Reports

Encapsulation of Proteins in Transparent Porous Silicate Glasses Prepared by the Sol-Gel Method

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Novel sol-gel synthetic techniques were used to immobilize copper-zinc superoxide dismutase (CuZnSOD), cytochrome c, and myoglobin (Mb) by encapsulation in stable, optically transparent, porous silica glass matrices under mild conditions such that the biomolecules retained their characteristic reactivities and spectroscopic properties. The resulting glasses allowed transport of small molecules into and out of the glasses at reasonable rates but nevertheless retained the protein molecules within their pores. Chemical reactions of the immobilized proteins could be monitored by means of changes in their visible absorption spectra. Silica glasses containing the immobilized proteins were observed to have similar reactivities and spectroscopic properties to those found for the proteins in solution. For example, encapsulated CuZnSOD was demetallated and remetallated, encapsulated ferricytochrome c was reduced and then reoxidized, and encapsulated met Mb was reduced to deoxy Mb and then reacted either with dioxygen to make oxy Mb or with carbon monoxide to make carbonyl Mb.

THE SPECIFICITY OF ENZYMES AND other proteins for binding of substrates, inhibitors, or related molecules makes them attractive candidates for molecular sensors if they can be incorporated into a matrix that stabilizes them and does not block signal transmission. Enzymes immobilized in or on inert matrices have been studied extensively as catalysts, but the matrices in general have not been suitable for use in optically based molecular sensors because they are opaque (*1, 2*). The sol-gel process for the preparation of transparent glasses is an attractive method for preparing optically based molecular sensors, but the normal synthetic conditions are too harsh for most proteins (*3, 4*). In order to overcome this problem, we have developed a new sol-gel procedure in which milder conditions are used and have discovered that proteins encapsulated in glasses through this new procedure retain their native spectroscopic properties and characteristic reactivities (*5*).

The sol-gel process is a technique that can be used to prepare transparent oxide glasses by hydrolysis and polycondensation of alkoxides (*3, 4*). Little or no heating is required, and consequently the gels can be doped with molecules whose poor thermal stability precludes their incorporation in traditional inorganic hosts (*6*). Such molecules become entrapped in the growing covalent gel network rather than being chemically bound to the inorganic matrix. The process can be divided into the following steps: (i) forming a solution, (ii) gelation, (iii) aging, and (iv) drying (*3, 4, 7*). In the typical preparation of a silica glass by the sol-gel method, one starts with an appropriate alkoxide, in our case $Si(OCH_3)_4$ (tetramethylorthosilicate or TMOS), which is mixed with water, an acidic catalyst such as HCl, and a mutual solvent, such as methanol, to form a solution. Hydrolysis leads to the formation of silanol groups (Si–OH) and methanol. The silanol and methoxy groups react further to form siloxane (Si–O–Si) groups, with water and methanol as additional reaction products. The process of condensation to form siloxane groups continues to occur during aging. The drying stage involves careful removal of the solvent phase, and the resulting dried gels, termed xerogels, are rigid, transparent materials with good thermal and dimensional stability. The fine pore networks in dried gels (<100 Å) do not scatter visible radiation and allow the diffusion of small molecules.

Conventional sol-gel procedures are not generally suitable for encapsulation of proteins because high acidity and high concentrations of alcohol lead to denaturation of most proteins. We therefore modified the methods described by Esquivias and Zarzycki (*8*). The first and most important modification was the addition of buffer after HCl-catalyzed hydrolysis of the TMOS and before the protein to be encapsulated was added. The addition of buffer brought the pH above 5.0 and thus prevented acid denaturation or aggregation or both. We also found that we could omit the addition of extra alcohol from the preparation of the silica sol. We sonicated the preparation, as in the procedure of Esquivias and Zarzycki (*8*), but only prior to the addition of protein, because sonication is likely to cause denaturation or shearing or both of some proteins.

