

Effects of Encapsulation in Sol–Gel Silica Glass on Esterase Activity, Conformational Stability, and Unfolding of Bovine Carbonic Anhydrase II

Jovica D. Badjić and Nenad M. Kostić*

Department of Chemistry, Iowa State University, Ames, Iowa 50010

Received August 6, 1999

Bovine carbonic anhydrase II retains its overall conformation when encapsulated in silica monoliths by the sol–gel method. Upon gradual heating the enzyme in solution precipitates at 64 °C, whereas the encapsulated enzyme does not; it unfolds, with the nominal melting temperature of 51 ± 3 °C. Even at 74.0 °C, the encapsulated enzyme is only ~77% unfolded, but it does not refold upon cooling. Upon treatment with guanidinium chloride, the degree of enzyme unfolding is 100% in solution but only ~83% within the silica matrix. Again, the enzyme does not refold upon removal of the denaturing agent from the glass. The glass matrix constrains the motions of the encapsulated protein molecules and prevents both their full unfolding and refolding. The former may be taken for “stabilization”, the later for “destabilization” of the native conformation. Evidently, the effect of the glass on the encapsulated protein cannot be described in these general terms. The encapsulated enzyme obeys the Michaelis–Menten kinetic law as it catalyzes hydrolysis of *p*-nitrophenyl acetate. The apparent Michaelis constant (K_M) is practically the same in the glass and in solution, but the apparent turnover number (k_{cat}) and specific activity for the encapsulated enzyme are only 1–2% of these values for the enzyme in solution. Because the substrate diffuses slowly into silica pores, most of the catalysis is due to the enzyme embedded near the surfaces of the glass monolith. Effect of encapsulation on structure and activity of proteins should be studied in quantitative detail before protein-doped glasses can be used as reliable biosensors, heterogeneous catalysts, and composite biomaterials.

Introduction

Controlled hydrolysis of alkoxides and polymerization of the resulting oxyacids, the sol–gel method, is very useful in many branches of chemistry and materials science. This method is used for the making of sensors, catalyst supports, optical elements, coatings, and special polymers.^{1–4} Enzymes, catalytic antibodies, and other proteins may be entrapped in robust silica glasses under mild conditions, so that they retain their chemical activity. Because the resulting bioceramics can be fabricated as monoliths, thin films, powders, and fibers, these recent achievements open many possibilities for research and application in bioanalytical chemistry, biocatalysis, biotechnology, and environmental technology.^{5–12} Hydrogels and xerogels are often referred to simply as sol–gel glasses and, colloquially, as sol–gels.

Despite widespread interest in various applications of protein-doped glasses, relatively little is known about their fundamental properties and, especially, about interactions between the protein molecules and the matrix in which they are embedded. These interactions govern the reactivity of the embedded protein, and they must be understood before useful devices can be made. Particularly important are the possible effects of encapsulation on conformation of enzymes, their interactions with substrates, and their catalytic ability.

Research in our laboratory showed that proteins and also small molecules may behave quite differently in traditional solutions and in the pores of a hydrogel.^{13,14a} We took advantage of transparency of silica monoliths to study photoinduced chemical reactions. The triplet state of zinc-substituted heme proteins cytochrome *c* and

(1) Avnir, D.; Braun, S.; Lev, O.; Ottolenghi, M. *Chem. Mater.* **1994**, *6*, 1605–14.

(2) Avnir, D. *Acc. Chem. Res.* **1995**, *28*, 328–34.

(3) Rosenfeld, A.; Blum, J.; Avnir, D. *J. Catal.* **1996**, *164*, 363–368.

(4) Wojcik, A. B.; Klein, L. C. *Appl. Organomet. Chem.* **1997**, *11*, 129–135.

(5) Livage, J. *C. R. Acad. Sci., Ser. II: Mec., Phys., Chim., Astron.* **1996**, *322*, 417–27.

(6) Dunn, B.; Zink, J. I. *Chem. Mater.* **1997**, *9*, 2280–2291.

(7) Braun, S.; Rappoport, S.; Shtelzer, S.; Zusman, R.; Druckmann, S.; Avnir, D.; Ottolenghi, M. *Design and properties of enzymes immobilized in sol–gel glass matrices*; Kamely, D. C., Ananda M, Ed.; Kluwer: Boston, Mass, 1991; pp 205–18.

(8) Reisfeld, R.; Joergensen, C. K. *Struct. Bonding (Berlin)* **1992**, *77*, 207–56.

(9) Braun, S.; Shtelzer, S.; Rappoport, S.; Avnir, D.; Ottolenghi, M. *J. Non-Cryst. Solids* **1992**, *147–148*, 739–43.

(10) Miller, J. M.; Dunn, B.; Valentine, J. S.; Zink, J. I. *J. Non-Cryst. Solids* **1996**, *202*, 279–289.

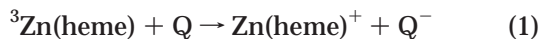
(11) Zheng, L.; Reid, W. R.; Brennan, J. D. *Anal. Chem.* **1997**, *69*, 3940–3949.

(12) Lan, E. H.; Dunn, B.; Valentine, J. S. V.; Zink, J. I. *J. Sol-Gel Sci. Technol.* **1996**, *7*, 109–116.

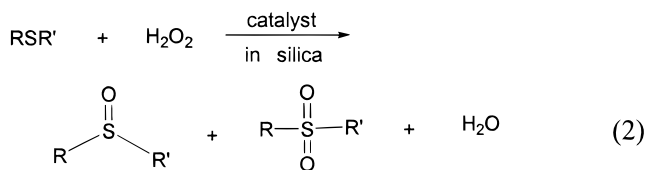
(13) Shen, C.; Kostić, N. M. *J. Electroanal. Chem.* **1997**, *438*, 61–65.

(14) (a) Shen, C.; Kostić, N. M. *J. Am. Chem. Soc.* **1997**, *119*, 1304–1312. (b) Kadnikova, E. N.; Kostić, N. M. Unpublished work.

myoglobin, designated Zn(heme), is oxidatively quenched by charged metal complexes and neutral organic compounds, designated Q, as in



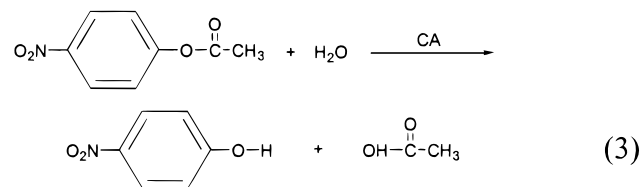
Because the protein was encapsulated and the quenchers were dialyzed in, both reactants were already inside the glass before the light pulse. Therefore, the problem of macroscopic diffusion was avoided, and precise kinetic experiments were possible. They revealed interesting electrostatic and structural properties of the glass interior, which make silica matrixes quite different from pure solvent as a reaction medium. The same pair of reactants behaves differently when confined in the matrix pores and when free in solution. We also examined the ability of peroxidases encapsulated in silica and in alkylated silica to catalyze oxidation of various sulfide compounds,^{14b} as in



Our studies^{13,14a} of the first kind disproved the popular notion that because the pores in silica are relatively large, small molecules diffuse freely in and out of the glass matrix. In fact, even prolonged soaking of silica monoliths in electrolyte solutions may not ensure equal partitioning of a given ion between the monolith and solution. At pH value at which the pore walls are negatively charged, anions, such as $[\text{Fe}(\text{CN})_6]^{3-}$, are only partially taken up, whereas cations, such as $[\text{Ru}(\text{NH}_3)_6]^{3+}$, are preferentially taken up. In either case, internal and external concentrations will remain unequal after the equilibrium is reached. Since many analytes are ions, such interactions must be understood before reliable biosensors for these analytes may be designed.

