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Chemical chaperones: influence of carboxylate orientation on the refolding of glucose oxidase

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Enzymes, due to their high specificities, low toxicities, and ability to function at room temperature, are excellent candidates for catalysis in chemistry [1–3]. Chemical applications of enzymes are currently limited due to their high cost, instability, and the difficulty in recovering active enzyme for reuse. Specific inorganic solids stabilize enzymes, and such enzymes have potential applications in carrying out organic transformations with high selectivity and efficiency [4,5]. The nature of the surface functions of the inorganic solid, however, profoundly influences the enzyme behavior [6]. Well defined, homogeneous, solid surfaces decorated with specific organic functional groups are needed to study these interactions.

The two-dimensional surfaces of layered α-Zr(IV) phosphonates [7–10] (α-Zr$(RPO_3)_2$, denoted as α-ZrRP, R = carboxymethyl (CM), carboxyethyl (CE), OH, Scheme 1) present such an opportunity to evaluate these interactions. The galleries of α-ZrRP are topologically, and chemically uniform, and hence, the bound enzyme behavior can be readily correlated with the protein–surface interactions. Selected organic functionalities, further more, are introduced at these surfaces to evaluate the contributions of these functions to the protein–surface interactions. The orientation of COO plane in α-ZrCMP is nearly parallel to the inorganic surface while in α-ZrCEP it is perpendicular (Scheme 1) [11,12] and current results indicate that this subtle difference has a large impact on α-ZrRPs ability in promoting protein folding.

The efficiency of refolding of glucose oxidase (GO), an enzyme of considerable synthetic importance [13–17] is used here as a sensitive measure of the nature of the enzyme–surface interactions. Reversible thermal denaturation of met-hemoglobin (Hb) at α-ZrRP, in this context, was recently reported [6] and refolding was most efficient at α-ZrCMP but not at α-ZrCEP. The interaction of surface lysyl functions of Hb with α-ZrCEP may result in covalent adduction of the protein with the solid surface, in principle, and prevent protein folding. Similar reaction with the carboxyl functions of α-ZrCMP is difficult due to the unfavorable orientation of the carbonyl group at these surfaces. Intrigued by these results, we decided to examine the refolding of GO, richly decorated with surface carboxyl functions, at α-ZrRP surfaces

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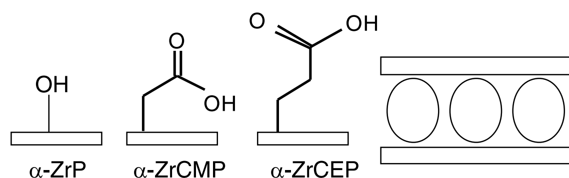

Scheme 1. The differences in the orientation of the OH/carbonyl functions of α-ZrP, α-ZrCMP and α-ZrCEP. Monolayer coverage of GO (large spheres) at α-ZrRP layers (long rectangles) is shown.

such that protein adduction is less likely, and refolding efficiencies may reflect on the abilities of these surfaces in aiding protein folding.

The minor differences between the carboxylate functions of the above two solids, current results indicate, profoundly influence GO refolding at these surfaces. GO immobilized on electrodes, controlled pore glass, polymers, sol–gels, polymer gels, and polyions led to enhanced enzyme activity and improved thermal stability, but no reports on the nature of these surfaces on GO behavior are known [18,19]. The strong role of α-ZrCEP in the renaturation of GO is evident from four different lines of evidence.

Intercalation of GO into the galleries of α-ZrP, α-ZrCMP, and α-ZrCEP was achieved, using methods reported previously [20]. [1] Intercalation was supported by the observed *d*-spacings of 67 Å for GO/α-ZrCMP, and 66 Å for GO/α-ZrCEP. These are much larger than the *d*-spacings of α-ZrCMP, and α-ZrCEP (11.1 and 12.9 Å, respectively [7–10]) prior to GO intercalation. The observed increases in *d*-spacings correspond well with the protein diameter (54 Å) [21], suggesting a monolayer packing of GO in the galleries (Scheme 1). The stoichiometries for the binding of GO to α-ZrP, α-ZrCMP, and α-ZrCEP are 700, 1700, and 1500 phosphate/phosphonate groups, respectively. Such large values for the stoichiometry suggest loose packing of GO, assuming that the GO molecules are randomly distributed in the galleries.