We focused initially on three proteins, bovine copper-zinc superoxide dismutase (CuZnSOD), horse heart cytochrome c, and horse heart myoglobin (Mb). These metalloproteins were chosen because they have characteristic visible electronic absorption spectral properties that may be monitored

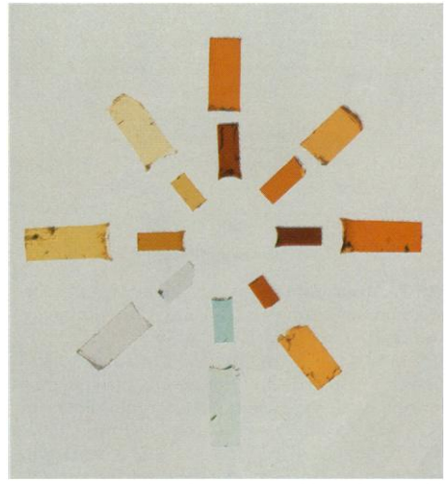

Fig. 1. Cytochrome c, copper-zinc superoxide dismutase, and myoglobin in aged and xerogel pairs. The inner circle consists of xerogels, and the outer circle contains the corresponding aged gels. Clockwise, starting from the top, are gels containing two different concentrations of ferrocytochrome c, two different concentrations of ferricytochrome c, CuZnSOD, CuZnSOD with cyanide bound, and two different concentrations of met myoglobin. The gels were prepared as described in the text and in (*12*). The solutions were poured into 1.5-ml disposable polystyrene cuvettes and sealed with Parafilm. Gelation occurred within 5 min. The process of aging commences after gelation is complete and is accompanied by shrinkage of the gel. Aging is complete after ~2 weeks. Xerogels (glasses) were formed from the aged gels by slow evaporation of the solvents (methanol and water). This process generally took from 1 to 4 weeks, depending on the rate of evaporation. The resulting xerogels have 1/8 of the volume of the aged gel, and the protein concentration is therefore increased by a factor of 8 relative to the starting solution. [Photograph by L. Meluso]

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readily in optically transparent glasses. CuZnSOD has a molecular weight of ~31,200 and is comprised of two identical subunits, each containing one Cu^{2+} and one Zn^{2+} ion in close proximity, held 6.3 Å apart by the imidazolate ring of a His residue (*9, 10*). Cytochrome c is a heme-containing electron transfer protein with a molecular weight of ~12,400, and Mb is a heme-containing dioxygen-binding protein with a molecular weight of ~18,800 (*11*). These proteins were immobilized by microencapsulation in stable SiO_2 glasses with our new technique (*12*). The resulting gels and glasses are shown in Fig. 1, the inner circle consisting of the xerogels and the outer circle consisting of aged gels. The effects of encapsulation on the spectroscopic properties of the proteins were monitored throughout the processes of gelation, aging, and drying of the gels.

Optically transparent materials containing CuZnSOD retained the blue-green color characteristic of the protein throughout the various stages of the sol-gel process. The visible absorption spectrum of the encapsulated enzyme was monitored throughout this process and was unchanged from that observed for the protein in solution (*9, 10*). The xerogel was a blue-green–colored piece of transparent glass with a volume 1/8 of that of the initial solution (see Fig. 1). The blue-green color of the protein is due to *d-d* transitions of the Cu^{2+} d^9 metal ion. Both in the aged gel and in the xerogel, the visible absorption band due to the characteristic Cu *d-d* transition remained unchanged. The position of this band is sensitive to the metal ion occupancy of the protein and the protein conformation (*9, 10*). The similarity of the spectra of the gel-encapsulated protein with those of the protein in solution is thus a good indication that the structure of the protein is not substantially changed upon encapsulation.