Several elegant, recent studies found that proteins are stabilized by encapsulation in silica.^{15–17} But the notion of “stability” has various meanings. We decided to examine one aspect of protein stability, namely propensity for controlled unfolding and refolding. The few kinetic studies of encapsulated enzymes to date all have been done with powdered glasses, to minimize the problem of substrate diffusion.^{18–21} We decided to study this diffusion in glass monoliths. An advantage of monoliths over powders is transparency, which permits the use of spectrophotometry.

We chose a well-characterized enzyme, isozyme II of bovine carbonic anhydrase, and a simple reaction, hydrolysis of *p*-nitrophenyl acetate:^{22–25}



By comparing the protein unfolding behavior, and also the kinetics of the reaction in eq 3 inside the glass and in solution, one can learn about the unexpected and important differences between the glass matrix and solvent as reaction media.

Experimental Procedures

Chemicals. Bovine carbonic anhydrase II, ultrapure guanidinium chloride, 3-(*N*-morpholino)propanesulfonic acid (MOPS), and its sodium salt were obtained from Sigma Chemical Co. Tetramethyl orthosilicate (also called tetramethoxysilane and designated TMOS), *p*-nitrophenyl acetate, *p*-nitroanisole, and acetonitrile were obtained from Aldrich Chemical Co. The salts $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ were obtained from Fischer Chemical Co. The salt $[\text{Ru}(\text{NH}_3)_5\text{Cl}]\text{Cl}_2$ was obtained from Strem Chemical Co. Distilled water was demineralized to electrical resistivity greater than 17 MΩ cm. A MOPS buffer having the ionic strength 11.6 mM was prepared by dissolving 1.70 g of the acid and 0.97 g of its sodium salt in 400 mL of water and adjusting the pH to 7.00 with a 0.100 M solution of NaOH. Phosphate buffer with ionic strength of 10.0 mM was prepared by dissolving 0.050 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 0.86 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ in 1.000 L of water and adjusting the pH to 8.00 with a 0.100 M solution of NaOH. Ionic strength, not concentration, is specified in this article. The Accumet 925 pH meter was equipped with a glass electrode. Glass monoliths (SLABS) intended for different experiments were prepared with, and finally kept in, different buffers. For the monoliths intended for studies of carbonic anhydrase conformation and unfolding, 11.6 mM MOPS buffer at pH 7.00 was used; for the monoliths intended for enzymatic catalysis and diffusion of substrate analogues, 10.0 mM phosphate buffer at pH 8.00 was used.

The Sol-Gel Process. Silica sol was prepared by a published procedure.¹⁸ A mixture of 15.25 g of $\text{Si}(\text{OCH}_3)_4$, 3.38 g of water, and 0.300 g of 0.040 M HCl was ultrasonicated in an ice bath for 30 min. A 4.64-mL portion of the resulting sol was mixed with 5.64 mL of the appropriate buffer (see above) and kept in an ice bath. A stock solution of carbonic anhydrase II containing a variable amount (2.0–14 mg) of the enzyme in 3.4 mL of the appropriate buffer (see above) was added to the sol, and the clear liquid was transferred to polystyrene cuvettes sized 10 × 10 × 40 mm. The cuvettes were sealed with Parafilm and kept at 4 °C. During the 14 days of aging the monoliths were washed twice a day with the same buffer with which they had been made. Then the Parafilm was removed for partial drying, still at 4 °C, and in additional 14 days the monoliths shrunk to 8 × 8 × 27 mm. The final volume was ~50% of that of the aged monoliths. The partially dried monoliths were stored in the same buffer with which they had been prepared, depending on the purpose.

Determining the Enzyme Concentration. Concentration of bovine carbonic anhydrase II in free solution and in solution

(15) Chen, Q.; Kenausis, G. L.; Heller, A. *J. Am. Chem. Soc.* **1998**, *120*, 4582–4585.

(16) Heller, J.; Heller, A. *J. Am. Chem. Soc.* **1998**, *120*, 4586–4590.

(17) Shtelzer, S.; Rappoport, S.; Avnir, D.; Ottolenghi, M.; Braun, S. *Biotechnol. Appl. Biochem.* **1992**, *15*, 227–35.

(18) Yamanaka, S. A.; Nishida, F.; Ellerby, L. M.; Nishida, C. R.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Chem. Mater.* **1992**, *4*, 495–7.

(19) Yamanaka, S. A.; Dunn, B.; Valentine, J. S.; Zink, J. I. *J. Am. Chem. Soc.* **1995**, *117*, 9095–6.

(20) Braun, S.; Rappoport, S.; Zusman, R.; Avnir, D.; Ottolenghi, M. *Mater. Lett.* **1990**, *10*, 1–5.

(21) Dosoretz, C.; Armon, R.; Starosvetzky, J.; Rothschild, N. *J. Sol-Gel Sci. Technol.* **1996**, *7*, 7–11.

(22) Pocker, Y.; Meany, J. E. *Biochemistry* **1967**, *6*, 239–46.

(23) Pocker, Y.; Storm, D. R. *Biochemistry* **1968**, *7*, 1202–14.

(24) Anderson, J.; Byrne, T.; Woelfel, K. J.; Meany, J. E.; Spyridis, G. T.; Pocker, Y. *J. Chem. Educ.* **1994**, *71*, 715–18.

(25) Thorslund, A.; Lindskog, S. *Eur. J. Biochem.* **1967**, *3*, 117–23.

confined in the pores of silica monoliths was determined on the basis of absorbance at 280 nm and the plot of that absorbance versus the enzyme concentration in a 11.6 mM MOPS buffer at pH 7.00. For accuracy, liquids were measured by weight rather than volume; because the buffer solutions were dilute, their density was taken to be unity. This approximation did not introduce a significant error in subsequent experiments. The enzyme concentration in the stock solution was 3.84 mg/mL; from it, by dilution, were made six more solutions, and their absorbances were measured. The slope of the plot gave the conversion factor of 1.48 mL/mg. Monolith doped with the enzyme was soaked in the buffer appropriate for the subsequent experiments, as explained in the preceding subsection. Both the doped monolith (in the sample beam) and the undoped monolith of the same dimensions (in the reference beam) were held in standard cuvettes, filled with the buffer. The ultraviolet absorption spectrum was recorded, and the concentration was calculated with the conversion factor, taking into account the path length of 0.80 cm. Concentration so determined always equaled that calculated from the amount of the enzyme encapsulated, considering the increase in concentration caused by shrinkage of the partially dried monolith.

Circular Dichroism Spectra. These spectra were recorded with the instrument J-710 by JASCO, equipped with a peltier thermoelectric cell. When the samples were solutions, the sensor was placed inside the cuvette, and temperature was kept within ± 0.05 °C; when the samples were silica monoliths immersed in solution, the sensor had to be placed next to the cuvette, and temperature was kept within ± 0.10 °C. In experiments not concerning thermal unfolding and refolding, temperature was 20.0 °C, and monoliths were kept at it for 30 min before the measurements were started. Far-UV CD spectra, in the region 200–240 nm, were recorded in 1-mm cuvettes, with silica monoliths (1 mm thickness) and free solutions that were both 12 μ M in carbonic anhydrase. Near-UV CD spectra, in the region 240–340 nm, were recorded in 10-mm cuvettes; the enzyme concentration was 28 μ M in silica and 25 μ M in free solution. The soaking buffer was always 11.6 mM MOPS at pH 7.00. In calculation of molar residue ellipticity,²⁶ the molecular mass of the enzyme was 28 900 Da; the path length was 0.80 cm in the case of doped silica monoliths (because of their shrinkage); and the enzyme concentration was determined as explained above.

