Efficient Renaturation of Immobilized Met-hemoglobin at the Galleries of α-Zirconium Phosphonate

C. V. Kumar* and Anita Chaudhari

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

Received September 12, 2000
Revised Manuscript Received November 18, 2000

Enzymes are excellent biological catalysts with high selectivity and efficiency, but their use in chemistry is severely restricted because of a number of factors, such as their poor thermal stability, high price, limited availability, and chemical sensitivity.[1] Immobilization of proteins at inert/stable solid surfaces can overcome some of these problems, and immobilized enzymes can be used for chemical transformations in organic and aqueous media.[2] Immobilized proteins, however, are often less active than the native proteins but these may be more stable than the free protein in solution.[3] Heat denaturation of proteins in aqueous media, or immobilized proteins, may result in irreversible loss of structure/activity, and hence, enzymatic reactions are often restricted to ambient temperatures.[4] Thermal stability and the ability to refold efficiently after thermal denaturation are desired attributes that are not often encountered with immobilized proteins. Refolding of small (single-domain) proteins in solution and on selected solid supports is reported. Ubiquitin C-terminal hydrolase, cytochrome *c*, and myoglobin, for example, undergo reversible thermal denaturation.[5a−d] Sol−gel encapsulated cytochrome *c* exhibited enhanced thermal stabilities[5e] as well as reversible thermal denaturation concomitant with inhibition of aggregation of the denatured proteins.[5f] Matrixes such as sol−gels were suggested to stabilize the native conformation and promote recovery of protein structure after thermal denaturation.[5g] Denaturation of chymotrypsinogen immobilized on poly(2-hydroxyethyl methacrylate) was followed using fluorescence methods and cooling of the immobilized denatured protein resulted in recovery of protein fluorescence.[6] In this context, we report here, for the first time, the efficient, stoichiometric, reversible thermal denaturation of multi-subunit functional met-hemoglobin (Fe(III) Hb) intercalated in the galleries of carboxymethyl Zr(IV) phosphonate. In contrast, the recovery of the Hb structure in solution is poor. The extent of recovery of the intercalated protein structure depended on the nature of the support matrix surface functions.

Hb consists of four subunits and each subunit has one noncovalently bound heme prosthetic group. Hb is interesting from the protein-refolding point of view, and this is because proper folding of each subunit, incorporation of the prosthetic group at its native site, and assembly of subunits to form the appropriate quaternary structure are necessary for the recovery of the Hb native form.[7] Although Hb does not function as an enzyme in biological systems, in the presence of hydrogen peroxide, Hb is converted to a reactive Fe(IV)oxo form capable of oxidizing substrates such as phenols, amines, and olefins (peroxidase activity).[8] This high valent form may have an important role in injury resulting from ischemic reperfusion, myocardial infarction, stroke, and internal hemorrhage.[9] We have used spectroscopic methods combined with activity studies to probe the recovery of the Hb structure. Hb is known to denature at 73 °C and the denaturation is accompanied by the loss of peroxidase activity, loss of α-helical content, and the production of random coils.[10] The enzymatic, thermal, and spectroscopic properties of immobilized Hb are monitored before and after heat denaturation to examine the extent of recovery of the protein native structure.

Immobilization of Hb at the galleries of the inorganic layered material, α-ZrP (α-$Zr(O_3POH)_2 \cdot nH_2O$), has been previously demonstrated[11] and protein immobilization resulted in expanded gallery spacings (55 Å for Hb/α-ZrP vs 7.6 Å for α-ZrP[12]). In a similar manner, Hb is readily immobilized at the galleries of α-zirconium carboxymethyl phosphonate, α-ZrCMP (α-$Zr(O_3PCH_2$-

(1) (a) Gubitz, G.; Kunssberg, 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, pp 129. (b) Gorton, L.; Marko-Varga, G.; Dominguez, E.; Emneus, J. In *Analytical Applications of Immobilized Enzyme Reactors*; Lam, S., Malikin, G., Eds.; Blackie Academic & Professional: New York, 1994; p 1.