We tested the effect of encapsulation on the reactivity of CuZnSOD by reacting the gels containing CuZnSOD with cyanide. In solution, CN^- reacts readily with CuZnSOD and causes the color to change from blue-green to violet and the visible absorption band at 680 nm to disappear and a new band at 530 nm to appear. Soaking both the aged gels and xerogels in solutions of KCN resulted in color and spectroscopic changes similar to those found in solution (see Figs. 1 and 2) (*9, 10, 13*). Another characteristic

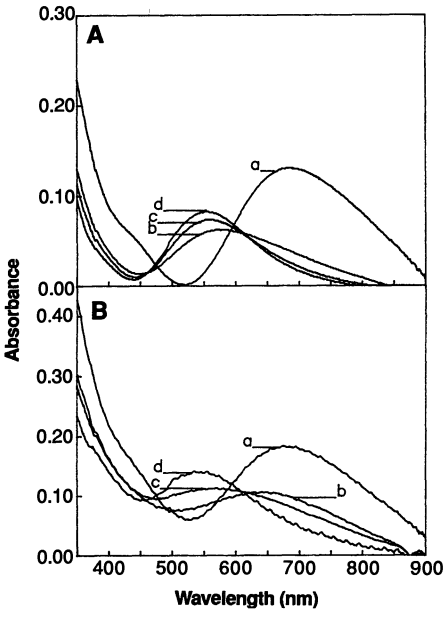

Fig. 2. Absorption spectra of CuZnSOD in solution and in aged gels and after reaction with cyanide ion (*16*). (**A**) Curve a shows CuZnSOD (0.5 mM, 0.1 M tris, pH 7.3) in solution and curves b, c, and d show the results of sequential titration with cyanide ion (two equivalents of KCN per addition). (**B**) Curve a shows CuZnSOD (0.65 mM, 0.1 M tris, pH 7.3) in aged gels, and curves b, c, and d show the results of sequential titration with cyanide ion (1.7 equivalents of KCN per addition).

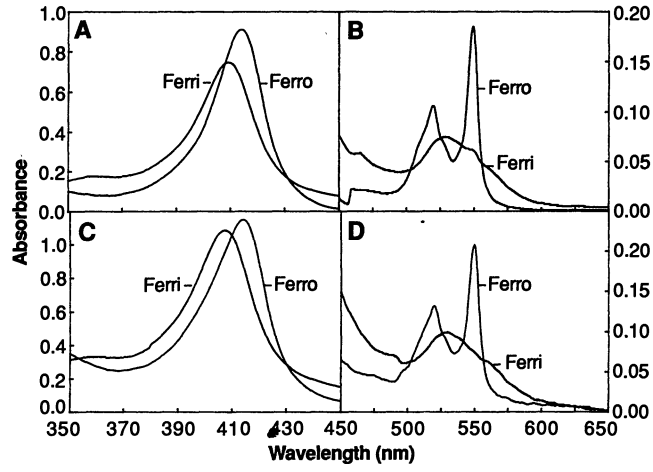

Fig. 3. Absorption spectra of ferricytochrome c and ferrocytochrome c in solution and in aged gels. (**A** and **B**) Ferricytochrome c (0.01 mM, 0.01 M phosphate, pH 7.0) in solution and its subsequent reduction with excess dithionite (1 to 2 mg) to form ferrocytochrome c. (**C** and **D**) Ferricytochrome c (0.012 mM, 0.01 M phosphate, pH 7.0) in aged gels and its subsequent reduction with excess dithionite (1 to 2 mg) to form ferrocytochrome c. Peaks between 450 to 500 nm are instrumental artifacts.