Unfolding of Carbonic Anhydrase in Free Solution. In studies of thermal denaturation, the solution of the enzyme in 11.6 mM MOPS buffer at pH 7.00 had a concentration of 0.761 mg/mL. Ellipticity at 245 nm in a 10-mm cuvette was recorded continuously, during slow heating at the rate of 0.50 °C/min. In addition, the CD spectrum in the range 240–340 nm was recorded at 20.0, 40.0, 55.0, and 75.0 °C. A stock solution of guanidinium chloride (GdmCl) was prepared carefully, by dissolving 15.1591 g of this denaturant in 25.0251 g of the 11.6 mM MOPS buffer at pH 7.00.²⁶ Concentration was also checked by measuring the refraction index of the solution. A stock solution of bovine carbonic anhydrase II was prepared by adding 1.0704 g of NaCl to 6.0227 g of the solution containing 3.84 mg of this enzyme per milliliter of 11.6 mM MOPS buffer at pH 7.00. The protein concentration was checked by measuring absorbance at 280 nm. To 0.5000 g of the stock solution of the protein (and NaCl) were added required weights of the GdmCl stock solution and of the MOPS buffer, to obtain a solution that was 0.600 M in NaCl and between 0 and 3.488 M in GdmCl. A series of these solutions differing only in the denaturant concentration was equilibrated at room temperature for 12 h before their near-UV CD spectra were recorded. The denaturation was reversed (i.e., the protein was refolded) by exhaustive dialysis with Slide-A-Lyzer 10K cassettes, obtained from Pierce Chemical Co. A 3.00-mL sample of the solution after the CD spectroscopic measurements was dialyzed against 400 mL of the 11.6 mM MOPS buffer at pH 7.00 that was 0.100 M in NaCl.²⁷ Then the near-UV CD spectrum was recorded again.

(26) Giletto, A.; Pace, C. N. *Protein Stability*; Price, N. C., Ed.; Academic: San Diego, CA, 1996; pp 233–239.

Unfolding of Encapsulated Carbonic Anhydrase by Heating. A transparent silica monolith sized $8 \times 8 \times 27$ mm containing 28 μ M bovine carbonic anhydrase II was soaked in a 11.6 mM MOPS buffer at pH 7.00 inside a quartz cuvette sized $10 \times 10 \times 40$ mm. The temperature of the cell holder was raised from 20.0 to 74.0 °C in 3.0-deg steps. After a 30-min period of equilibration, the near-UV CD spectrum was recorded (for an additional 20 min). The mean residue ellipticities at 245 and 269 nm were plotted against temperature. In control experiments performance of the CD instrument was verified by recording near-UV spectra of the monolith not doped with the protein at 20.0 and 74.0 °C; the heating was done gradually, with the doped monolith. The doped monolith was then cooled, at a rate of 3.0 °C/h, back to 20.0 °C, and the CD spectrum was recorded again. The apparent melting (denaturation) temperature of the protein, T_m , was obtained by fitting the plots at 245 and 269 nm to the simple model of direct conversion of the folded state into an unfolded state.^{28,29}

Unfolding of Encapsulated Carbonic Anhydrase with Guanidinium Chloride. Because the denaturing agent in this salt is the cation, we first determined the conditions under which cations can be introduced into sol-gel silica glass. In these preliminary experiments we used the salt $[\text{Ru}(\text{NH}_3)_5\text{Cl}]\text{Cl}_2$ because its cation is colored and because its charge is higher than that of the Gdm^+ ion. A silica monolith sized $8 \times 8 \times 27$ mm was soaked, for 24 h at 25 °C, in a 5.2×10^{-4} M solution of the complex salt in a 11.6 mM MOPS buffer at pH 7.00 that was 0.600 M in NaCl. Diffusion was assisted by gentle shaking. The UV-vis spectrum of the monolith infused with $[\text{Ru}(\text{NH}_3)_5\text{Cl}]^{2+}$ cations was recorded against an "empty" monolith in the reference beam and compared with the spectrum of the fresh soaking solution. Concentration of the complex cation was determined on the basis of the absorptivity $\epsilon_{326} = 1850 \text{ M}^{-1}\text{cm}^{-1}$ and the optical path length of the monolith of 0.80 cm. Transparent silica monoliths sized $8 \times 8 \times 27$ mm containing 28 μ M bovine carbonic anhydrase II were each soaked in 10.0 mL of 11.6 mM MOPS buffer at pH 7.00 that was 0.600 M in NaCl and between 0 and 4.35 M in GdmCl. Diffusion of the denaturant and NaCl into the doped glass was assisted by gentle shaking for 24 h at 25 °C. Circular dichroism spectra at 240–340 nm of all the monoliths were recorded, and molar residue ellipticity at 269 nm was plotted against the GdmCl concentration. The monolith containing 4.35 M GdmCl was soaked in 400 mL of the 11.6 mM MOPS buffer at pH 7.00 that was 0.600 M in NaCl, for 24 h at 4 °C. This same procedure was repeated with a fresh buffer. Near-UV CD spectra were recorded at 24 and 48 h, after each soaking.

Degree of Unfolding of Carbonic Anhydrase. This degree, designated f_u , varies from 0.0 to 1.0 and is proportional to the molar residue ellipticity at 269 nm. The limiting values correspond to the protein dissolved in a 11.6 mM MOPS buffer containing no GdmCl and 3.488 M GdmCl.

This denaturant completely unfolds bovine carbonic anhydrase.³⁰ The same f_u scale was used for the enzyme in free solution and encapsulated in silica.

Diffusion of Small Molecules into Silica Glass. The substrate for carbonic anhydrase (acting as an esterase) is *p*-nitrophenyl acetate. An unreactive analogue of it is *p*-nitroanisole. The solvent was a 9:1 mixture by volume of a 10 mM phosphate buffer at pH 8.00 and acetonitrile. Solutions were made in volumetric flasks. Their concentrations were 1.00 mM in the substrate and 0.14 mM in its analogue. Silica monoliths were sized $8 \times 8 \times 27$ mm. Three monoliths doped with 28 μ M carbonic anhydrase II were each soaked in 30.0 mL of the substrate solution. The yellow color of *p*-nitrophenoxide anion advanced into the monoliths for 10, 60, and 600

(27) Wong, K.-P.; Tanford, C. *J. Biol. Chem.* **1973**, *248*, 8518–23.

(28) Biltonen, R. L.; Lumry, R. *J. Am. Chem. Soc.* **1969**, *91*, 4256–64.

(29) Lee, J. C.; Timasheff, S. N. *J. Biol. Chem.* **1981**, *256*, 7193–201.

(30) Uversky, V. N.; Ptitsyn, O. B. *J. Mol. Biol.* **1996**, *255*, 215–28.

min. From each soaked monolith a $8 \times 8 \times 7$ mm slice was obtained by cutting off 10-mm pieces from both ends, and this slice was photographed with a Canon T-60 camera equipped with a macro lens having FD of 50 mm and 1:3.5 ratio and with an extension tube having FD of 25 mm. An undoped (enzyme-free) monolith sized $8 \times 8 \times 27$ mm was soaked first in the solvent (the aforementioned 9:1 mixture) and then in 20.0 mL of the *p*-nitroanisole solution for 18 h. Occasionally the monolith was removed from the solution, and its absorbance at 316 nm was recorded against an unsoaked monolith in the reference beam. Absorbance at 316 nm was plotted versus the soaking time.