(2) (a) Goncalves, A. P. V.; Lopes, J. M.; Lemos, F.; Ribeiro, F. Ramoa, Prazeres, D. M. F.; Nagamune, T. *Kagaku Kogaku* **1996**, *60* (11), 797. (b) Akita, H. *Biocatal. Biotransform.* **1996**, *13* (3), 141. (c) Nakamura, K.; Takano, S.; Terada, K.; Ohno, A. *Chem. Lett.* **1992**, *6*, 951. (d) Bullock, C. *Sci. Prog.* **1995**, *78* (2), 119.

(3) (a) Vidal, M. M.; Gil, M. H.; Delgadillo, I.; Alonso, J. *Biomaterials* **1999**, *20* (8), 757. (b) Lan, E. H.; Dave, B. C.; Fukuto, J. M.; Dunn, B.; Zink, J. I.; Valentine, J. S. *J. Mater. Chem.* **1999**, *9* (1), 45. (c) Kozlov, L. V. *Bioorg. Chem.* **1980**, *6* (8), 1243.

(4) Gupta, M. N. *Thermostability of Enzymes*; Springer-Verlag: New York, 1993; p 124.

(5) (a) Larsen, C. N.; Price, J. S.; Wilkinson, K. D. *Biochemistry* **1996**, *35* (21), 635. (b) Ikai, A.; Tanford, C. *Nature* **1971**, *230* (5289), 100. (c) Acampora, G.; Hermans, J. *J. Am. Chem. Soc.* **1967**, *89* (7), 1543. (d) Defaye, A. B.; Ledward, D. A.; MacDougall, D. B.; Tester, R. F. *Food Chem.* **1994**, *52* (1), 19. (e) Lan, E. H.; Dave, B.; Fukuto, J. M.; Dunn, B.; Zink, J.; Valentine, J. S. *J. Mater. Chem.* **1999**, *9* (1), 45. (f) Dave, D.; Miller, J. M.; Valentine, J. S. *J. Sol-Gel Sci. Technol.* **1997**, *8*, 629. (g) Shen, C.; Kostic, N. M. *J. Am. Chem. Soc.* **1997**, *119*, 1304.

(6) Brynda, E.; Bleha, M. *Collect. Czech. Chem. Commun.* **1979**, *44* (10), 3090.

(7) Hernan, R. A.; Sligar, S. G. *J. Biol. Chem.* **1995**, *270* (44), 26257.

(8) Fujita, A.; Senzu, H.; Kunitake, T.; Hamachi, I. *Chem. Lett.* **1994**, 1219.

(9) (a) Taiei, N.*Chikusan no Kenyu* **1994**, *48* (12), 1283. (b) Osawa, Y.; Darbyshire, J. F.; Meyer, C. A.; Alayash, A. I. *Biochem. Pharmacol.* **1993**, *46* (12), 2299.

(10) Niwa, S.; Izumimoto, M. *Anim. Sci. Technol.* **1994**, *65* (7), 668.

(11) (a) Kumar, C. V.; Chaudhari, A. *J. Am. Chem. Soc.* **2000**, *122* (5), 830. Hb/α-ZrRP composites were prepared by mixing Hb (10 mM K_2HPO_4, pH 7.2) and exfoliating α-ZrRP in a 3:1 volume ratio such that the final concentrations were 51 μM Hb/13−20 mM α-ZrRP. Samples were allowed to dry on slides and heat denatured in a nitrogen atmosphere for 5 min. For the XRD measurements, air-dried films were heated at the corresponding temperature (under nitrogen purge) for 5 min and then cooled for 1 min, following which the XRD was recorded (5-min scan). The same sample was heated and cooled at 20, 40, 60, 80, and 90 °C and the loss in the XRD pattern was monitored. α-ZrP, α-ZrCMP, and α-ZrCEP were synthesized by the reflux method according to published procedures.[12] Powder X-ray diffraction patterns matched those reported in the literature. α-ZrP was refluxed in 9 M phosphoric acid for 24 h whereas α-ZrPAA and α-ZrCEP were refluxed in 9 N HCl for extended times (3 days for α-ZrPAA and 14 days for α-ZrCEP).

