir, D.; Keinan, E. *Chem. Mater*. **1997**, 9, 2258.
(11) Kumar, C. V.; Chaudhari, A. *Chem. Commun.* **2002**, 2382.
(12) Kumar, C. V.; Chaudhari, A. *Chem. Mater*. **2001**, *13*, 238.
(13) Jagannadham, V.; Bhambhani, A.; Kumar, C. V. *Microporous Mesoporous Mater*. **2006**, *88*, 275−282.
(14) (a) Alberti, G.; Constantino, U. In *Intercalation Chemistry*; Whittingham, M. S., Jacobson, A. J., Eds.; Academic Press: New York, 1982. (b) Alberti, G.; Constantino, U.; Alluli, S.; Tomassini, N. J. *Inorg. Nucl. Chem*. **1990**, *29*, 2112. (c) Dines, M. B.; DiGiacomo, P. M. *Inorg. Chem*. **1981**, *20*, 92.
(15) Kumar, C. V.; Chaudhari, A. *Micorporous Mesoporous Mater*. **2003**, *57*, 181. Chaudhari, A.; Thota, J.; Kumar, C. V. *Microporous Mesoporous Mater*. **2004**, *71*, 251.
(16) Chaudhari, A.; Kumar, C. V. *Micorporous Mesoporous Mater*. **2005**, *77*, 175.

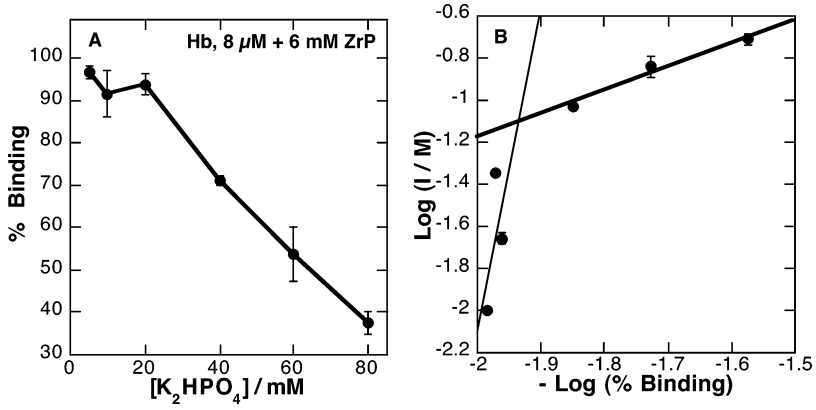


Figure 1. (A) Influence of ionic strength on the binding of Hb to α-ZrP (pH 7.2, room temperature) over a narrow range of ionic strengths (5−80 mM K_2HPO_4). (B) Plot of log(ionic strength) versus −log(% binding) according to the polyelectrolyte theory.

Hb Activity. The peroxidase-like activity of Hb was monitored by a reported procedure,[17] after minor modifications. Stock solutions of Hb (100 μM) and the substrate (*o*-methoxyphenol, 127 mM) were mixed with hydrogen peroxide (50 mM) to the desired final concentration. Control experiments contained no peroxide (diluted with an equivalent volume of phosphate buffer). The product formation was monitored as a function of time by measuring the absorbance of the product at 470 nm. Spectra were recorded every second, for 250 s, and the kinetic traces were constructed. Hb/α-ZrP samples, containing 6−7 μM Hb and 6 mM α-ZrP in phosphate buffer of different ionic strengths were diluted fivefold and mixed with substrate (final concentrion, 12.5 mM) and hydrogen peroxide (final concentration, 5 mM). The activity of free Hb was monitored in a similar way.

Differential Scanning Calorimetric (DSC) Measurements. The DSC experiments were performed on a Calorimetry Sciences Corporation (CSC) 6100 Nano II differential scanning calorimeter, sample cell volume of 0.299 mL, interfaced with a personal computer (IBM-compatible). In a series of DSC scans, both cells were first loaded with buffer, equilibrated at 10 °C for 10 min, and scanned from 10 to 120 °C at a scan rate of 2 °C/min. The buffer versus buffer scan was repeated once more, and upon cooling, the sample cell was emptied, rinsed, and loaded with Hb/α-ZrP suspension. The sample was equilibrated at 10 °C for 10 min prior to the scan.

Upon denaturation, Hb is known to undergo aggregation.[18] As a consequence the heat absorption is altered, and the position of baseline after denaturation becomes uncertain. Hence, a capillary cell and higher rate of heating (2 °C/min) are used in the current studies to reduce the extent of aggregation. Cells were carefully cleaned before each experiment. The samples and reference solutions were degassed for at least 5 min at room temperature and carefully loaded into the cells to avoid bubbles. Typically, the protein/α-ZrP suspensions (phosphate buffer, pH 7.2) at various ionic strengths were scanned against a reference solution from 10 to 120 °C. A constant pressure of 3 atm was maintained to prevent possible degassing of the samples on heating. A background scan recorded with the buffer in both cells was subtracted from each test scan. Control experiments with α-ZrP suspensions (no protein) indicated no evidence of endothermic or exothermic transitions over the entire temperature range studied. The reversibility of thermal transitions was checked by examining the reproducibility of the calorimetric trace in the second heating cycle, immediately after cooling the sample.

