Stability of Oxidases Immobilized in Silica Gels

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Abstract: The gain or loss of stability of three flavoprotein oxidases, glucose oxidase, lactate oxidase, and glycolate oxidase, upon immobilization in a hydrated silica gel by a sol−gel process was quantified. Glucose oxidase (isoelectric point, IP, pH 3.8) retained most or all of its initial activity, while lactate oxidase and glycolate lost most of theirs. The half-life of glucose oxidase at 63 °C increased upon immobilization 200-fold; the half-lives of lactate oxidase and of glycolate oxidase were not extended beyond those of the water-dissolved enzymes. After lactate oxidase (IP pH 4.6) was electrostatically complexed with the weak base poly(*N*-vinylimidazole) prior to its immobilization, most of its activity was retained and its half-life at 63° C increased 150-fold. Lactate oxidase was also stabilized when electrostatically complexed with the stronger base poly(ethyleneimine) prior to immobilization. Glycolate oxidase (IP pH 9.6) was not stabilized by poly(*N*-vinylimidazole) but was stabilized by poly(ethyleneimine) complexing prior to immobilization. The complexed enzyme retained its initial activity upon immobilization, and its half-life at 60 °C also increased 100-fold. The results show that encaging an oxidase in a silica gel can lead either to gain in stability or to loss of activity and that electrostatic complexing is required for stabilization by encapsulation of some, but not all, flavoprotein oxidases.

Introduction

This article and the one that follows[1] consider effects of the chemical environment of hydrated silica on the stability of oxidases. The first describes experiments showing that, while immobilization in silica gel improves the stability of glucose oxidase, identical immobilization causes loss of activity of lactate oxidase and of glycolate oxidase. Nevertheless, when the destabilized enzymes are coimmobilized in the silica gel with countercharged polymers that form electrostatic adducts with the enzymes, their stabilities also improve. The accompanying article proposes a model, based on the resolved structures of the enzymes, explaining the observed stabilities. It postulates that stability is determined by a combination of the global electrostatic interactions, which are the subject of this article, and specific local interactions considered in the article that follows.

Enzymes have been stabilized by their binding to silica surfaces for at least a quarter of a century. The rich literature on such stabilization[2−6] describes mostly successes rather than failures, even though much could be learned from the latter. Two central concepts have been invoked in explaining the observations of improved stability. According to the first, restricting the segmental motion of protein chains reduces the likelihood of an irreversible structural change that may result, for example, from a hydrophobic interaction. Here, two protein segments, hydrated by translationally and rotationally restricted water molecules, transiently and reversibly lose water, collide, then irreversibly bind. This binding is driven by the gain of entropy upon freeing of water molecules to translate and rotate. According to the second, a similar irreversible change in the protein structure occurs by segmental collision with the surface on which the enzyme is immobilized or on which it is adsorbed. When the motion of protein chain segments is restricted through attachment to the surface and when the surface is well hydrated, protein denaturing segmental collisions are unlikely.[7] For silica, the free energy of hydration is quite negative, and encapsulation in hydrated silica also adds a third stabilizing factor, the prevention of access of proteolytic enzymes. Cases of protein stabilization by sol−gel encapsulation in hydrated silica were elegantly presented by Avnir, Braun, Ottolenghi, and their colleagues and by Dunn, Valentine, Zink, and their colleagues, as well as by other researchers.[8−14] These and other groups described immobilization procedures, where proteins lost little, if any, of their biological activity. These involved mixing of the aqueous solution of the protein with that of a silicon tetra- or trialkoxide dissolved in an alcohol, hydrolysis of the alkoxide to the silanol, polymerization of the silanol to form a sol, dehydration of the sol to form a gel, and gradual densification

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of the gel with further loss of water. Through this procedure, the protein was encased by the hydrated silica in a cage tailored to its size. Transport of substrate and product to and from the enzyme was maintained, at least initially, through water-rich pores or pores formed by the co-incorporation of a water-soluble nonprotein-binding polymer, such as poly(ethylene glycol), and the maintenance of these channels after substantial densification of the gel. The literature on enzyme stabilization also provides examples where the stability of an enzyme was improved through electrostatic interaction with a countercharged macromolecule.[15−18] Conceptually, such stabilization was related to confinement or restriction of the segmental motion of protein chains. We show in this article and in the one that follows that these concepts explain the observed gains and losses in stability when oxidases are immobilized in hydrated silica by the sol−gel method only when complemented by understanding of the local electrostatic interactions.