Heating of the GO/α-ZrCEP (at 62°C, N_2 purge, 5 min) results in the loss of the XRD peak (Fig. 1a, dashed line) and the peak recovered upon cooling to room temperature for 48 h (Fig. 1a, thick line). [2] Recovery of the original *d*-spacings are suggestive of recovery of protein size, and they indicate possible recovery of GO native structure. In contrast, GO/α-ZrCMP and GO/α-ZrP, when subjected to thermal denaturation and cooling, under similar conditions, did not show recovery of *d*-spacings to a significant extent.

Recovery of GO secondary structure was followed by recording the circular dichroism (CD) and FTIR spectra of the renatured samples.[1] The CD spectrum of GO/α-ZrCEP (2 μM GO/3 mM α-ZrCEP, Fig. 1b) shows characteristic minima at 210 and 222 nm, and these bands are similar to those of the native GO in solution. After denaturation (at 60°C) and cooling for 48 h, the CD spectrum of the heat treated GO/α-ZrCEP closely resembles that of the sample prior to denaturation. The intensity of 222 nm band is slightly larger than the untreated sample, and this indicates small increases in the α-helical content of GO or stiffening of the helices. The CD bands of GO/α-ZrCMP at 210 and 222 nm (native form), however, are replaced by a new minimum at 230 nm (denatured/cooled, unordered form). The CD bands of heat treated GO/α-ZrP recovered less than 10% of their initial intensities, and showed only weak bands at 210 and 222 nm. The CD data suggest efficient recovery of GO bound to α-ZrCEP but not α-ZrP or α-ZrCMP. Renaturation of GO/α-ZrRP was further tested using FTIR, and activity studies.

The amide I and amide II vibrational bands of proteins are useful to monitor protein denaturation, and renaturation [22]. Heat denaturation of GO (at 58°C, under N_2 purge) resulted in the shift of the amide I and II band positions from 1649 and 1536 to 1646 and 1527 cm^{-1}. On cooling the sample to 25°C, the band positions are not re-

[1] The powder X-ray diffraction pattern of GO/α-ZrP at low concentrations of GO indicated expanded interlayer *d*-spacings of 60 Å while, our earlier studies with higher concentrations of GO yielded a bilayer formation with a *d*-spacing of 116 Å.

[2] GO/α-ZrCEP pellets were heated under N_2 at 60°C in an oil bath for 5 min after which samples were equilibrated at 25°C for 48 h. The CD spectra were recorded after resuspension of the solid in 5 mM K_2HPO_4, pH 7.2. GO/α-ZrCMP, GO/α-ZrP, and GO were treated similarly.

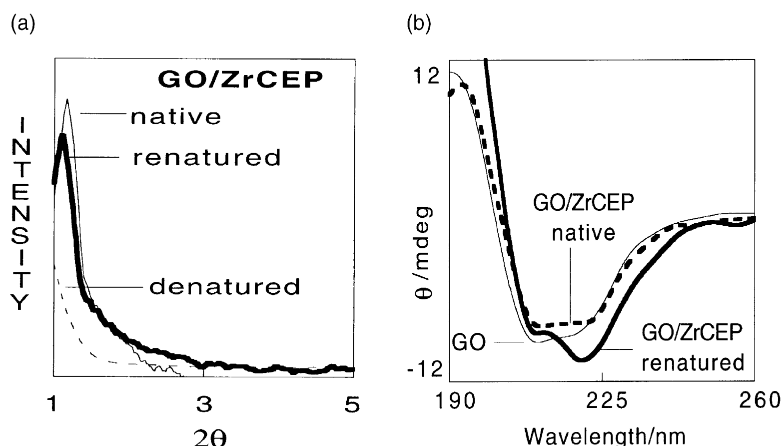

Fig. 1. 1(a) Powder X-ray diffraction patterns of GO/α-ZrCEP (3.6 μM/12 mM) native, heat denatured, and renatured; (b) circular dichroism spectra of GO (2 μM), GO/α-ZrCEP (2 μM/3 mM) before heating and after renaturation.

covered, even after 48 h. [3] The FTIR bands of GO/α-ZrCEP, however, observed at 1640, and 1538 cm^{-1}, before heat treatment, compare well with the band positions after renaturation. GO/α-ZrP, when heat denatured and cooled, shows only partial recovery of its secondary structure as inferred from the FTIR and CD spectra.