Kinetics of Catalysis by the Encapsulated Carbonic Anhydrase. These experiments were done with 32 very similar silica monoliths sized $8 \times 8 \times 27$ mm, chosen from a set of 64 monoliths prepared under identical conditions. These 32 were divided into four sets of 8; members of each set were doped with the same concentration of carbonic anhydrase II: 10, 31, 56, or 79 μM . Each monolith was first soaked in 100 mL of a 10 mM phosphate buffer at pH 8.00 to establish the pH value of the interior. After this equilibration, each monolith was soaked in 2.0 mL of the solvent in which the hydrolysis was to be done, a 9:1 mixture by volume of this buffer and acetonitrile; the containers polystyrene spectrophotometric cuvettes sized $10 \times 10 \times 40$ mm, were thermally equilibrated in a water bath at 25 °C for 15 min. The reaction was started by addition to each cuvette of a small volume of a solution of *p*-nitrophenyl acetate in the aforementioned solvent. The substrate concentrations in the reaction mixture spanned the range 1.0 to 10.0 mM. Absorbance at 400 nm of *p*-nitrophenoxide anion, a sum of contributions from the monolith and the surrounding solution, was measured over time. Absorptivity of this product, $\epsilon_{400} = 16\,840 \text{ M}^{-1} \text{ cm}^{-1}$, was determined by measuring absorbances of a series of solutions in the same solvent having concentrations in the relevant range, 1.0–10.0 mM. After the incubation period of 50 s, dependencies of absorbance at 400 nm (*A*) on time (*t*) were fitted to

$$A = V_0 t + c \quad (4)$$

with the linear least-squares method embodied in the program SigmaPlot 1.0. In similar experiments with undoped silica monoliths, initial rates for "background" hydrolysis at the same concentration of *p*-nitrophenyl acetate were found to be less than 10% of the rates of hydrolysis catalyzed by the enzyme-doped monoliths. After the subtraction of the "background" rates, the initial rates (slopes V_0 in eq 4) for the enzymatic hydrolysis of the substrate at concentration *S* were fitted to the Lineweaver–Burk equation

$$\frac{1}{V_0} = \frac{K'_M}{V_{\max} S} + \frac{1}{V_{\max}} \quad (5)$$

and the following kinetic parameters were obtained: apparent Michaelis constant, K'_M ; maximum velocity, V_{\max} ; and apparent turnover number, k'_{cat} .³¹ The primed quantities are termed apparent because the concentration of the enzyme doing catalysis is not known exactly.

Specific activity of carbonic anhydrase is 10^{-3} times the number of milligrams of the substrate (*p*-nitrophenyl acetate) hydrolyzed by 1.0 mg of the enzyme per 1.0 min.

Results and Discussion

Encapsulation of Carbonic Anhydrase. The bovine isozyme II is a well-characterized protein, with the molecular mass of 28 900 Da and the isoelectric point $pI = 5.9$. Because none of its 259 amino acid residues is cysteine, chemical and spectroscopic studies are not complicated by oxidation and formation of disulfide

bridges.^{27,30,32–34} Because the sol–gel method yields silica glass that is transparent at wavelengths as low as 250 nm, concentration of the encapsulated protein was accurately determined on the basis of the absorbance at 280 nm; shrinkage upon partial drying of the glass monoliths was taken into account in determining the optical path length. Concentration of the enzyme in the monolith was 0.80 mg/mL on the basis of the UV absorption spectrum and 0.79 mg/mL on the basis of the amount of the enzyme used in the encapsulation experiments. Equality of these values within the error margins, seen in Figure S1 in the Supporting Information, proves that frequent rinsings of the monoliths over 2 weeks during the aging of silica does not leach the trapped enzyme. Indeed, the enzyme was not detected by UV absorption in the rinsing buffer.

Monitoring of Carbonic Anhydrase Unfolding. Although diffusion of relatively small molecules through the porous sol–gel silica glass has been studied before,^{35,36} subtler kinds of motions, such as conformational changes of encapsulated molecules, have only begun to be investigated.³⁷ These investigations are needed, because conformational states of proteins determine their biological activity and chemical reactivity. Carbonic anhydrase is well-suited for these studies in the glass because its unfolding in solution has been examined. Because gradual heating causes irreversible precipitation at 64 °C and higher temperatures, this denaturation could not be monitored by spectroscopic methods; a more involved method had to be used.³⁸ Denaturation by guanidinium chloride (GdmCl) in solution, however, is a well-characterized, reversible process, conveniently followed by circular dichroism (CD) spectroscopy.³⁰

The CD bands of bovine carbonic anhydrase II in the far-UV region are relatively weak, because α -helical content is low. A minimum in ellipticity at 217 nm is diagnostic of the high content of antiparallel β -sheet. In the near-UV region, 240–320 nm, the spectrum is complex, with multiple Cotton effects. There are major bands at 245 and 270 nm and two minor ones above 280 nm. Because isozymes II of human and bovine carbonic anhydrases have very similar near-UV CD spectra, the same chain length, the same tryptophan residues (nos. 5 and 16 in one of the two clusters of aromatic residues, and nos. 97, 123, 192, 209, and 245 in the central β -sheet region), and many other similarities, reported properties of the mutants of the human enzyme are relevant to the bovine enzyme that we used.³⁹ All the tryptophan residues make positive

(31) Segel, I. H. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*; Wiley-Interscience: New York, NY, 1975.

(32) Wong, K.-P.; Hamlin, L. M. *Biochemistry* **1974**, *13*, 2678–83.

(33) Christianson, D. W.; Fierke, C. A. *Acc. Chem. Res.* **1996**, *29*, 331–339.

(34) Silverman, D. N.; Lindskog, S. *Acc. Chem. Res.* **1988**, *21*, 30–6.

(35) Koone, N.; Shao, Y.; Zerda, T. W. *J. Phys. Chem.* **1995**, *99*, 16976–81.

(36) Sieminska, L.; Zerda, T. W. *J. Phys. Chem.* **1996**, *100*, 4591–7.

(37) Edmiston, P. L.; Wambolt, C. L.; Smith, M. K.; Saavedra, S. *J. Colloid Interface Sci.* **1994**, *163*, 395–406.

(38) McCoy, L. F., Jr.; Wong, K.-P. *Biochemistry* **1981**, *20*, 3062–7.

(39) Freskgaard, P.-O.; Maartensson, L.-G.; Jonasson, P.; Jonsson, B.-H.; Carlsson, U. *Biochemistry* **1994**, *33*, 14281–8.