The excess molar heat capacity of the sample was calculated using the mean molecular mass of 64.5 kDa of Hb and the partial specific volume of the protein. This latter quantity was calculated to be 0.73 mL/g from the known amino acid sequence of Hb by a reported method.[19] Each thermodynamic value reported here is an average of at least three separate measurements.

3. Results

The influence of ionic strength on the bound protein behavior at the solid−liquid interface was examined over a small range of ionic strengths (5−80 mM K_2HPO_4, pH 7.2, room temperature). Even small increases in the ionic strength improved the structure and activities of the intercalated protein to a significant extent. Our observations are the following.

Binding Affinities. The amount of protein adsorbed on a solid is affected not only by the properties of protein and the solid surface but also by the environmental conditions such as pH, ionic strength, and temperature.[20] Electrostatic interactions are expected to play a minor role in the binding of Hb at pH 7.2, because Hb is expected to be nearly neutral or slightly negatively charged (isoelectric point (pI) = 6.8) at this pH. Increased ionic strength is expected to decrease this charge repulsion, if any, with the negatively charged α-ZrP. The binding, therefore, is not likely to be effected by ionic strength,[21] and only minor changes are anticipated.

Binding of Hb to α-ZrP was carried out by contacting Hb solutions (8 μM) at increasing ionic strengths (5−80 mM K_2HPO_4, pH adjusted to 7.2). The mixture was equilibrated overnight and centrifuged to collect the intercalated protein, and the amount of bound protein was assayed by monitoring Soret absorbance at 412 nm (Figure 1A). The *y* scale on Figure 1 represents the percent protein bound out of the total protein available and percent binding = {[Hb bound]/[total Hb]} × 100. Initial additions of the electrolyte (up to 20 mM) had little or no influence on the extent of binding. Further increase in the ionic strength resulted in rapid decrease in the amount of protein bound to the solid.

The initial plateau region, where the binding did not depend on the ionic strength, clearly illustrates that the

(17) Maehly, A. C.; Chance, B. *Methods Biochem. Anal.* **1954**, *1*, 357.
(18) Sochava, I. V.; Belopolskaya, T. V.; Smirnova, O. I. *Biophys. Chem.* **1985**, *22*, 323.
(19) Perkins, S. J. *Eur. J. Biochem.* **1986**, *157*, 169.
(20) Kazuhiro, N.; Sakiyama, T.; Imamura, K. *J. Biosci. Bioeng.* **2001**, *91* (3), 233.
(21) Ramsden, J. J. *Chem. Soc. Rev.* **1995**, *24*, 73.

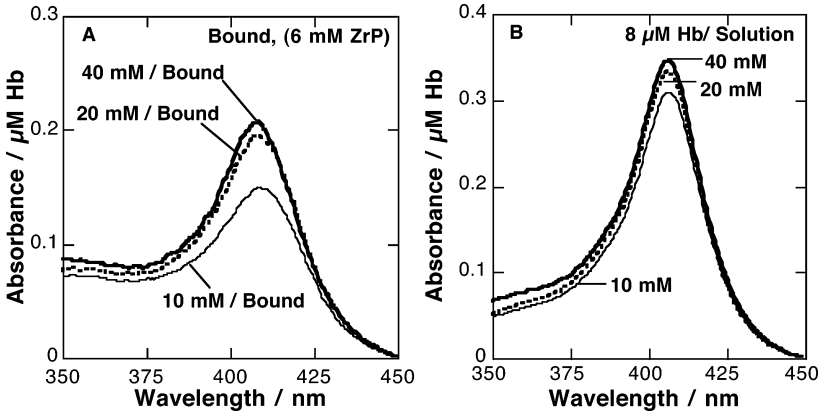


Figure 2. Effect of ionic strength on the Soret absorbance (409 nm), at 10 mM (thin continuous line), 20 mM (thick dotted line), and 40 mM (thick line) K_2HPO_4, pH 7.2, of (A) bound Hb (6−8 μM)/α-ZrP (6 mM) and (B) free Hb (8 μM).

binding in this range of ionic strength is dominated by nonelectrostatic interactions. Because pI of Hb is 6.8, its overall charge is expected to be negative at pH 7.2. Therefore, we suspect that a substantial component of binding is driven by the dehydration of the macromolecular surfaces that are brought together. Binding may also be driven by gain in conformational entropy from some unfolding of the bound protein. The calorimetric titrations (10 mM K_2HPO_4, pH 7.2, room temperature) showed that Hb binding to α-ZrP, at least at low ionic strengths, is accompanied by decreases in entropy.[16] The strong role of electrostatic interactions at moderate ionic strengths (40−80 mM K_2HPO_4) is clear from the current data.

