# Stabilization of Creatine Kinase Encapsulated in Silicate Sol−Gel Materials and Unusual Temperature Effects on Its Activity

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The enzyme creatine kinase (CK) is stabilized by encapsulation in silicate sol−gel monoliths. Its activity is measured as a function of long-term storage time at both room temperature and at elevated temperatures and is compared to that in solution. At room temperature, the activity of the encapsulated enzyme decreases to 50% of its initial value after more than 5 months, whereas in solution it decreases to 50% after 10 days. At 47 °C, the immobilized enzyme retains 50% of its maximum activity after 5 days of constant heating compared to that at 13 h in solution. At 60 °C, the immobilized enzyme retains 50% of its maximum activity after 5 h of heating as compared to that for less than an hour in solution. Surprisingly, a 4-fold *increase* in activity is observed after short exposures to the elevated temperatures. This increase is explained by structural changes in both the enzyme and the sol−gel matrix. The structural integrity and conformational changes of the encapsulated enzyme are observed by circular dichroism spectroscopy. The spectrum shows that the initially encapsulated enzyme has a structure different from that in solution but that upon heating the enzyme reverts to a conformation similar to that in solution. In addition, the encapsulated enzyme does not completely denature at temperatures up to 90 °C while in solution the midpoint temperature of the unfolding transition is 75 °C. These effects are interpreted in terms of electrostatic interactions between the positively charged patches on the enzyme's surface and the sol−gel matrix and to conformational changes within the pores upon heating. Heat treatment also affects the silica matrix by increasing the pore size as measured by gas absorption/desorption isotherms. The increase may allow small changes in the enzyme's structure, but in general the pore constrains the enzyme and inhibits denaturation.

## Introduction

The stabilization of biomolecules in silicate sol−gel materials is attracting increasing attention.[1−25] The physical encapsulation and entrapment of enzymes and other proteins in the solid support can inhibit deleterious changes to the protein structure and activity, protect the active site, prevent unfolding, and stabilize the electrostatic interactions. The inorganic silicate matrix formed by the sol−gel method involves room temperature solution based hydrolysis and condensation chemistry and can be fabricated in the form of optically transparent monoliths, films, and fibers and opaque

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powders.[26] Under acidic conditions, linear polymers with low branching density, small pores, and narrow pore size distributions are formed; these materials are best suited for optical studies, such as those reported in this paper. Under base catalysis conditions, highly branched structures with wide pore size distributions are formed; these materials are powdery and opaque. The initial demonstrations of encapsulation of biomolecules employed both types of material synthesis, alkaline phosphatase[1] in opaque materials and myoglobin, copper−zinc superoxide dimutase, and cytochrome *c* in transparent materials.[3] Since then, many biological molecules have been incorporated into both pure silica glass as in the initial studies and also into organically modified silica. This active field has been the subject of recent reviews.[27−30]

The stabilization of the entrapped biomolecules by the sol−gel matrix is frequently studied by monitoring the biomolecules using spectroscopic methods such as electronic absorption, luminescence, and circular dichroism. Fluorescence, UV, and resonance Raman have been used to probe human and bovine serum albumin,[7,24] myoglobin,[7,31−33] monellin,[12,34] hemoglobin,[19,23] cytochrome *c*,[3,13,14,17,35,36] copper−zinc superoxide dismutase,[3,31] and bacteriorhodopsin.[6,11] Circular dichroism was used to study lysozyme,[22] α-lactalbumin,[22] oncomodulin,[16] and bovine carbonic anhydrase.[20] The biomolecules retained their structural integrity, comparable to that of solution, when they were embedded in the sol−gel matrix. Dopants have been included to help further stabilize the biomolecules.[4,8,10,15,18,37−40]

In the case of enzymes, the most important criterion for stabilization (or destabilization) in sol−gel matrixes is the measurement of activity. A stringent test of the stabilizing effect of the matrix on the enzyme is to compare the reactivity of the encapsulated enzyme to that of the enzyme in buffer solution as a function of time. An even more severe test is to subject the enzyme to elevated temperatures for designated periods of time and compare the reactivities of the encapsulated enzyme to that of the enzyme in the buffer solution. In the latter studies, it is important that the rates be compared at the same temperature (usually room temperature or 37 °C) after the heating period. The effects of extended periods of elevated temperatures on the activities of enzymes encapsulated in sol−gels have not been widely studied. Glucose oxidase, the most studied enzyme as a biosensor, has been incorporated into the sol−gel and the activity observed after heating to 63 °C. The powdered silica gel-immobilized glucose oxidase retained 60% of its initial activity after 20 h.[15] Lipase was entrapped in a hybrid methyltrimethoxysilane and tetramethoxysilane sol−gel matrix and heated at 65 °C. The retained activity was about 10 times greater than that of lipase deposited on Celite support.[41] Trypsin and acid phosphatase were also trapped in silicate sol−gel glass with poly(ethylene glycol). The half-life of the entrapped enzymes was 100-fold greater than that of the enzymes in solution after both had been heated to 70 °C.[4]