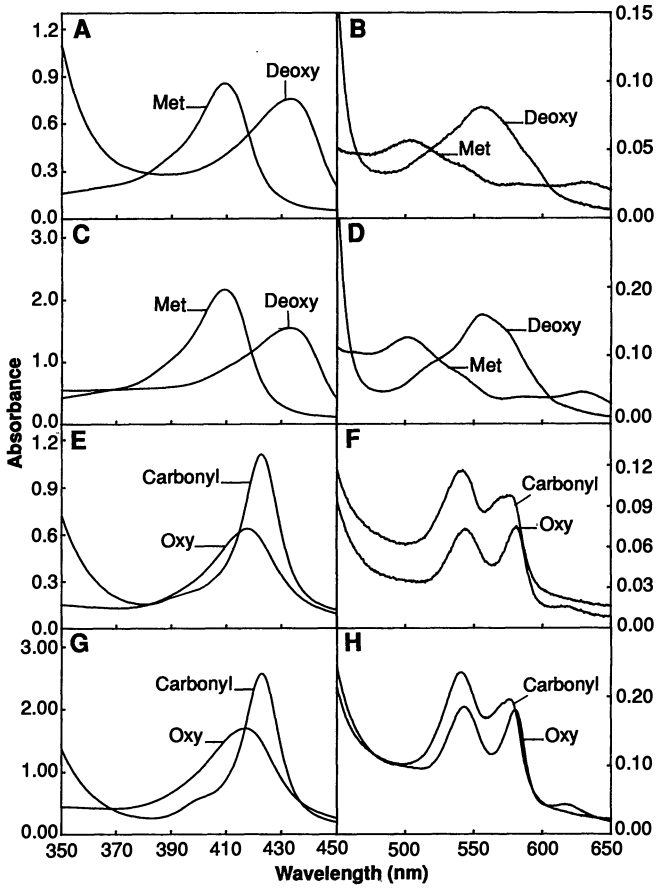

Fig. 4. Absorption spectra of met Mb, deoxy Mb, carbonyl Mb, and oxy Mb in solution (5 μM) and in aged gels (13 μM). (**A** and **B**) Met Mb (0.01 M phosphate, pH 7.0) and deoxy Mb prepared by reduction of met Mb with dithionite (1 to 2 mg) under argon. (**C** and **D**) Aged gels containing met Mb and deoxy Mb. The conditions of the reaction were the same as described for (A) and (B) except that the excess dithionite was washed out of the deoxy sample. (The absorbance below 370 nm is due in part to dithionite.) (**E** and **F**) Carbonyl Mb prepared from deoxy Mb by bubbling with CO for 1 hour. Oxy Mb generated by centrifugation of the carbonyl Mb solution through a Sephadex G-25 column (5 ml of packing material, 0.01 M phosphate, pH 7.0). This treatment resulted in the removal of dithionite and exposure of the sample to O_2 with which it reacted. (**G** and **H**) Aged gels containing carbonyl Mb and oxy Mb. The conditions of the reaction were the same as described for (E) and (F) except that the dithionite was removed from the gel by washing with phosphate buffer.

reactivity of CuZnSOD in solution is the removal of the metal ions by treatment with the metal-ion chelating reagent EDTA (*9, 10*). The metal ions were removed from the encapsulated protein in xerogels under similar conditions, resulting in a colorless glass. The encapsulated apoprotein (metal-free protein) was remetallated by addition of metal ions, restoring to the glass both the color and the visible absorption spectrum, in a manner also identical to the solution process (*14*). This process, that is, removal and addition of metal ions, was repeated without any apparent change in the spectroscopic properties of the encapsulated CuZnSOD.

We also encapsulated ferricytochrome c and found no detectable change in its spectroscopic properties as a result of encapsulation (see Fig. 3). When the red-brown ferricytochrome c aged gels or xerogels were soaked in a solution of sodium dithionite, the glasses became salmon colored and the resulting spectra were virtually identical to that of reduced ferrocytochrome c in solution (see Figs. 1 and 3). The encapsulated ferrocytochrome c spontaneously reoxidized when exposed to air. This oxidation-reduction process was repeated without deterioration of spectroscopic properties.

The third protein system chosen for study was Mb. Met Mb, that is, containing iron in the Fe^{3+} state, was encapsulated, and the resulting brown gels (see Fig. 1) were soaked in a solution of sodium dithionite to give deoxy Mb, which contains iron in the Fe^{2+} state. The deoxy Mb in the gels was then reacted with O_2 by exposure to air to give oxy Mb, MbO_2, and with CO to give carbonyl Mb, MbCO. The spectroscopic changes associated with all of these reactions in the aged and the xerogels (*15*) were similar to those which occur under the same conditions in solution (see Fig. 4).