10.1021/cm0007398 CCC: $20.00
Published on Web 01/06/2001

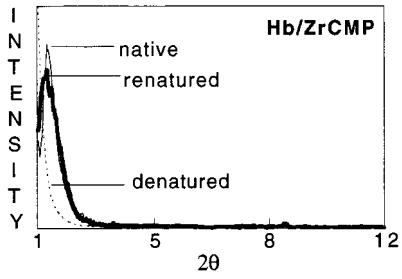


Figure 1. Powder X-ray diffraction patterns of Hb/α-ZrCMP (18 μM Hb/13 mM α-ZrCMP), heat-denatured Hb/α-ZrCMP, and renatured Hb/α-ZrCMP.

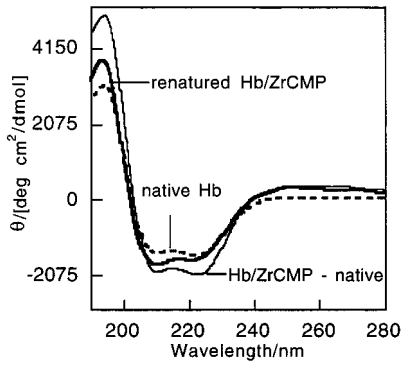


Figure 2. Circular dichroism spectra of Hb (3 μM), Hb/α-ZrCMP (3 μM Hb/13 mM α-ZrCMP), and renatured Hb/α-ZrCMP.

$COOH)_2 \cdot nH_2O$), as indicated by the expanded gallery spacings from 11 Å for α-ZrCMP to 64 Å for Hb/α-ZrCMP (Figure 1). Heating the immobilized Hb/-ZrCMP under nitrogen to 99 °C for 5 min resulted in the loss of the XRD peak, suggesting the collapse of the layer spacings due to protein denaturation (Figure 1). When the sample is cooled to room temperature, the *d* spacings were recovered within 1 h (Figure 1), indicating the recovery of the protein size. In contrast, Hb immobilized on α-zirconium carboxymethylphosphonate (α-$Zr(O_3PCH_2CH_2COOH)_2 \cdot nH_2O$ or α-ZrCEP[12]) did not recover its XRD pattern (see Supplemental #1 in the Supporting Information) under similar conditions. Hb/α-ZrP also indicated irreversible denaturation, in a similar way, and the denatured protein did not recover its layer spacings, even after 3 days at room temperature. Renaturation of immobilized Hb is further monitored in spectroscopic studies.

The recovery of the protein secondary structure was followed by monitoring the circular dichroism (CD) and infrared absorption (FTIR) spectra of the renatured proteins. Native Hb has nearly 80% α-helical content and these features are assigned to strong negative CD bands at 210 and 223 nm.[13] The CD spectrum of the Hb/α-ZrCMP is similar to that of the native protein (Figure 2), and heating of the protein to 83 °C for 5 min results in the loss of most of its molar ellipticity at 210 and 223 nm. Cooling the immobilized sample to room temperature (36 h) resulted in near complete recovery (90%) of its initial molar ellipticity at these wavelengths. The CD data are complemented by the FTIR spectra and these are recorded using the attenuated total internal reflection accessory (see Supplemental #2 in the Supporting Information). The amide I and amide II band positions of Hb/α-ZrCMP are determined from the second derivative of the absorbance data. The amide I peak appeared at 1651 cm^{-1} for Hb/α-ZrCMP and this value matches that of the free protein. Upon denaturation at 95 °C and cooling, the amide I peak recovers its position at 1651 cm^{-1}. The amide II band of Hb/α-ZrCMP appeared at 1548 cm^{-1} (superimposable with the free protein), and this matches the spectrum of the heat-treated samples (see Supplemental #2 in the Supporting Information). Overall, the CD and FTIR data indicate recovery of the Hb/α-ZrCMP secondary structure.