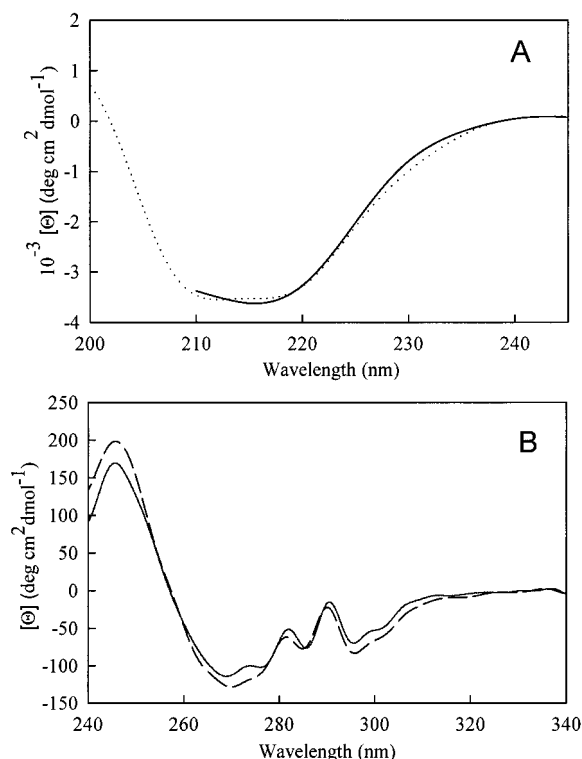


Figure 1. Far UV (A) and near-UV (B) circular dichroism spectra of bovine carbonic anhydrase II dissolved in a MOPS buffer having ionic strength 11.6 mM and pH 7.00 (dashed or dotted line) and encapsulated in silica glass (solid line). The protein concentration and path length were 5 μ M and 1.0 mm (A) or 28 μ M and 8.0 mm (B). Molar ellipticity is normalized to the mean residue value.

contributions to the CD in the region 230–250 nm, but tyrosine residues make a minor negative contribution. Tryptophan residues nos. 5, 123, 209, and 245 contribute mainly to the band at 269 nm. Because the relatively many aromatic residues are distributed in various regions of the carbonic anhydrase molecule, its CD spectrum reflects the conformation of the whole protein, not only a part of it. (This advantage is absent in studies that use only one chromophore or fluorophore for monitoring of conformational changes.) Our CD spectra of the enzyme in solution agree with those reported earlier.²⁷ As Figure 1 shows, encapsulation in silica causes only small changes in band intensities and shapes. Similar small changes were found upon encapsulation of bacteriorhodopsin and zinc cytochrome *c* in silica glasses.^{14,40} Studies of these two proteins showed that they are not significantly perturbed in the glass, and we conclude that neither is carbonic anhydrase. Both the backbone (far-UV) and the relevant side chains (near-UV) seem to retain upon encapsulation the general structure that they had in solution.

We monitored unfolding by measuring molar residue ellipticity, θ , at 245 and 269 nm, wavelengths at which the spectral changes were largest. The corresponding plots obtained at these two wavelengths were always very similar because each reflected the changes in the tertiary structure of the whole enzyme molecule.

Electrostatic Effect of the Silica Glass on Uptake of Ionic Species. When a monolith of sol–gel

silica is immersed in a solution, the solute will diffuse into the monolith. Even though the solute molecules may be much smaller than the pores in the glass, when an equilibrium is reached after long soaking concentrations of the solute inside the glass and in the surrounding solution may not be equal. This inequality will persist if the solute is an ionic species and the surface of the glass pores is charged. Since the isoelectric point of silica is 2.1, the pores of sol–gel glass are negatively charged at pH values 7.00 and 8.00, used in our experiments.⁴¹ Research in this laboratory showed partial uptake of the solute when the charges of the solute and the glass are like and excessive uptake when the charges are opposite. These electrostatic effects can be abolished by raising ionic strength of the solution. In the presence of 0.600 M NaCl, at pH 7.00, concentrations of $[\text{Fe}(\text{CN})_6]^{3-}$ ion and other ionic solutes “inside” the monolith and in the solution “outside” do become equal after sufficient time.^{13,14a} We confirmed this result in experiments with $[\text{Ru}(\text{NH}_3)_5\text{Cl}]\text{Cl}_2$, a salt containing a colored complex cation. As Figure S2 in Supporting Information shows, concentration of this doubly charged cation inside and outside the silica monolith became equal in the buffer at pH 7.00 that was also 0.600 M in NaCl.

In studies of unfolding of encapsulated carbonic anhydrase with guanidinium chloride, concentration of the colorless Gdm^+ cation in the glass could be known only if it were kept equal to that in the external solution. The 0.600 M NaCl, which proved sufficient even for the multiply charged ions, was kept in all solutions. To permit correct comparisons of the unfolding process in the glass and in solution, 0.600 M NaCl was always used, although this salt was not necessary for precisely knowing the concentration of GdmCl in free solution.

Unfolding of Carbonic Anhydrase in Solution.

A previous study³⁸ found that heating of the enzyme solution to 64 °C and higher temperatures causes precipitation that cannot be reversed by cooling. Denaturation had to be followed by monitoring of pH as a function of temperature.³⁸ The enzyme unfolds in the interval 55.0–70.0 °C; precipitation begins at 55.0 °C, and the precipitate remains up to 95.0 °C. The melting temperature $T_m = 64.3$ °C.³⁸ As Figure S3 in the Supporting Information shows, our experiments confirm that onset of precipitation at 64 °C is accompanied by a large change of ellipticity and an increase in the spectral noise.

Unfolding by guanidinium chloride at room temperature proceeds via two intermediates. At the denaturant concentration of 3.83 M, bovine carbonic anhydrase II is completely unfolded.³⁰ Our results, shown in Figure 2A, confirm these results. We repeated the experiments with the enzyme in solution to allow comparisons with our experiments with the enzyme in glass, which will be described next.

Unfolding of Carbonic Anhydrase in Silica Glass by Heating. To determine the T_m value, CD measurements in Figure 3 were fitted to an approximate model containing two limiting states, folded and unfolded.⁴² The result is independent of the wavelength, as it should

(40) Wu, S.; Ellerby, L. M.; Cohan, J. S.; Dunn, B.; El-Sayed, M. A.; Valentine, J. S.; Zink, J. I. *Chem. Mater.* **1993**, *5*, 115–20.

(41) Yamanaka, S. A.; Nguyen, N. P.; Dunn, B.; Valentine, J. S.; Zink, J. I. *J. Sol-Gel Sci. Technol.* **1996**, *7*, 117–121.

(42) Pace, C. N. *Methods Enzymol.* **1986**, *131*, 266–80.

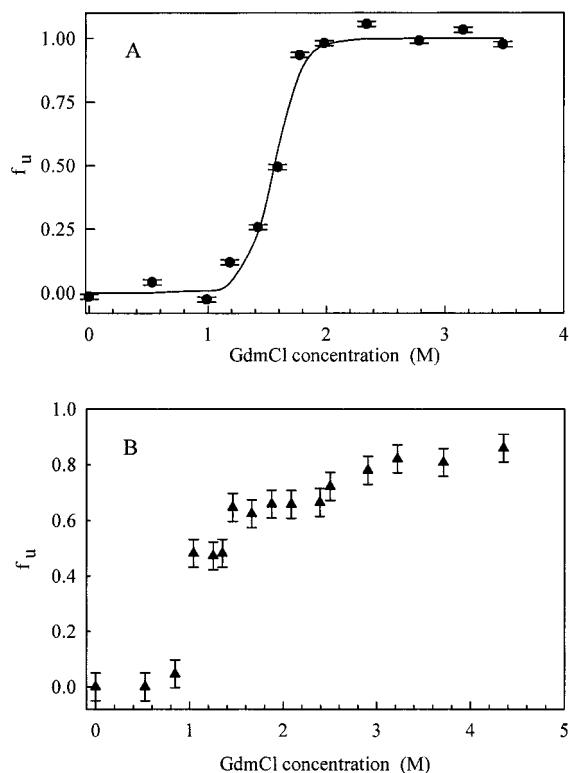


Figure 2. Unfolding by guanidinium chloride of bovine carbonic anhydrase II dissolved in a MOPS buffer having ionic strength 11.6 mM and pH 7.00 that was also 0.600 M in NaCl (A) and encapsulated in silica glass (B). The degree of unfolding, f_u , was calculated from the ellipticity at 269 nm. Each point was obtained with a different sample, but all the samples were prepared under the same conditions.