Experimental Section

Chemicals. Lactate oxidase (LOx) (Genzyme, Cambridge, MA, Lot No. D50293, Catalog No. 70-1381-01, EC 1.1.3.2 from *Aerococcus viridans* 37.0 units/mg of powder), glucose oxidase (GOx) (Sigma Chemical Inc., St. Louis, MO, EC 1.1.3.4 from *Aspergillus niger* type X-S, 198 units/mg of solid, 75% protein), glycolate oxidase (GLyOx) (Sigma, EC 1.1.3.15 from *Spinacia oleracea*, 10 units/mg of solid), tetramethylorthosilicate (TMOS) (Aldrich, Milwaukee, WI, Catalog No. 34,143-6), and Bermocoll EHM-100 (a block copolymer of ethyl hydroxyethyl ether and ethyl ether cellulose) (Berol Nobel AB, Stenungsund, Sweden) were used as received. The poly(1-vinylimidazole) (PVI) was synthesized as described previously.[19] Poly(ethyleneimine) (PEI) (50% w/v aqueous solution, average MW 50 000) was purchased from Sigma.

Oxidase-Doped Silica Gel Powder Preparation. Tetramethyl orthosilicate (TMOS) (0.15 g) was placed in a small vial, cooled in an ice bath, and stirred at about 600 rpm. HCl (36 μL, 2.44 mM) was added, and the solution was stirred for 10 min. A vacuum was then pulled on the vial for an additional 10 min to evaporate the methanol produced in the sol step. The vacuum was released, and the pH of the solution was adjusted to pH 5.1 by adding 20 μL of 20 mM sodium phosphate buffer (pH 7.4). In a separate small vial, 6 mg of the oxidase (LOx, GOx, or GLyOx), 180 μL of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (sodium salt) buffer solution (pH 7.5), and, when polymer was added, 20 μL of 20 mg/mL aqueous PVI or PEI were mixed. The lactate oxidase-containing solution was added to the TMOS-containing solution, and the resulting mixture was stirred for 1 min. A vacuum was applied to the stirred mixture until a gel formed. The vacuum was released, and the gel was rinsed with 2 mL of distilled water three times. The gel was then soaked in 2 mL of distilled water overnight at 4 °C. The water was removed from the vial, and the gel was allowed to dry at room temperature overnight. The dried gel was first collected and then ground to a powder with a mortar and pestle.

SEM of the silica gels revealed featureless surfaces at 1-μm resolution.

Qualitative Activity Assay for Enzyme-Doped Silica Gel Powder. For qualitative spot tests at room temperature of the oxidase-doped silica gel powder, peroxide test strips (Reflectoquant 16974 Peroxide Test, Merck, Darmstadt, Germany) were used. Enzyme-doped silica gel powder (1−2 mg) was applied to the active area of the test strip followed by 20 μL of 1 M substrate (prepared in phosphate buffer solution, pH 7.4). Enzymatic activity resulted in the appearance of a blue spot.

In qualitative tests of the stability of the glucose oxidase-doped silica gel powder at elevated temperatures, the powder was immobilized on a glass slide by the following procedure. Bermocoll EHM-100 (26 mg), 1 mL of cyclohexanone, and 1 mL of water were mixed in a small vial. The vial was heated and agitated over a flame for 5 min. The solution was allowed to settle, and the white micelle fraction was collected. Micelles (90 μL) and 10 mg of the glucose oxidase-doped silica gel powder were mixed and then stirred for 5 min. Ten microliters of the mixture was applied to a glass slide and allowed to dry overnight. The glass slide was immersed in a controlled temperature bath for 5 min at the relevant temperature. The slide was removed and allowed to dry. Ten microliters of 2 M glucose was applied to the immobilized micelles. The active area of the peroxide testing strip was placed on top of the immobilized micelle area. The appearance of a blue color on the strip indicated the presence of glucose oxidase activity.