The ability of these three solids to aid in the renaturation was also monitored in enzyme activity studies. [4] The initial velocity for the oxidation of glucose (1 mM) by GO/α-ZrCEP (1 μM enzyme) before denaturation was 14 s^{-1} and after renaturation the initial velocity was 13 s^{-1} (Fig. 2), essentially unchanged. GO/α-ZrP also recovered its activity with initial rates that are the same before heat treatment, and after cooling (7.2 s^{-1}, 1 μM enzyme). Heat treated GO/α-ZrCMP recovered only 75% of its initial activity (0.6 s^{-1} before heating, vs. 0.45 s^{-1} after heating and cooling, 1 μM enzyme). [5] Activity data clearly indicate the renaturation of GO/α-ZrCEP, con-

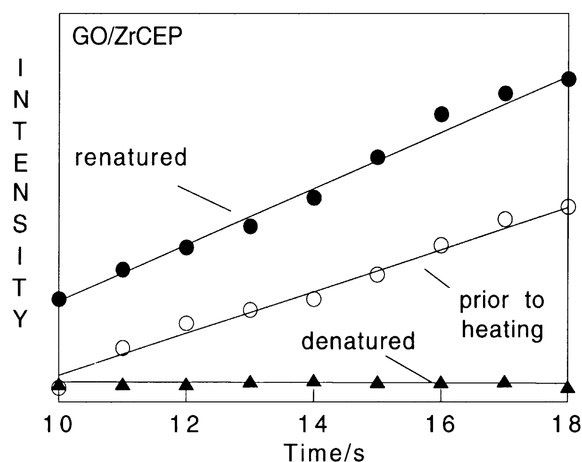

Fig. 2. Increase in fluorescence intensity of pyrenebutyric acid, as oxygen is consumed during the catalytic oxidation of glucose by GO. GO/α-ZrCEP (1 μM/2 mM) before heating (○), after denaturation (▲), and after renaturation (48 h, ●). Glucose concentration was 1 mM in all experiments.

[3] For ATIR-FTIR studies, GO/α-ZrCEP (1.7 μM/11 mM) and GO/α-ZrP (112 μM/20 mM ZrP) were heat denatured, cooled to 25°C, and lyophilized. GO immobilized on self-assembled monolayers underwent conformational changes as a function of surface hydrophilicity, leading to greater β-sheet content [23].

[4] Activities of GO/α-ZrP (0.85 μM/1 mM), GO/α-ZrCMP (1 μM/2.3 mM), and GO/α-ZrCEP (1 μM/2 mM), before and after heat treatment were followed in fluorescence experiments using pyrenebutyric acid (0.5 μM) as the probe. As oxygen is consumed during the oxidation of glucose by GO, the fluorescence increased. Typical errors in these measurements were less than ±7%.

[5] The differences in the activities of GO at these solids may also reflect on the structure of GO prior to thermal denaturation, and accessibility of the active site to the substrate.

sistent with the above spectral data, but the recovery of activities of GO/α-ZrP and GO/α-ZrCMP indicates that activity recovery may not always indicate complete structure recovery.

Taken together, the XRD, CD, FTIR, and activity studies clearly indicate efficient renaturation of GO at α-ZrCEP. This is the first example of reversible thermal denaturation of GO at a solid surface, despite the fact that increased thermal stability of immobilized GO is known [24,25]. Denatured proteins of the inclusion bodies are successfully refolded using reverse micellar media, [26] and Ribonuclease A is renatured in the presence of thiol derivatized microspheres [27]. Isolation of individual protein molecules, and preventing protein aggregation, in these cases, appears to be an important first step in facilitating refolding. Similarly, aggregation of GO is inhibited in α-ZrRP galleries due to the slow diffusion [28]. Isolation of individual enzyme molecules is important, clearly, but it is not the only factor as the efficiency of GO refolding depended on the surface functions. Current results demonstrate the subtle differences in the interactions between these solids and GO. The orientation of the carbonyl functions of α-ZrCEP is more favorable to hydrogen bond with the protein, and this may be significant in the guided folding of GO.

The favorable orientation of the carboxyl functions of α-ZrCEP render it more reactive towards primary amines when compared to α-ZrCMP [11,12]. Such orientation in α-ZrCEP is also expected to enhance its H-bonding with bound proteins, and H-bonding can stabilize GO, prevent aggregation, and/or assist in the refolding. The surface functions of the matrix, we conclude, play an important role in protein refolding, and such surfaces may function as chemical chaperones.

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