The number of ZrP units per bound Hb (stoichiometry) are estimated from these data, and they are 773, 820, 800, 1060, 1395, and 2000 for 5, 10, 20, 40, 60, and 80 mM K_2HPO_4 (pH 7.2), respectively. When the footprint of each phosphate group in α-ZrP galleries was taken as 24 Å,[2,22] the average area occupied by Hb was calculated from these stoichiometries. The average area occupied per Hb increased from 15 700 Å^2 at 5 mM K_2HPO_4 to ∼40 500 Å^2 at 80 mM K_2HPO_4.

The data are analyzed further by plotting log(I) versus −log(% binding) (Figure 1B), according to the polyelectrolyte theory.[23] The data were fitted to the equation $\log(K) = -n'\psi \log [I] + \delta \log K_{1M}$, where $n'\psi$ is the number of monovalent counterions released as a result of the binding of Hb to a site containing n' phosphates on the solid, ψ is the extent of counterion binding to the solid prior to Hb binding, and K_{1M} is the binding constant at 1 M ionic strength.[24] The binding constant is expressed in terms of the percent Hb bound, and the data are analyzed. Biphasic behavior of the binding with ionic strength is clearly illustrated in Figure 1B. The slopes and intercepts of the linear fits to the two regions of the data are distinct, and these indicated the release of 15 (thin line) and 1.1 (thick line) counterions per Hb. At higher ionic strengths, a larger number of counterions are released. These ionic strength data clearly illustrate the role of electrostatic interactions in the binding even at moderate ionic strengths. Binding at a pH near the pI of the protein is expected to be nearly independent of ionic strength,[25] and the current observations are interesting.

Another important observation in the above experiments was that the time required for achieving the binding equilibrium also increased with ionic strength (1 h @ 5 mM vs 24 h @ 80 mM K_2HPO_4, pH 7.2). As the ionic strength increases, the surface potential of α-ZrP (negative) becomes smaller as a result of counterion binding, and this results in a decreased rate for the binding of the oppositely charged ions. Also, an increase in ionic strength promotes binding of counterions to Hb, and this can lower the net charge on Hb (pI = 6.8). These factors contribute to slower adsorption at higher ionic strengths when two binding surfaces are oppositely charged. One possibility is that positively charged patches on Hb interact with the oppositely charged α-ZrP. In such a case, the increased ionic strength is expected to weaken the protein−solid interactions. Such weaker interactions with the solid are expected to result in less distortion of bound protein structure,[26] which was examined next.

Soret Absorption. The Soret absorption band of Hb ($\epsilon_{410\ nm}$ = 303 960 M^{-1} cm^{-1}) is a sensitive measure of the protein structure surrounding the heme moiety, and it is also sensitive to changes in the coordination environment of the heme iron. Therefore, the Soret band was used to monitor the bound Hb structure as a function of ionic strength.[27] The protein intercalation was carried out at increasing ionic strengths, and the Soret absorption spectra of Hb/α-ZrP (∼7 μM Hb/6 mM ZrP, at increasing ionic strengths, pH 7.2) have been recorded (Figure 2). It is clear from the Soret absorption spectra that the intensity of this transition increases steadily with ionic strength. Note that the effect of ionic strength on the Soret absorption of the free Hb is quite small (Figure 2B). Therefore, the major portion of the changes

(22) Clearfield, A. *Inorganic Ion Exchange Materials*; CRC Press: Boca Raton, FL, 1982.
(23) Manning, G. S. *J. Chem. Phys.* **1969**, *51* (3), 924.
(24) (a) Manning, G. S. *Q. Rev. Biophys.* **1978**, *11*, 179−246. (b) Record, M. T., Jr.; Anderson, C. F.; Lohman, T. M. *Q. Rev. Biophys.* **1978**, *11*, 103−178. (c) Barton, J. K.; Goldberg, J. M.; Kumar, C. V.; Turro, N. J. *J. Am. Chem. Soc.* **1986**, *108*, 2081−2088.
(25) Ramsden, J. J.; Prenosil, J. E. *J. Phys. Chem.* **1994**, *98*, 5376.
(26) Nakanishi, K.; Sakiyama, T.; Imamura, K. *J. Biosci. Bioeng.* **2001**, *91* (3) 233.
(27) Acampora, G.; Hemrans, J. *J. Am. Chem. Soc.* **1967**, *89*, 1543.