Quantitative Activity Assay of the Enzyme-Doped Silica Gel Powder. Lactate oxidase, glycolate oxidase, and glucose oxidase catalyze the air oxidation of their substrates by oxygen in a reaction where hydrogen peroxide is stoichiometrically formed. The hydrogen peroxide was assayed using the peroxidase catalyzed oxidation of a leuco-dye or of a dye precursor (e.g., a quinoneimine), the optical density of which is measured. The assay, of Genzyme,[20] utilizes a set of stock solutions. A solution (I) was prepared by combining (a) 6.0 mL of 0.2 M 3,3-dimethylglutarate [$NaO_2CCH_2CH_2C(CH_3)_2CO_2Na$] buffer, pH 6.5; (b) 3.0 mL of horseradish peroxidase, 50 units/mL in water; (c) 3.0 mL of 15 mM 4-aminoantipyrine in water; (d) 3.0 mL of 0.5 M substrate; and (e) 9.0 mL of distilled water. A second stock solution (II) contained 0.5% *N*,*N*′-dimethylaniline in water. A third solution of the enzyme diluent (III) was prepared by adding 66 mg of FAD into 4 mL of a 10 mM KH_2PO_4−NaOH (pH 7.0) buffer solution. The enzyme solutions of 1 mg/mL were prepared by dissolving the enzyme in III. A solution of the enzymatic reaction stopper (IV) was prepared by dissolving 0.25 g of dodecylbenzenesulfonic acid sodium salt [$C_{12}H_{25}C_6H_4SO_3Na$] in 100 mL of distilled water. All chemicals were purchased from Aldrich unless otherwise mentioned.

In the activity test, 1.0 mg of the powder was weighed into a 3-mL cuvette. Distilled water (100 μL) was used to wash the powder down from the inside walls to the bottom of the cuvette. The cuvette was then capped with Parafilm and placed in a temperature bath set at 63 °C for the testing period. After this period, the cuvette was removed from the bath and 100 μL of solution III was added so as to restore activity if activity was lost by leaching of the cofactor. The cuvette was then placed in a 37 °C temperature bath for another 5 min. Solutions I and II at a 4:1 ratio were then allowed to equilibrate at 37° C. One milliliter of this solution was added to the cuvette. This mixture was aerated by strong agitation and allowed to react for exactly 10 min at 37 °C, then 2.0 mL of solution IV was added and the absorbance at 564 nm (A_s) was measured.

The procedure was repeated, using 1.0 mg of enzyme-free silica gel powder in place of the oxidase-doped silica gel powder. The same procedure was followed thereafter, and the blank absorbance (A_b) was measured. The net absorbance of the oxidase-doped silica gel powder solution was determined from D ($D = A_s - A_b$). When D was greater than 0.6, the assay was repeated and the weight of the lactate oxidase-doped silica gel powder was adjusted accordingly.

Results

Silica gels in which enzymes and other biological macromolecules were immobilized are classified as either xerogels (dried gels) or aged gels (wet gels).[2,3] The latter have water-filled pores that are much larger than those of the xerogels. Most of the earlier work on sol−gel immobilization of enzymes, including the pioneering studies of Avnir, Ottolenghi, Braun[3,8] and Lev at the Hebrew University and of Dunn, Valentine,

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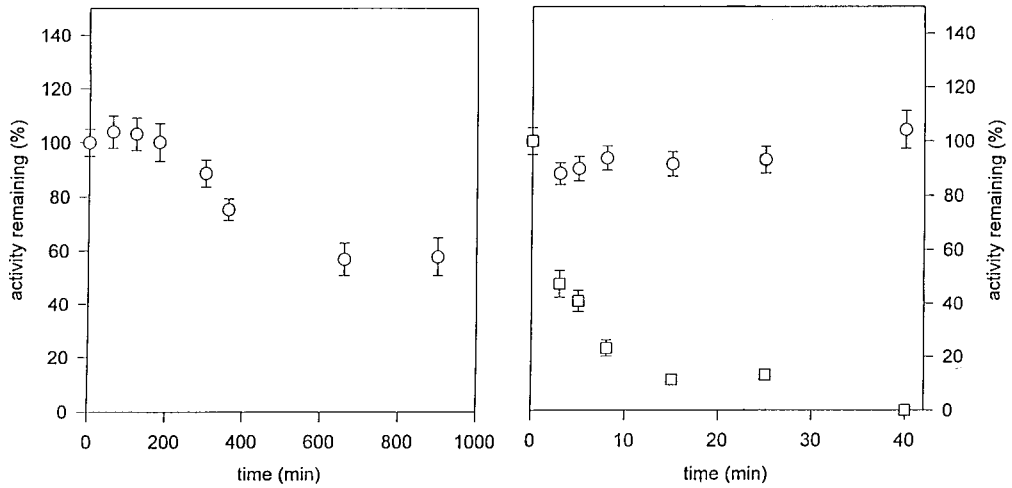

Figure 1. Decay of the activity of LOx immobilized in silica gels and suspended in water at 63 °C. Circles: PVI complexed LOx. Squares: noncomplexed LOx. Each data point represents the average value of three measurements.