be: 54 ± 3 °C at 245 nm and 49 ± 3 °C at 269 nm are practically the same temperature. We take the average value, $T_m = 51 \pm 3$ °C. On a generally accepted assumption that the random-coil conformations obtained by chemical denaturation (Figure 2A) and by heating (Figure 3B) are indistinguishable, it turns out that the encapsulated enzyme becomes only 77% unfolded. That the plot in Figure 3B levels off at $f_u = 0.77$ is an indication that the encapsulated enzyme unfolds incompletely. Thermal unfolding of the enzyme causes precipitation in solution, as discussed above, but not in the silica glass. Both incomplete unfolding and lack of precipitation can be attributed to the influence of the silica matrix. The enzyme molecules are relatively constrained by this matrix, which restricts the mobility of the protein molecules that is required for complete unfolding. Because the enzyme molecules are isolated in separate "cages" in porous silica, they cannot aggregate together and precipitate. The relatively small slope (i.e., broad melting range) of the plots in Figure 3 may be an indication that the protein molecules exist in different microenvironments and have somewhat different stabilities toward unfolding.

As Figure 4 shows, the encapsulated enzyme at 74.0 °C shows an essentially featureless CD spectrum. Upon gradual cooling to 20.0 °C, the spectrum remains almost the same, except for the nonspecific change in the region 240–250 nm. Clearly, the unfolded protein does not refold upon cooling. This finding, again, indicates that the protein molecules are constricted by the surrounding silica matrix, which precludes the mobility required for

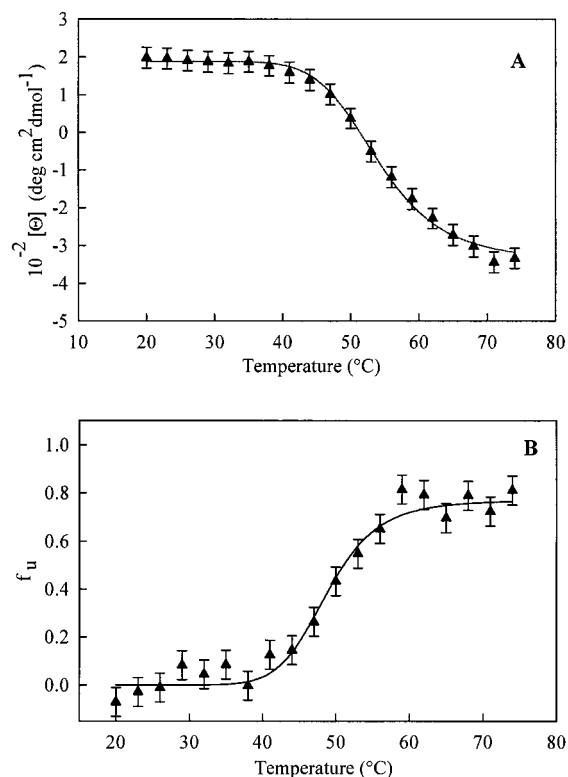


Figure 3. Unfolding by heating of bovine carbonic anhydrase II encapsulated in silica glass at the concentration of 28 μ M, monitored at 245 nm (A) and 269 nm (B). Molar ellipticity was normalized to the mean residue value. The degree of unfolding, f_u , was calculated from the ellipticity at 269 nm.

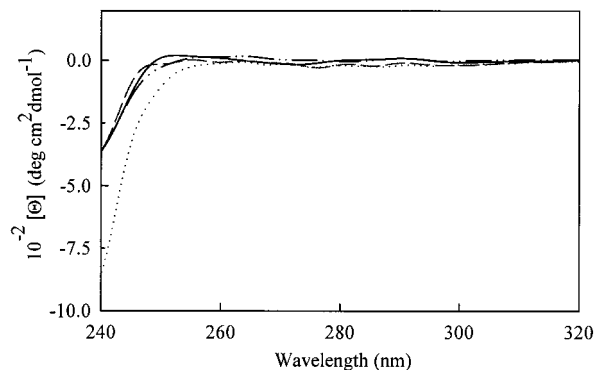


Figure 4. Near-UV circular dichroism spectra of bovine carbonic anhydrase II encapsulated in silica glass at the concentration of 28 μ M. One sample, immersed in a MOPS buffer having ionic strength 11.6 mM and pH 7.00, was heated at the rate of 3.0 °C/h to 74.0 °C (dotted line) and then cooled at the same rate to 20.0 °C (dashed line). Another sample was infused with the MOPS buffer that was also 0.600 M in NaCl and 4.35 M in guanidinium chloride (solid line), and then guanidinium chloride was removed by dialysis against the aforementioned buffered NaCl solution (chain line), all at 20.0 °C.

refolding. Previous studies of alkaline phosphatase, acid phosphatase, and flavoprotein oxidases showed these enzymes to be thermally more stable in silica glass than in solution. Although the T_m values for carbonic anhydrase in solution (64.3 °C) and in silica glass (51 ± 3 °C) were obtained by different methods, their comparison may be instructive. *This enzyme, unlike the aforementioned ones, seems to be somewhat destabilized toward unfolding by its encapsulation in silica.* Evidently, encapsulation in sol-gel glasses may differently

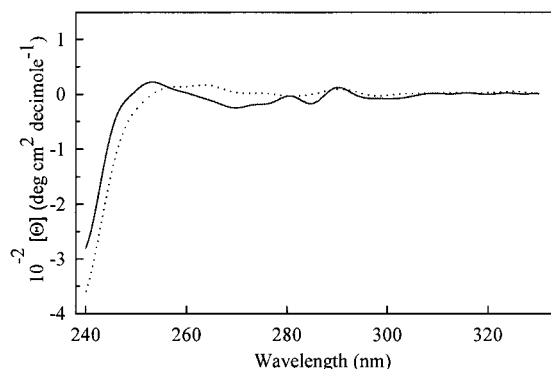


Figure 5. Near-UV circular dichroism spectra of bovine carbonic anhydrase II encapsulated in silica glass at the concentration of $28 \mu\text{M}$ (solid line) and dissolved at the concentration of $26 \mu\text{M}$ (dotted line). The glass monolith was immersed, and the enzyme was dissolved, in a MOPS buffer having ionic strength 11.6 mM and $\text{pH } 7.00$ that was 0.600 M in NaCl and that also contained guanidinium chloride, 3.710 M in the case of encapsulated and 3.488 M in the case of the dissolved enzyme.

affect stability of different proteins. More research is needed before generalizations can be made.

Unfolding of Carbonic Anhydrase in Silica Glass by Guanidinium Chloride. Figure 2B shows, again, that the encapsulated enzyme is incompletely unfolded ($f_u = 0.83$) at the denaturant concentrations higher than 3.71 M , at which the enzyme in solution was completely unfolded ($f_u = 1.00$ in Figure 2A). We refrained from fitting the results in Figure 2B to some model because any fitting would require assumptions about the mechanism of unfolding. As Figure 5 shows, the CD spectrum retains some features even at the 3.71 M concentration of the denaturant. Although the spectral bands are very weak, their number and general shape clearly resemble those in Figure 1. Even in the presence of 4.35 M guanidinium chloride, the ellipticity does not reach the value characteristic of the unfolded conformation. The encapsulated enzyme seems to retain some conformational features in the presence of the denaturant.