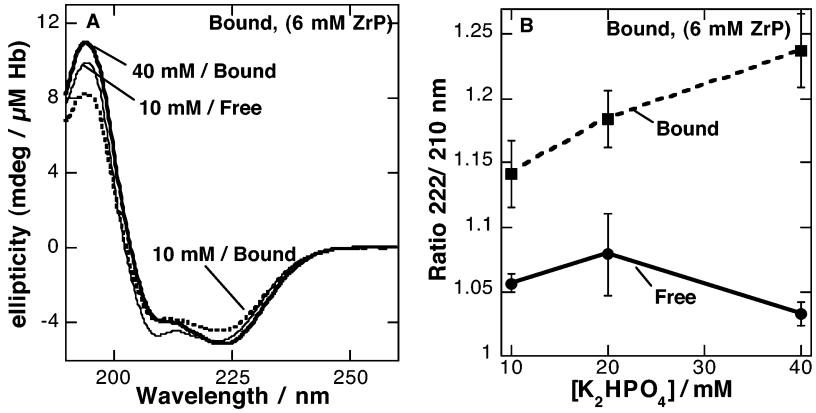


Figure 3. Effect of ionic strength on (A) secondary structure of bound protein (Hb, 6−8 μM/ZrP, 6 mM). The spectra are normalized with respect to the concentration of the bound protein. (B) Plot of the ratio of the molar ellipticities at 210−222 nm as a function of ionic strength for the free and bound Hb. Changes in the secondary structure of the immobilized protein depend on ionic strength.

noted in Figure 2A are due to the effect of the ionic strength during the adsorption process and/or ionic strength effect on the adsorbed protein. This conclusion is further investigated by examining the protein secondary structure in CD studies.

CD Studies. The CD spectra of proteins in the 190−250 nm region are used to examine protein secondary structure. For example, α-helices exhibit double minima at 210 ($\pi-\pi^*$ transitions) and 222 nm (n−π^* transitions), whereas a single, broad, negative peak at 212 nm is characteristic of β sheets. Random coils show a strong, sharp, negative peak at 195 nm, and these three types of common protein secondary structures can be readily distinguished in CD studies.[28] In addition to the peak positions, the ratio of the intensities at 222 to those at 210 nm is indicative of the helix−helix interactions. While the molar ellipticity at 222 nm ($[\theta]_{222}$) is related to the helical content, the intensity of the 210 nm band is sensitive to whether the α-helix is monomeric or engaged in tertiary contacts with other helices/sheets. Therefore, the $[\theta]_{222}/[\theta]_{210}$ ratio corresponds to the degree of coiling of the helices.[29,30] Furthermore, a comparison of the CD spectra of the free and bound proteins can provide valuable insight about the extent of structure retention after adsorption. Such studies have already been reported earlier.[31,32]

The CD spectra of Hb/α-ZrP prepared at increasing ionic strengths are shown in Figure 3, and these indicate only minor changes. The observed spectrum at 10 mM K_2HPO_4 is superimposable with that of free Hb (Figure 3A), and it is in excellent agreement with previous reports.[8,15] In addition to the excellent retention of the secondary structure, after intercalation, the spectra did not undergo considerable changes with increased ionic strength.

The ratio $[\theta]_{222}/[\theta]_{210}$ increased with ionic strength for bound Hb, and similar data for the free Hb are also shown here (Figure 3B). The ratio, a characteristic of the degree of coiling, increased from 1.14 (±0.026) to 1.24 (±0.028), which suggests that the ionic strength promotes some improvement in the inter-helical contacts between the helices.[30] Such changes are not noted with the free Hb. Thus, only minor changes in the secondary structure of the bound Hb are evident, and some improvement in the degree of coiling may also be present (Figure 3B). This was surprising, given the substantial changes noted in the binding stoichiometries and the Soret absorption band. Therefore, we examined the CD spectra associated with the Soret absorption of Hb/α-ZrP to further confirm the above-suggested environmental changes around the heme cofactor.

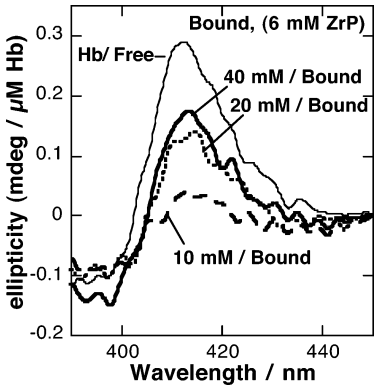


Figure 4. Soret CD of the Hb/α-ZrP (Hb, 6−8 μM/α-ZrP, 6 mM) as a function of ionic strength using a 0.2 cm cuvette. Changes in the tertiary structure of the Hb/α-ZrP, as it depends on ionic strength, is evident from the Soret CD of immobilized protein.