Table 1. Percent of Remaining Activity of Glucose Oxidase at 63 °C in Water

dissolved particles				suspended particles			
time (min)	% remaining	no. of samples	RSD (%)	time (min)	% remaining	no. of samples	RSD (%)
0	100	3	5.02	0	100	3	6.75
5	61.67	3	4.67	20	97.28	3	6.87
10	41.73	3	6.54	60	98.91	3	4.35
20	15.71	3	8.69	140	88.17	3	9.65
30	11.03	3	7.97	180	76.85	3	9.65
40	7.22	3	9.07	240	67.05	3	8.76
50	7.36	3	8.78	1163	61.32	3	7.94

Zink[4,5,9] and colleagues at UCLA, was carried out on aged gels. This study was performed, however, on xerogels, where the smaller pore size was likely to enhance the interactions, including the electrostatic interactions between the silica and the protein, and thereby the stabilization or destabilization of enzymes. Loss of activity was likely to be more severe also because capillary forces, on the order of several MPa, may develop upon drying of the xerogels.

Qualitative activity assays of water-dispersed glucose oxidase in silica gel showed that the enzyme retained at least part of its activity after 20 min of exposure to a temperature as high as 92 °C, where the powdered silica gel was hydrolyzed and fell off the substrate. At 75 °C activity was retained for at least 60 min.

Results of the quantitative stability assays at 63 °C on water-dispersed, powdered silica gel-immobilized glucose oxidase and on water-dissolved glucose oxidase are presented in Table 1. The silica gel-immobilized GOx retained 60% of its initial activity after 20 h. In contrast, the half-life of dissolved GOx was only 6.5 min. Also at 63 °C, but in 2 M glucose, the immobilized glucose oxidase retained half of its initial activity after 6 h.

While glucose oxidase activity remained intact, lactate oxidase lost at least 70% of its activity upon immobilization in silica gel. Furthermore, the silica gel-immobilized lactate oxidase was totally inactive after being in water at 63 °C for 40 min. At 50 °C, the water-dispersed, silica gel-immobilized, lactate oxidase and the water-dissolved lactate oxidase had respective half-lives of 50 min and 10 h. (Table 2). At 63 °C the dissolved and immobilized enzymes had similar 6-min half-lives.

When lactate oxidase, a polyanion at pH 7,[21] was complexed with polycationic PVI or with PEI prior to its mixing with the

Table 2. Percent of Remaining Activity of Lactate Oxidase at 50 °C in Water

dissolved particles				suspended particles			
time (h)	% remaining	no. of samples	RSD (%)	time (h)	% remaining	no. of samples	RSD (%)
0	100	3	8.72	0	100	3	7.63
1	44.46	3	9.36	1	95.13	3	6.74
2	37.76	3	7.46	3	71.29	3	8.46
3	0.24	3	5.31	20	12.65	3	9.23
4	0.02	3	3.12	45	0.35	3	6.36

silica gel-forming alkoxide precursor, the enzyme retained most of its activity. Figure 1 shows the decay of the activity of the water-dispersed silica gel-immobilized PVI-lactate oxidase at 63 °C. The immobilized enzyme retained, after 15 h at 63 °C, 55% of its initial activity, a 150-fold improvement over the half-life of the free enzyme and the enzyme immobilized without PVI. The immobilized PVI-LOx also retained its full initial activity after 11 days of soaking in water at room temperature. In the presence of 1 M lactate, the immobilized PVI-LOx retained half of its initial activity after 6 h at 63 °C.

PEI binds electrostatically to lactate oxidase and has been shown to stabilize enzymes.[22,23] Silica gel-immobilized PEI-LOx was prepared by a procedure similar to that used for PVI-LOx. Figure 2 shows the decay of the activity of the silica gel-immobilized PEI-LOx at 63 °C. After 15 h, 18% of the initial activity remained.