These experiments demonstrate that it is possible to create inorganic materials that contain biochemically active macromolecules. Specifically, we find that: (i) a wide variety of proteins may be encapsulated in sol-gel matrices giving optically transparent glasses; (ii) the proteins are remarkably stable in such matrices; (iii) these proteins undergo characteristic reversible reactions in the gel glasses; and (iv) spectroscopic changes occurring in the gel-glasses can be readily quantified with optical spectroscopy. The prospects are excellent for the use of these novel materials in biosensors.

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5. These results complement those of another study, which showed that a number of enzyme systems (glucose oxidase, peroxidase, chymotrypsin, and trypsin) encapsulated in transparent silica glass matrices by this method remained enzymatically active. [S. A. Yamanaka *et al.*, unpublished results; L. M. Ellerby *et al.*, unpublished results].
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12. The silica sol was prepared by mixing tetramethylorthosilicate (TMOS, 15.22 g) and deionized water (3.38 g) followed by the addition of HCl (0.04 M, 0.22 g), which was used to catalyze the reaction. After sonication of the reaction mixture had proceeded for 20 minutes, sonicated sol (2 ml) was mixed with buffer (2 ml, 0.01 M sodium phosphate, pH 6.0). The protein solution (0.01 to 5 mM. 1 ml, 0.01 M sodium phosphate, pH 6.0) was then added directly to the buffered sol.
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14. CuZnSOD in aged gels showed different behavior. Removal of the metal ions to prepare the apoSOD in aged gels proceeded smoothly. However, addition of Cu^{2+} and Zn^{2+} to these apoSOD aged gels resulted in the appearance of a visible absorption band at 650 rather than at 680 nm. We do not understand the source of this difference since demetallation and remetallation of the CuZnSOD xerogel (that is, prepared by drying of an aged gel) gave spectral changes virtually identical to those occurring in solution for the same processes. One possible explanation is that the apoprotein was trapped in a nonnative configuration, because polymerization of the silanol groups is still proceeding actively at that stage in the aged gel, and remetallation thus cannot restore the native conformation. In contrast, polymerization in the xerogel should be much closer to completion.
15. In the case of Mb, the aged gel was washed with 0.05 M tris, 0.1 M sodium sulfate, pH 7.5, prior to drying.
16. In some instances, the aged gels show a sloping base line, which we tentatively attribute to scratching of the surface during handling. For xerogels, which are rigid glasses, this phenomenon is usually not observed.

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Rectification of STM Current to Graphite Covered with Phthalocyanine Molecules

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The scanning tunneling microscope (STM) can be used to measure current-voltage characteristics on an atomic scale. The attachment of copper phthalocyanine molecules, in contrast to a variety of other molecules, to graphite changes the electrical characteristics of the STM from relatively symmetric to highly asymmetric or rectifying. Evidence is presented to show that the asymmetry arises because of the electronic energy levels of the copper phthalocyanine. The organic molecules were bonded to the graphite by an acid-base reaction that may have wide applicability.

THE STM INCORPORATES A SHARP metallic tip that is used to observe and affect the properties of surfaces on an atomic scale. The tip has been used as an electrode with which asymmetric, or rectifying, electrical characteristics have been observed on some surfaces. [Examples include pure semiconductors (*1, 2*), a metal (*3*), and semiconductors with atomic or small molecular adsorbates (*4*).] Reviews of STM experiments on organic and biological molecules (*5, 6*) do not mention unusual electrical characteristics, such as rectification. We have been studying the effects of organic molecules because it is expected that, when the electronic energy levels of the adsorbates are close to the Fermi level E_F of the electrodes, the molecules might strongly affect the tunneling characteristics (7). This offers the possibility of measuring the molecular energy levels. Also, because the energy levels of organic molecules depend on their structure and composition, there is the possibility of choosing the energy levels to obtain desired electrical properties. It is advantageous to choose molecules that are large enough so that it is possible to make chemical substitutions, in order to chemically bond them to particular electrodes, with minimal changes in the electronic structures of the molecules. We report here that the asymmetry of the STM current-voltage (*I-V*) characteristics of graphite surfaces (Fig. 1A) is changed by an order of magnitude when copper phthalocyanine molecules are chemically attached (Fig. 1, B and C). Other molecules that we attached to graphite did

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