The environment around the heme is important in controlling its catalytic activity, and, hence, changes in the Soret CD of the Hb/α-ZrP are monitored as a function of ionic strength (Figure 4). These indicated significant improvements in the Soret band intensities, as a function of increased ionic strength. In contrast, there is little or no effect of ionic strength on the Soret CD of the free Hb (Supporting Information, S1). Thus, the above data (Figures 1−4) show that the bound Hb structure, particularly around the heme, can be improved significantly by simply raising the ionic strength. These improvements in the structure suggested that similar enhancements in the activity of bound Hb can be expected with increasing ionic strength. Hence, the activities of the bound Hb are tested as a function of ionic strength.

(28) (a) Woody, R. W. In *Circular Dichroism and Conformational Analysis of Biomolecules*; Fasman, G. D., Ed.; Plenum Press: New York, 1996; pp 25−27. (b) Gratzer, W. B.; Cowburn, D. A. *Nature* **1969**, *222*, 426
(29) Cooper, T.; Woody, R. W. *Methods Enzymol.* **1990**, *240*, 478.
(30) Zhou, N. E.; Kay, C. M.; Hodges, R. S. *Biochemistry* **1992**, *31*, 5739.
(31) Johnson, W. C., Jr. *Annu. Rev. Biophys. Biophys. Chem.* **1988**, *17*, 145.
(32) Johnson, W. C., Jr. *Proteins* **1990**, *7*, 205.

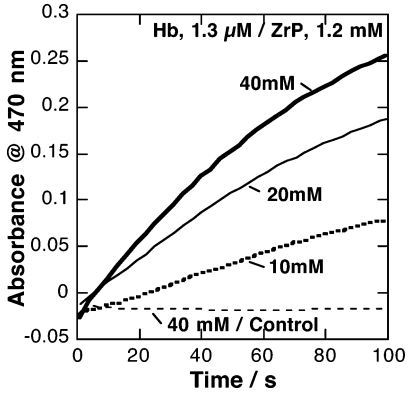


Figure 5. Activities of Hb (1.3 μM)/α-ZrP (1.2 mM) in the presence of 10 mM (thick dotted line), 20 mM (thin continuous line), and 40 mM K_2HPO_4, pH 7.2 (thick continuous line), were followed in the presence of guaiacol (12.5 mM) and hydrogen peroxide (5 mM). The control experiment contained Hb (1.3 μM)/α-ZrP (1.2 mM) in 40 mM K_2HPO_4, pH 7.2 (12.5 mM guaiacol, thin dashed line), but no peroxide.

Activity Studies. Although Hb is not an enzyme, its ability to oxidize a number of biological substrates, in the presence of hydrogen peroxide, is well-known.[17] Hb catalyzes the oxidation of *o*-methoxyphenol (guaiacol) by hydrogen peroxide, and the resulting colored product has an absorption maximum at 470 nm, which provides a convenient handle to examine Hb's catalytic activity.[33]

Activities of bound Hb in 10, 20, and 40 mM K_2HPO_4, pH 7.2, have been followed using this approach (Figure 5), and these indicated rapid increases in the rate of the product formation as well as increases in the total product yield (absorption at 100 s). The initial rate (calculated from the first 50 data points) was 1.2×10^{-3}/s at 10 mM K_2HPO_4. This value increased to 3.5×10^{-3}/s when the ionic strength was raised (40 mM K_2HPO_4, pH 7.2, Figure 5). Over this small range of ionic strengths, the rate was improved by a factor of 3. This increase in the rate with ionic strength is consistent with increases in the intensities of the Soret absorption and Soret CD bands of the bound Hb. Similar ionic strength studies with free Hb did not show appreciable improvements in rates or the total product yields (data not shown).

DSC Studies. To further quantify the influence of ionic strength on the bound Hb properties, we examined the thermal denaturation of Hb/α-ZrP in DSC studies. DSC provides the most direct experimental data to resolve the energetics of protein denaturation,[34] and it allows continuous measurement of apparent specific heat of a system as a function of temperature.[34] It is well-known that mutation of a single residue in a protein can have a dramatic effect on its thermal stability.[34,35]

The thermal stability of the bound protein was investigated by DSC at increasing ionic strengths. First, the DSC profile of free Hb (10 mM K_2HPO_4 buffer, pH 7.2, no α-ZrP) is shown in Figure 6A. Analysis of these data, by following published methods, resulted in the thermal denaturation parameters for free Hb (Table 2), and these parameters are in agreement with reported values.[34,36] Furthermore, the denaturation parameters of the free Hb indicated a weak dependence on ionic strength of the medium (Table 2, Supporting Information, S2).