Free Hb, Hb/α-ZrP, and Hb/α-ZrCEP, in contrast, did not recover their structures when subjected to heat denaturation and cooling. Only 20% of the CD signal was recovered for the free Hb after 24 h and no recovery was observed for Hb/α-ZrP. The recovery of the FTIR spectrum was not observed. The amide I band of Hb/α-ZrP at 1651 cm^{-1} shifted to 1637 cm^{-1}, indicating the loss of its α-helical structure. Free Hb in the solid state also did not recover its native spectrum with 50% loss of intensity of the peaks at 1651 and 1548 cm^{-1}. The FTIR spectrum of denatured Hb/α-ZrCEP showed shifts in the amide I peak position (1655 vs 1651 cm^{-1}), corresponding to irreversible loss of structure. These data suggest the key role of α-ZrCMP in the renaturation of denatured Hb while the other two supports examined do not permit efficient renaturation. The refolding of immobilized Hb was further examined in activity studies.

Immobilized Hb/α-ZrCMP showed peroxidase activity and this provided a simple method for monitoring the renaturation process.[14] The activities of immobilized Hb/α-ZrCMP before and after heat treatment are shown in Figure 3 and the data clearly indicate the complete recovery of activity for the heat-treated samples, and the activities are nearly equal, within our experimental error (initial slopes of the curves are $5 \pm 0.04 \times 10^{-5}$/s and $8 \pm 0.94 \times 10^{-5}$/s, respectively).[15] The recovery of activity after denaturation was also examined by measuring the activity as a function of cooling time (Figure 3, inset).[16] Hb/α-ZrCMP showed 50% recovery of its initial activity after 10 min and 100% recovery after 1 h. This is in contrast to the free protein that when thermally denatured recovers <30% of its activity, even after several hours. Hb/α-ZrP recovered only 50% of its

(12) (a) Clearfield, A.; Stynes, J. A. *J. Inorg. Nucl. Chem.* **1964**, *26*, 117. (b) Alberti, G.; Constantino, U. In *Intercalation Chemistry*; Whittingham, M. S., Jacobson, A. J., Eds.; Academic: New York, 1982. (b) Alberti, G.; Constantino, U.; Allulli, S.; Tomassini, N. *J. Inorg. Nucl. Chem.* **1990**, *29*, 2112. (c) Dines, M. B.; DiGiacomo, P. M. *Inorg. Chem.* **1981**, *20*, 92.

(13) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin In Their Reactions With Ligands*; North-Holland Publishing Co.: New York, 1971; p 102.

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

(15) Samples were denatured at 90 °C in a nitrogen atmosphere for 5 min and after 24 h at room temperature were resuspended in 10 mM K_2HPO_4; the activity was measured using 3 mM guaiacol and 5 mM H_2O_2.

(16) The percentage of recovery of immobilized Hb activity was calculated in terms of the ratio of the activity (initial rates in terms of A/time) of the heat-denatured Hb sample after a specific time of cooling (at room temperature) per activity of native-immobilized Hb before denaturation. Exactly the same concentrations of immobilized Hb before and after denaturation were used for the activity measurements. The free solution Hb recovered none of its activity after heat denaturation and room-temperature equilibration of the solid over extended time periods.