Thorough dialysis of the enzyme-doped monolith against buffer that was 0.600 M in NaCl removed the denaturant, but the near-UV CD spectrum of the encapsulated enzyme did not change, did not “recover” its intensity and features. Evidently, the enzyme cannot refold inside the glass matrix. It remains almost completely unfolded in the absence of the denaturant.

Effect of Encapsulation on Conformational Stability of Carbonic Anhydrase. Upon partial drying of the aged silica glass, the pores partially collapse, and the monolith shrinks. This collapse may affect the conformational stability, but we did not investigate this process. Very recent studies^{15,16} showed that encapsulation in silica prolongs the lifetime of enzymes at higher temperatures. This important finding can be attributed to the enhanced thermodynamic stability of the enzymes, and our study may help explain this enhancement. Constrained in the silica matrix, protein molecules lack the mobility required for complete unfolding. Separated by this matrix, the molecules cannot aggregate and precipitate. Both of these effects may be considered stabilization with respect to the state of these proteins in solution. We note, however, that a simple property such as the melting point (T_m) may not be a

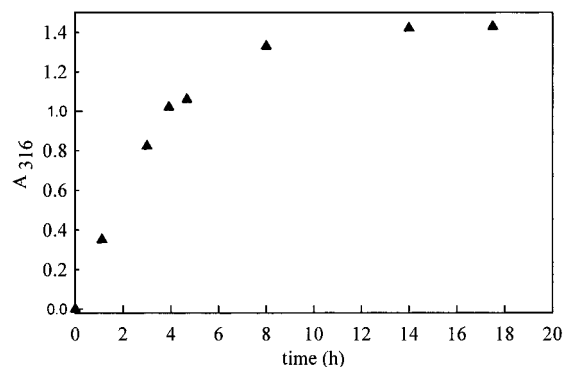


Figure 6. Absorbance at 316 nm of a silica monolith sized $8 \times 8 \times 27 \text{ mm}$ immersed for various times in a 0.14 mM solution of *p*-nitroanisole in a 9:1 mixture by volume of a phosphate buffer having ionic strength 10 mM $\text{pH } 8.00$ and acetonitrile. As the guest compound enters the glass, absorbance of the glass increases.

correct criterion of the protein “stability” because the concept of stability has multiple meanings, especially when applied to enzymes.

So far we discussed conformational stability, i.e., resistance of the protein to its unfolding by heating and by guanidinium ions. Next, we will discuss the enzymatic activity of carbonic anhydrase encapsulated in silica.

Diffusion of Organic Compounds through Silica Glass. Retardation by the glass matrix of movement of small molecules (such as acetone, acetonitrile, and chloroform) has been quantified by the so-called hindrance factors.³⁵ Because *p*-nitrophenyl acetate, the substrate for carbonic anhydrase (eq 3), is a larger molecule, it is expected to diffuse relatively slowly through silica. Partial hydrolysis of this ester would occur in this time and complicate the study of its diffusion. Therefore, we used *p*-nitroanisole, $p\text{-CH}_3\text{O-C}_6\text{H}_4\text{-NO}_2$, which resembles *p*-nitrophenyl acetate in size, composition, and shape but does not hydrolyze under the conditions of the diffusion experiments. Figure 6 shows that ca. 10 h are required for full penetration of this guest compound in the monolith, that is, for equalization of the guest concentrations inside the glass and in the external solution. The negative charge of the sol-gel glass at $\text{pH } 8.00$ does not affect the extent of uptake of this electroneutral guest molecule.

Hydrolysis of *p*-Nitrophenyl Acetate by Encapsulated Carbonic Anhydrase. The biological function of carbonic anhydrase is to catalyze interconversion of CO_2 and HCO_3^- , but this enzyme can also catalyze hydrolysis of esters.^{24,33} The reaction in eq 3 in transparent monoliths of silica doped with the enzyme produced the yellow *p*-nitrophenoxide anion, and we followed its growing absorbance. Figure 7 shows that this yellow product of hydrolysis appears in minutes along the faces of the monolith and only after hours in its core. Evidently, the substrate is hydrolyzed as it diffuses into the monolith and first encounters the enzyme in the surface layers of silica. Agreement between the results in Figures 6 and 7 confirms that *p*-nitroanisole is a good mimic of *p*-nitrophenyl acetate so far as the diffusion rate is concerned.

Michaelis-Menten Kinetics and Effect of Encapsulation on Activity of Carbonic Anhydrase. Although our results do not show whether mass transfer

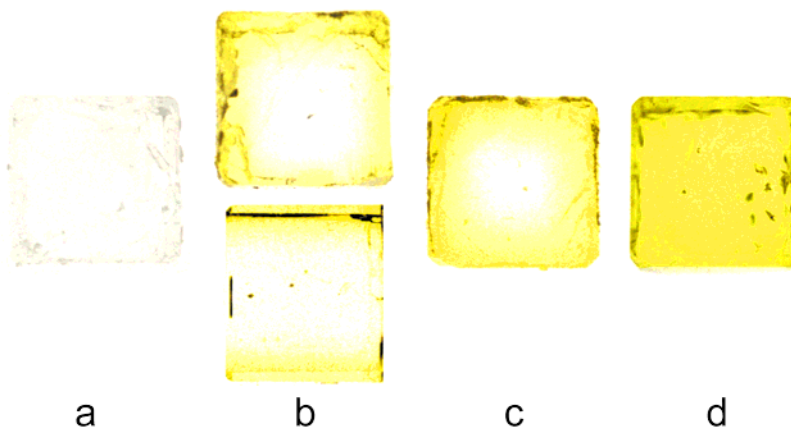


Figure 7. Cross sections of a silica monolith sized $8 \times 8 \times 27$ mm that was doped with $28 \mu\text{M}$ bovine carbonic anhydrase II and immersed in 30 mL of a 1.00 mM solution of *p*-nitrophenyl acetate in a 9:1 mixture by volume of a phosphate buffer having ionic strength 10 mM pH 8.00 and acetonitrile. The encapsulated enzyme catalyzes hydrolysis of *p*-nitrophenyl acetate and formation of *p*-nitrophenoxide anion, which is yellow. The immersion times were (a) 0, (b) 10 min, (c) 1.0 h, and (d) 10 h. As the ester gradually penetrates the doped monolith, it encounters the enzyme and becomes hydrolyzed.

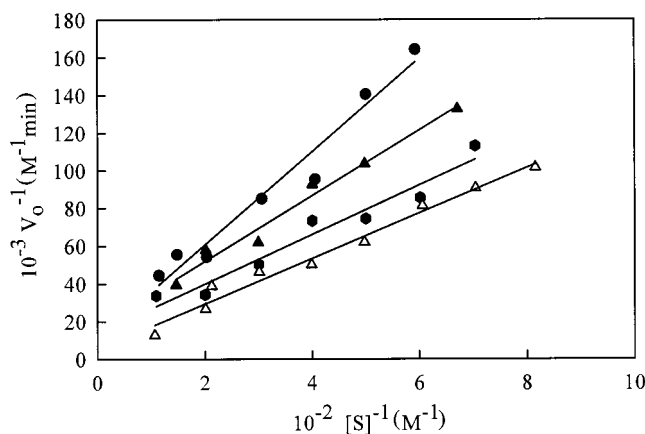


Figure 8. Lineweaver-Burk plots for hydrolysis at 25.0°C of *p*-nitrophenyl acetate catalyzed by bovine carbonic anhydrase II in silica monoliths sized $8 \times 8 \times 27$ mm. From the bottom to the top plot, concentrations of the enzyme in silica were 79, 56, 31, and $10 \mu\text{M}$. The ester was dissolved in a 9:1 mixture by volume of a phosphate buffer at ionic strength 10 mM and pH 8.00 and acetonitrile.