The DSC thermograms of Hb/α-ZrP (6.3 μM Hb/6 mM ZrP, in the presence of 10 and 40 mM K_2HPO_4, pH 7.2) are shown in Figure 6B. The DSC profiles of Hb/α-ZrP are also endothermic similar to that of free Hb, but these are quite distinct. The profiles of the bound Hb are broadened extensively, and the protein unfolding (denaturation) begins at or around 45 °C, much earlier than that of the free Hb. But the endothermic transition of the bound protein continued up to 120 °C, which is well beyond the complete denaturation point of free Hb. The peak maxima for the bound and free proteins are comparable, and a significant fraction of the bound protein denatures above this temperature. Furthermore, the overall area under the DSC thermogram increased substantially at higher ionic strength (40 mM K_2HPO_4, pH 7.2).

The enthalpies for the thermal denaturation of Hb/α-ZrP were estimated from these data by integrating the areas under the curves in Figure 6B. Note that ΔH is model independent, and its evaluation does not require that the thermal transition be reversible. The corresponding enthalpy changes for all the DSC profiles recorded are collected in Table 2. Note that the enthalpy changes estimated for the bound protein are significantly higher than those of the free protein (at all ionic strengths), and this is a significant improvement in raising the thermodynamic stability of the bound protein. The enthalpy changes of the bound Hb also indicated some dependence on the ionic strength, but this variation is well within our experimental error. Enhanced enthalpy changes of the bound Hb can be attributed, at least in part, to the protection offered by the solid matrix as a physical barrier to denaturation.

Extensive broadening of the thermal transitions of the bound Hb deserves some comment. Multipoint attachment of the protein to the solid via H-bonding, heterogeneity in the structures of the surface bound protein, and aggregation accompanying the denaturation at the solid are some of the factors that can account for the observed broad transition. The conformational heterogeneity of a protein often results in broadening of its thermal transition.[37−39]

Furthermore, the reversibility of the Hb/α-ZrP thermal denaturation was checked by cooling the sample to 10 °C, after the first cycle of heating, followed by heating in the second cycle. No endotherm was detectable in the second cycle, and this clearly indicated that Hb denaturation is irreversible. This was the case with both the free Hb and

(33) (a) Waks, M.; Yon, J.; Morett, J.; Jayle, F. *Biochim. Biophys. Acta* **1963**, *67*, 417. (b) Hayashi, T.; Hitomi, Y.; Ando, T.; Mizutani, T.; Hisaeda, Y.; Kitagawa, S.; Ogoshi, H. *J. Am. Chem. Soc.* **1999**, *121*, 7747.
(34) Jelesarov, I.; Bosshard, H. R. *J. Mol. Recognit.* **1999**, *12*, 3.
(35) Culajay, J. F.; Blaber, S. I.; Khurana, A.; Blaber, M. *Biochemistry* **2000**, *39*, 7153.
(36) Cho, K. C.; Choy, C. L. *Biochim. Biophys. Acta* **1980**, *622*, 320.
(37) Horbett, T. A.; Brash, J. L. Proteins at interface; Current issues and future prospects. In *Physicochemical and Biochemical Studies*; Horbett, T. A., Brash, J. L., Eds., American Chemical Society: Washington, DC, 1987; pp 1−33.
(38) Lenk, T. J.; Horbett, T. A.; Ratner, B. D.; Chittur, K. K. *Langmuir* **1991**, *7*, 1755.
(39) Steadman, B. L.; Thompson, K. C.; Middaugh, C. R.; Matsuno, K.; Vrona, S.; Lawson, E. Q.; Lewis, R. V. *Biotechnol. Bioeng.* **1992**, *40*, 8.

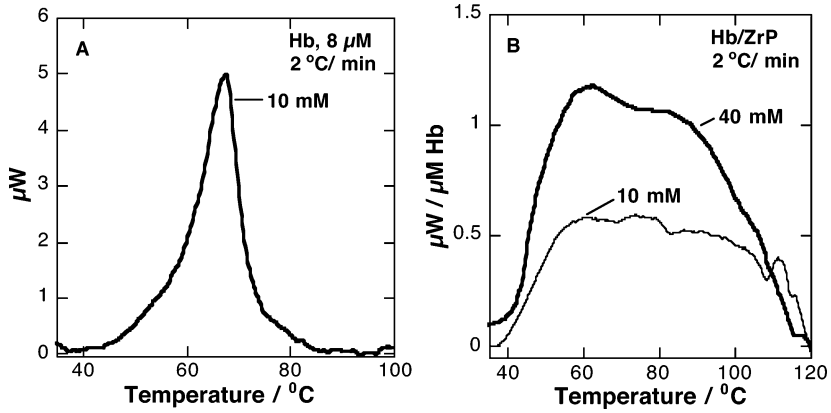


Figure 6. (A) DSC profile of free Hb (8 μM Hb, 10 mM K_2HPO_4, pH 7.2) indicated a sharp transition around 67.2 °C. (B) The DSC thermograms for Hb (6.5 μM)/α-ZrP (6 mM) in the presence of 10 and 40 mM K_2HPO_4, pH 7.2. The DSC data were normalized with respect to the concentration of Hb.