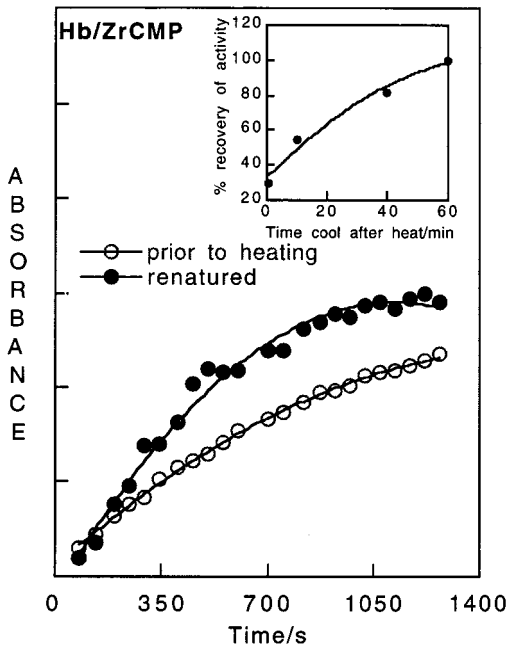
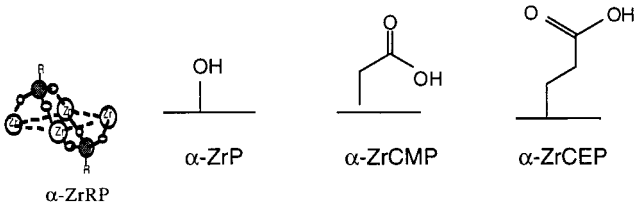


Figure 3. Peroxidase activity of Hb/α-ZrCMP (0.72 μM Hb/1 mM α-ZrCMP) at room temperature (open circles) and after heating/cooling (closed circles). The inset shows the recovery of enzyme activity as a function of time and the recovery was complete after 1 h.

activity in 1.5 h, and the activity did not improve any further with time. Hb/α-ZrCEP was even less efficient in activity recovery, with a maximum of 30% recovery, and no further improvement was observed with time.

Surface-specific refolding of Hb/α-ZrCMP, therefore, is demonstrated here for the first time. We attribute this selectivity to the nature of the surface functions of the support matrix. The CH_2CH_2COOH functions of α-ZrCEP, for example, are known to react with primary amines to produce amides while the CH_2COOH functions of α-ZrCMP are unreactive under similar conditions.[17] The orientation of the carbonyl functions with respect to the α-ZrRP surface (Chart 1) was suggested to be important in controlling the reactivity of these

Chart 1. Schematic Structures of α-ZrRP and the Surface Functions of α-ZrP, α-ZrCMP, and α-ZrCEP in the Galleries



surfaces. The reason for the partial reversibility with α-ZrP is not clear. Amide formation between the lysine side chains of the denatured Hb with α-ZrCEP can readily prevent the recovery of its native structure while such reactivity is not expected with α-ZrCMP. Only a small fraction of such amide functions can seriously impede protein structure recovery. Cross-linking of the ϵ-amino groups of lysine with corresponding aspartate and glutamate side chains, upon heat treatment of keratin (in the absence of coupling agents), resulting in intramolecular amidations, was documented.[18] Chemical reactivities of supports at elevated temperatures are an important consideration in evaluating the properties of immobilized proteins.

Immobilized proteins may react with the support matrix surfaces, in specific cases, and proper surface functions should, in principle, allow the refolding of proteins into their native structures. In this respect, zirconium phosphonate support matrixes with appropriate surface groups may function as artificial chaperones for protein folding. In future studies, we plan to probe the molecular details of these support surfaces for efficient refolding of proteins.

Acknowledgment. The authors thank the National Science Foundation (DMR- 9729178) for financial support of this work. We are grateful to Professor S. L. Suib for access to the XRD instrument.

Supporting Information Available: Figures of (1) powder X-ray diffraction plots of Hb immobilized on α-ZrP and (2) ATR-FTIR spectra of Hb and Hb/α-ZrCMP before and after heating (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

CM0007398

(17) (a) Burwell, D. A.; Thompson, M. E. *Chem. Mater.* **1991**, *3*, 14. (b) Burwell, D. A.; Valentine, K. G.; Timmermans, J. H.; Thompson, M. E. *J. Am. Chem. Soc.* **1992**, *114*, 4144. (c) Burwell, D. A.; Thompson, M. E. *Chem. Mater.* **1991**, *3*, 730.

(18) Asquith, R. S.; Otterburn, M. S. *J. Text. Inst.* **1970**, *61* (11), 569.