In this paper we report the strong temporal and thermal stabilization of CK in optical quality sol−gel matrixes. The results of three types of studies are reported: enzyme activity as a function of long-term storage at room temperature (>6 months), enzyme activity after exposure to elevated temperatures (37, 47, and 60 °C) for extended periods of time, and circular dichroism spectroscopy of the heat-treated enzymes to monitor the secondary structural changes. To decrease to 50% of its maximum activity, the CK in the monoliths requires 17, 15, 10, and 5 times more time than enzymes in solution at room temperature, 37 °C, 47 °C, and 60 °C, respectively. We study the structure and conformational changes of the enzyme by circular dichroism and the gel morphology by pore size distributions. Surprisingly, we observe a 4-fold *increase* in the activity after short heating intervals in the sol−gel. We propose that the stabilization is a result of the physical and electrostatic interplay between the matrix and the biomolecule, especially between the enzyme−surface and pore−surface and between the enzyme's structure and the material's pore structure.

## Experimental Section

**Reagents.** Tetramethyl orthosilicate (TMOS) was obtained from Aldrich Chemical Co. and used as received. Creatine kinase was purchased from Lee Scientific, Inc., and also used as received. The two-part liquid reagent solution used for the rate measurements contained creatine phosphate (≥30 mM), ADP (2 mM), D-glucose (16 mM), $NADP^+$ (2 mM), G-6-P-dehydrogenase (5 mM), *N*-acetyl cysteine (24 mM), AMP (≥1.5 KU/L), magnesium acetate (≥10 mM), and hexokinase (≥2.5 KU/L). The HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethane sulfonic acid]) buffer solution at pH 6.8 was prepared with 1% BSA, 10% sucrose, 150 mM NaCl, and 10 mM HEPES.

**Synthesis of Transparent Sol−Gel Glass Monoliths.** The silicate sol−gel was prepared by using TMOS as a precursor. TMOS (15.27 g), deionized water (3.36 g), and 0.04 M HCl (0.22 g) were mixed and sonicated over ice for 30 min to produce the sol. Creatine kinase (2.2 mg) was dissolved in 4 mL of 10 mM pH 6.8 HEPES buffer solution and placed over ice. Then 2.6 mL of the TMOS sol was added to the enzyme solution and the mixture was immediately transferred to cassettes (purchased from Novex, 1-mm thick) for gelation. Gelation occurred within 1 min and the resulting solid was clear and transparent. The final enzyme concentration of the sol−gel monoliths was 0.33 mg/mL (200 U/mL). After the gel formed, the HEPES buffer solution was added to the cassette

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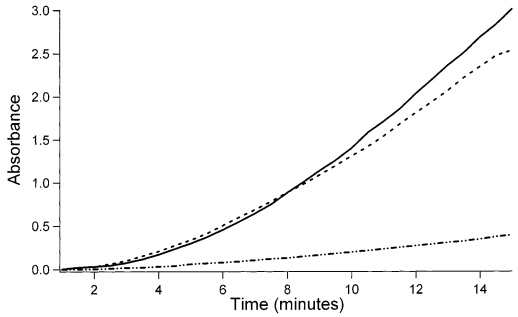


**Figure 1.** Plots of the absorbance of NADPH at 340 nm versus time to measure the activity of CK encapsulated in the monoliths. The activity is proportional to the slope in the linear region between 8.5 and 13 min. The measurements were made on monoliths that were stored for 9 days (—), 180 days (- - -), and 205 days (—•—•) at room temperature.

to keep the gel wet at all times. The cassette was covered with Parafilm and placed in the refrigerator overnight. The cassette was then opened and the monolith was cut into approximately 1 cm × 3 cm × 1 mm pieces and stored in the buffer solution.

For studies of the long-term stability at room temperature, the beaker containing the gels in buffer solution was covered with Parafilm and stored on the benchtop. For studies of the effects of elevated temperatures on the gels, the beakers were placed in a water bath at 47 and 60 °C or an incubator at 37 °C.

**Creatine Kinase Solution.** Solutions of creatine kinase were made for comparison studies of their activities versus those of the solid monoliths. Creatine kinase was dissolved in HEPES buffer solution at a concentration of 0.17 mg/mL (100 U/mL) for the experiments.