Like lactate oxidase and unlike glucose oxidase, glycolate oxidase (GLyOx) lost at least 70% of its activity upon immobilization in silica gel. Furthermore, the glycolate oxidase-doped silica gel powder was totally inactive after being heated in water to 63 °C for 20 min. Mixing of glycolate oxidase with PVI prior to immobilization did not improve the stability. However, when glycolate oxidase was complexed with PEI prior to immobilization, the enzyme lost little, if any, of its activity and its stability at 63 °C improved, with 27% of the initial activity retained after 8 h.

Discussion

Of the three flavoprotein oxidases only glucose oxidase was stabilized by immobilization in a hydrated silica gel. The extent

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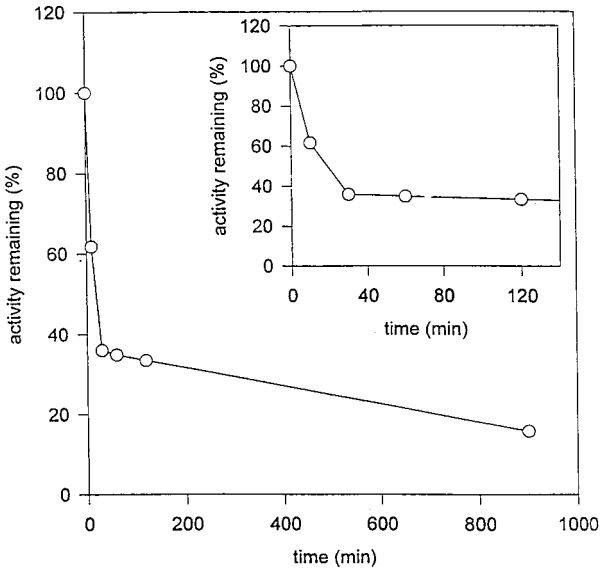

Figure 2. Decay of the activity of LOx complexed with PEI and immobilized in silica gel suspended in water at 63 °C. Each data point represents the average value of three measurements.

of stabilization of glucose oxidase is exemplified by a 200-fold increase in its half-life at 63 °C. In contrast, both lactate oxidase and glycolate oxidase lost most of their activity when immobilized in the silica gel. After immobilization, their residual activity was not better retained at 63 °C than that of the water-dissolved enzymes. The concept behind protein stabilization by immobilization, according to which restricting the segmental motion through encasement in a conforming cavity prevents irreversible structural changes, explains the results for glucose oxidase but not for lactate oxidase or glycolate oxidase.

Electrostatic interactions are known to affect the stabilities of enzymes,[14] with either polyanions and polycations stabilizing countercharged enzymes and enzymes with countercharged surface regions. The reported isoelectric points of glucose oxidase and lactate oxidase (that are anionic at neutral pH) and glycolate oxidase (that is cationic at neutral pH) are, respectively, 3.8,[24] 4.6, [21] and 9.6.[25] The polymers were polycations. The pH where one-half of the imidazoles of PVI are protonated (average pK_a) is well below that of monomeric *N*-alkylimidazoles, the shift resulting of the electrostatic repulsion between the cationic sites. For the same reason the average pK_a is also a strong function of the ionic strength. At physiological ionic strength, near 0.15 M NaCl, the average pK_a of PVI is near 6.[26] PEI is a stronger base, its electrophoretic mobility declining to one-half of its highest value near pH 8.[27] Thus, while PVI binds at neutral pH with glucose oxidase and lactate oxidase,[24] it is less likely to bind with glycolate oxidase, even though binding to a particular zone of this overall polycationic enzyme would be possible if a zone having a high density of anionic aspartate and glutamate residues existed. From the following paper[1] it is not apparent that such a zone exists. The more basic PEI binds, however, both with lactate oxidase and with glycolate oxidase. Such binding stabilizes lactate oxidase and glycolate oxidase, their half-lives at 63 °C increasing 150-fold in the case of LOx-PVI and 100-fold in the case of GLyOx-PEI upon immobilization in the hydrated silica gel.

We conclude that encasement of flavoprotein oxidases in hydrated silica cavities, tailored through silanol condensation to their shape and size, stabilizes the oxidases; that the extent of stabilization is massive in all cases; and that in some cases stabilization requires that the oxidase be shielded by precomplexing with a countercharged macromolecule. A microscopic model predicting when an oxidase requires electrostatic complexing for its stabilization and when it does not is presented in the paper that follows.

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