Table 2. Thermodynamic Parameters for Hb and Hb/α-ZrP in 10, 20, and 40 mM Phosphate Buffer, pH 7.2, As Obtained from DSC[a]

	$[K_2HPO_4]$ (mM)	ΔH (kcal/mol)	ΔS (kcal/K·mol)	curve maximum (°C)	[Hb]/[ZrP]
Hb	10	152 ± 2	0.447 ± 0.005	67.2 ± 0.2	8 μM
Hb/α-ZrP	10	510 ± 60	1.553 ± 0.35	55.05 ± 0.35	6.3 μM/6 mM
Hb	20	203 ± 4	0.596 ± 0.014	67.1 ± 0.2	8 μM
Hb/α-ZrP	20	545 ± 35	1.587 ± 0.042	55.5 ± 2.82	7 μM/6 mM
Hb	40	211 ± 4	0.621 ± 0.012	65.7 ± 0.16	8 μM
Hb/α-ZrP	40	503 ± 15	1.523 ± 0.05	56.8 ± 0.67	6.3 μM/6 mM
Hb/α-ZrP	40	518 ± 21	1.574 ± 0.11	57.2 ± 0.6	6.5 μM/12 mM

[a] Each value represents the average of at least three experiments.

bound Hb, on these time-scales. The DSC data provide model independent thermodynamic parameters including ΔH, ΔC_p, T_m, and the full-width at half-maximum of the peak. These parameters are reproducible in the current data set, and their evaluation does not require reversibility of the transition. Evaluation of model dependent parameters such as ΔS, however, requires reversibility of the transition. Early work has demonstrated that Hb bound to various layered materials re-folds,[12] after thermal denaturation over long time periods. Therefore, the thermal denaturation noted here is, at the least, partially reversible. Even so, many authors agree that data from irreversible transitions are usable to estimate the thermodynamic parameters such as ΔS and ΔG.[39,40] The DSC data, therefore, were used to estimate the thermodynamic parameters ΔS and ΔG (Table 2), as practiced in the literature.[40,41]

4. Discussion and Conclusions

The binding of proteins to solids has been known for decades.[42] However, general guidelines for the binding of proteins to solids such that they retain structure, maintain significant activity, and exhibit improved stability are still not clear. Our understanding of protein−solid interactions is rudimentary, and quantitative thermodynamic studies on protein−solid interactions, conducted at well-defined solid surfaces, are still sparse. Stabilization of the native state, and destabilization of the denatured state, relative to corresponding native/denatured states in solution, is expected to increase the thermodynamic stability of the bound protein, yet it is not clear how to achieve this goal. It is clear that binding of proteins to solids often results in diminished activities, and there are no general methods to improve the bound enzyme activities. Current data show that ionic strength of the medium plays an important role in the binding, and this is a simple method to improve the bound protein structure, activity, and stability.

Despite the complexity of the binding process, a random sequential model for protein adsorption focuses on two particularly important features: (1) irreversibility of binding on experimental time scales and (2) the dependence of the binding rate on the type of solid and ionic strength, as well as blockage from previously bound protein.[43] The transport and/or amount of protein bound at the surface and its variation with ionic strength can be explained by various factors such as diffusivity, surface potential, interfacial potential, net charge on the protein, area occupied per protein, number of counterions released per protein bound, and so forth.[25] The diffusivity of Hb in the present experiments is practically constant, and even at the most dilute buffer concentrations, the electrolyte is in a 1000-fold molar excess over the protein. If the ionic strength induces major conformational changes, then this may result in an increase in the surface area occupied per protein, but current data show that the structure and conformation of the free protein are not altered by ionic strength over this small range. Hence, current

(40) Galisteo, M. L.; Pedro, L. M.; Sanchez-Ruiz, J. M. *Biochemistry* **1991**, *30*, 2061.
(41) Steadman, B. L.; Trautman, P. A.; Lawson, E. Q.; Raymond, M. J.; Mood, D. A.; Thompson, J. A.; Middaugh, C. R. *Biochemistry* **1989**, *28*, 9653.
(42) (a) Kasemo, B. *Biol. Surf. Sci.* **2002**, *500*, 656. (b) Lvov, Y.; Mohwald, H. *Protein Architechture; Interfacing Molecular Assemblies and Immobilization Biotechnology*; Marcel Dekker: New York, 2000. (c) Chaga, G. S. *J. Biochem. Biophys. Methodol.* **2001**, *49*, 313. (d) Nakanishi, K.; Sakiyama, T.; Imamura, K. *J. Biosci. Bioeng.* **2001**, *91*, 233.
(43) Talbot, J.; Tarjus, G.; Van Tassel, P. R.; Viot, P. *Colloids Surf.* **2000**, *165*, 287.

observations indicate that such conformational changes in the free protein, if any, are not responsible for the improved properties of the bound protein with increased ionic strength.