of substrate and product or enzymatic catalysis determines the observed reaction rate, we assume that the latter is the case. Linearity of the plots in Figures 8 and 9 is evidence that the encapsulated enzyme obeys the Michaelis–Menten law of enzyme kinetics. Table 1 and Figure 10 show that specific activity of the encapsulated enzyme is lowered to ca. 1% of that of the dissolved enzyme and that it depends little on the extent of doping of silica by the enzyme. The small decrease in specific activity as the enzyme concentration in the monolith increases cannot be caused by partial loading, aggregation, or denaturation of the enzyme in glass. Evidence earlier in this article refutes all these three possibilities. If the decrease in activity is significant, it may be a consequence of a small decrease in accessibility of the enzyme to the substrate as the enzyme concentration increases. Similar, although somewhat larger, dependence of activity on concentration has been reported for encapsulated trypsin.¹⁷ More such studies are needed, but these early results are promising. For use of sol-gel glasses in catalysts or biosensors, it is encouraging that activity seems to be only little affected by the extent of doping these glasses with enzymes.

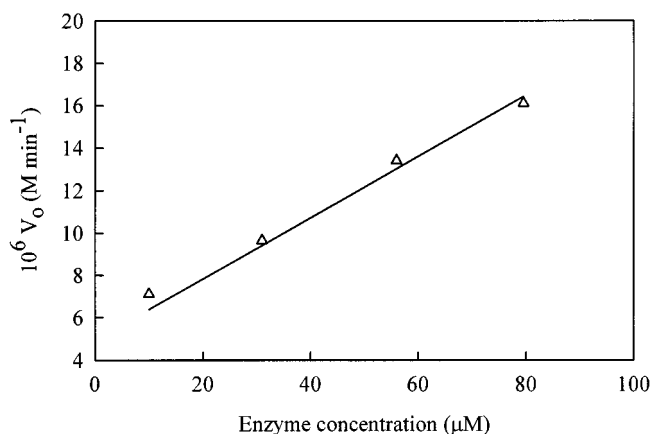


Figure 9. Initial rate at 25.0°C of for hydrolysis of *p*-nitrophenyl acetate catalyzed by bovine carbonic anhydrase II (eq 3) encapsulated at four concentrations in silica monoliths sized $8 \times 8 \times 27$ mm. The monoliths were immersed in a 2.00 mM solution of *p*-nitrophenyl acetate in a 9:1 mixture by volume of a phosphate buffer having ionic strength 10 mM pH 8.00 and acetonitrile.

Table 1. Michaelis–Menten Parameters for Hydrolysis of *p*-Nitrophenyl Acetate (Eq 3) Catalyzed by Bovine Carbonic Anhydrase II in Solution (the First Row) and Encapsulated in Silica Glass at Four Different Concentrations (the Next Four Rows)^a

enzyme conc (μM)	K'_M (mM)	K_{cat} (min^{-1})	K_{cat}/K'_M ($\text{M}^{-1} \text{min}^{-1}$)	10^3 spec activity ($\text{mg min}^{-1} \text{mg enzyme}^{-1}$)
3.3	14 ± 3	388	27 000	304
10	22 ± 15	9	409	4.45
31	10 ± 3	2	200	1.95
56	9 ± 4	1	111	1.52
79	22 ± 14	2	91	1.27

^a In all cases the solvent was a phosphate buffer at ionic strength of 10 mM and pH 8.00.

Table 1 allows comparisons of encapsulated and dissolved carbonic anhydrase as a catalyst for the reaction in eq 3. The apparent Michaelis constant for the four different concentrations of the encapsulated enzyme $K'_M = 16 \pm 8$ mM, is identical to the value $K'_M = 14 \pm 3$ mM for the dissolved enzyme. Evidently, the affinity of the active site for the substrate is unaffected by encapsulation in silica. Indeed, CD spectra in Figure

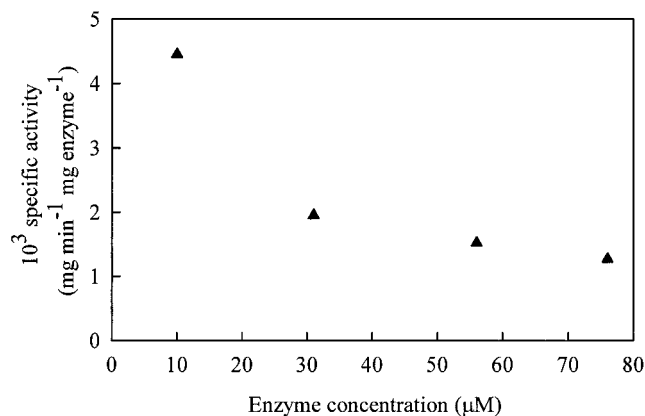


Figure 10. Specific activity at 25.0 °C of bovine carbonic anhydrase II encapsulated at four different concentrations in silica monolith sized 8 × 8 × 27 mm. The enzyme catalyzed hydrolysis (eq 3) of *p*-nitrophenyl acetate dissolved in a 9:1 mixture by volume of a phosphate buffer at ionic strength 10 mM and pH 8.00 and acetonitrile.

1 show that the overall enzyme conformation is preserved in the silica matrix. This finding about K_M value is interesting and possibly important, but its generality should be tested in studies of other enzymes.

The apparent turnover number, k_{cat} , is diminished upon encapsulation. The average value for the four different concentrations of the encapsulated enzyme is 4 min⁻¹. Since the substrate does not penetrate the inner core of the monolith in the 10-min period over which we studied the kinetics, the question arises as to what fraction of the encapsulated enzyme is responsible for catalysis. Unable to answer it accurately, we estimate on the basis of Figure 7 that only one-half of the total encapsulated enzyme actually catalyzes the observed reaction. Therefore, a better estimate may be $k_{cat} = 8 \text{ min}^{-1}$. Although this very approximate value is ~2%

of the corresponding value for the dissolved enzyme, it is sufficiently high to justify further studies of enzyme activity in sol–gel glass.

Conclusion

There is keen interest in encapsulation of enzymes in glasses by the sol–gel method and in use of these doped glasses as catalysts, sensors, and composite biomaterials. For these applications to succeed, however, much has to be learned about the effects of the encapsulation on structure and activity of enzymes. We showed that some properties of the enzyme (overall conformation and K_M value) are preserved, while others (propensity for unfolding and k_{cat} value) are changed upon encapsulation. Slow diffusion of potential substrates and their interactions with the glass matrix, if not corrected by raising ionic strength, would limit application of doped monoliths. Spatial and steric constraints of the protein molecules by the glass matrix have important and unexpected consequences—the protein does not precipitate upon denaturation and cannot be either fully unfolded or refolded after it had been unfolded. These constraints should be understood in order to control protein–matrix interactions in ways that may allow selective adsorption, chemical sensing, and shape-selective catalysis.

Acknowledgment. This work was supported by the U.S. National Science Foundation through grant MCB-9808392.

Supporting Information Available: Three figures, showing UV spectra of dissolved and encapsulated carbonic anhydrase, UV–vis spectra of $[\text{Ru}(\text{NH})_3\text{Cl}]^{2+}$ in silica monolith and in surrounding solution, and CD evidence for precipitation of dissolved carbonic anhydrase upon heating. This material is available free of charge via the Internet at <http://pubs.acs.org>.

CM990507C