At any given ionic strength, the structure, activity and stability of the bound protein are influenced to a large extent by protein−solvent, protein−solid, and protein−protein interactions. Ionic strength can modulate the electrostatic component of these interactions. Ionic strength may alter the amount of protein bound and even influence the orientation of the protein as it binds to the solid.[44,45] The so-called "soft" proteins such as Hb have low internal stabilities, and they are generally known to adsorb on numerous surfaces, irrespective of electrostatic interactions. This binding is attributed to gain in conformational entropy from some unfolding of the protein and/or dehydration of the macromolecular surfaces that are brought together during binding.[46,16] Binding of Hb to α-ZrP (10 mM K_2HPO_4), however, involved a overall decrease in entropy ($\Delta S = -77 \pm 8$ eu, $\Delta H_1 = -31.0 \pm 2.5$ kcal/mol).[15] Current data show that there are subtle conformational changes occurring at the heme binding pocket, under these conditions.

The structure of bound Hb at low ionic strengths (10 mM phosphate) is perturbed to a significant extent (Figures 2, 3, and 4). But this perturbation diminishes rapidly with ionic strength, and binding at moderate ionic strengths preserves protein structure and improves its activity to a significant extent. Data from previous studies[15] showed that Hb is not randomly oriented in the galleries of α-ZrP, and at least a significant fraction of the tetramer binds with its long axis oriented nearly perpendicular to the surface. This orientation requires the interaction of a large patch of the positively charged region of the tetramer with the solid. Current ionic strength studies clearly support these earlier conclusions, and the binding interactions are modulated by the ionic strength of the medium.

Dibasic potassium phosphate, K_2HPO_4, is a salting-out electrolyte; that is, an increase in the concentration of K_2HPO_4 results in its preferential exclusion from the proteins (preferential hydration).[47,48] This decreases the solubility of protein in the aqueous phase, and this may also promote protein binding to the solid. Moreover, as a rule, the conformations of globular proteins are stabilized (but not always) by salting-out salts.[47] Despite these expectations, note that the structure of the free Hb was not altered to a significant extent over this small range of ionic strength, but the bound protein was influenced to a larger extent (Table 2).

At higher ionic strengths, Hb binding to α-ZrP is modulated by ionic strength. A significant change in the surface pH, which differs from the bulk pH,[25] or a considerable decrease in the surface potential due to enhanced counterion condensation at the solid and a net decrease in the protein charge due to counterion binding to protein can account for the reduction in the binding of Hb with increased ionic strength. This reduction can result in enhanced protein−protein distances and decreased protein−protein interactions at the solid. At any rate, the increased ionic strength resulted in enhanced secondary structure, enhanced Soret absorption, improved retention of the environment around the heme, and considerable increment in peroxidase-like activity. These are welcome changes for biocatalytic applications.

The DSC data (Figure 6) show that the denaturation enthalpy is increased at least threefold upon binding to the solid. This can be viewed as increased resistance to denaturation, at least for a significant fraction of the bound protein. Current studies clearly show that binding of Hb to the solid improves the overall stability of a significant fraction of the bound protein. As binding of Hb to the solid is spontaneous (exoergonic, $\Delta G < 0$), the native state is stabilized. Binding also increased the energy gap between the native and denatured states. Therefore, α-ZrP is an appealing matrix for the stabilization of Hb. This study clearly demonstrates the use of ionic strength to fine-tune the protein−solid interactions, and such approaches are useful for the design of more effective methods to maximize enzyme function.

Acknowledgment. We thank the National Science Foundation (DMR-0300631) for the financial support of this work.

Supporting Information Available: Soret CD of free Hb (8 μM) and molar heat capacity of free Hb (8 μM) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

CM0521065

(44) Zoungrana, T.; Findernegg, G. H.; Norde, W. *J. Colloid Interface Sci.* **1997**, *190*, 437.
(45) Baron, M. H.; Revault, M.; Moinville, S. S.; Abadie, J.; Quiquqmpoix, H. *J. Colloid Interface Sci.* **1999**, *214*, 319.
(46) Bos, M. A.; Shervani, Z.; Anusiem, A. C. I.; Giesbers, M.; Norde, W.; Kleijn, J. M. *Colloids Surf., B* **1994**, *3*, 91.
(47) Arakawa, T.; Bhat, R.; Timasheff, S. N. *Biochemistry* **1990**, *29*, 1914.
(48) Green, A. A. *J. Biol. Chem.* **1931**, *93*, 495−516.