**Measurements of Activity and Stability.** The activity runs were carried out by monitoring the absorbance of NADPH at 340 nm using a Shimadzu UV-3101PC spectrophotometer. A monolith sample was weighed (≈0.05 g) and equilibrated in a plastic cuvette containing buffer solution at room temperature before each measurement. The instrument was zeroed with 2 mL of the reagent solution as a reference. The monolith was then carefully placed in the cuvette containing the reagent solution and the increase in absorbance as a function of time was monitored. The duration of the runs was 15 min for the monoliths and 10 min for the solutions. Readings were taken every 30 s. After every reading, the solution in the cuvette containing monolith was stirred to keep the reaction product evenly distributed in solution. The reagent solution was freshly made and kept on ice until the beginning of the run. All of the reactions of the monoliths (including those that were heat-treated) were measured in the reagent solution at room temperature. A plot of absorbance versus time was made for each run.

The relative activity of the enzyme is defined in this paper as the slope of the absorbance versus time plot between 8.5 and 13 min, where the slope is linear, divided by the weight of the monolith (Figure 1). The units for the relative activity are expressed as ΔAbs/mass. The relative activity was then plotted versus time (days after monolith formation or hours heated at a given temperature). The experimental uncertainty is dominated by the variability and total surface area of the monoliths caused by the presence of small cracks that occurred occasionally during the course of the experiments.

An equivalent measure of activity is the slope of the absorbance versus time plot between 8.5 and 13 min, where the slope is linear, divided by the mass of the enzyme in the monolith. In solution, the comparable activity is the slope of the absorbance versus time plot divided by the mass of the dissolved enzyme. Two comparisons of activities and activity changes are used in this paper. The first is the comparison of relative activities and relative activity changes where the maximum activity is normalized to 100% activity. The second is the comparison of activities per unit mass of enzyme in both gels and in solution.

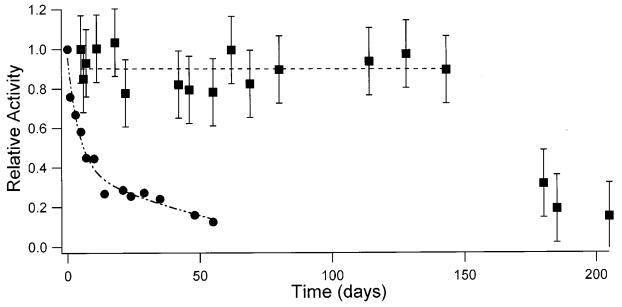


**Figure 2.** Plots of the relative activity versus time of creatine kinase at room temperature in HEPES pH 6.8 buffer solution (●) and in sol−gel monoliths (■). The maximum activities per mg of enzyme for the solution and monolith are 0.2 and 7 × $10^{-5}$, respectively.

An apparent activation energy for the increase in activities of the enzymes in the monoliths is calculated from a plot of the natural log of the initial slope of the activity rise at each temperature versus $1/T$. The initial slope is defined as the difference between the initial and maximum activities at a given temperature divided by the time required to reach the maximum activity.

**Pore Size Distribution.** The pore size distribution of the monolith was determined using the BJH model and nitrogen absorption and desorption isotherm data (Micromeritics ASAP 2010). The morphology of the wet gels was retained by using supercritical drying to avoid pore collapse during solvent removal. For these experiments, undoped gel samples were prepared using the same procedure as above. The solvent was exchanged with acetone for 5 days prior to supercritical drying with liquid $CO_2$.

**Circular Dichroism (CD) Spectra.** CD spectra were recorded on a J-715 Spectropolarimeter (Jasco Corporation). The measurements of solutions were taken using a 2-mm path length cuvette and those of the monoliths were taken in buffer solution by suspending them in the cuvette. The temperature was maintained at 20 °C for the runs. Thermal transition studies were performed between 20 and 90 °C, with a constant ramping of 2 °C/min. Spectra were taken before and after heating of the solution and monolith. The monoliths were heated at the specified temperature for 3 h and cooled, and the CD spectra were measured at 20 °C. The protein solution concentration was 0.5 mg/mL and the sol−gel concentration was 0.33 mg/mL (200 U/mL).

## Results

**Long-Term Storage at Room Temperature.** The gels were prepared as described above. The gelation time of the monoliths was on the order of 2−3 min. After gelation, the monoliths were aged in the cassette for a day and stored in HEPES pH 6.8 buffer solution. For the comparison solution samples, CK was dissolved in buffer solution and stored at the appropriate temperatures.

At first, the activity was measured every other day, but as time progressed, it was measured every few weeks. The plot of the raw data of the CK activity is plotted in Figure 1. The encapsulated enzyme retained 90% of its activity for about 5 months in buffer solution. After 5 months at room temperature, the activity started to decrease and reached 50% of its maximum value after 6 months. In contrast, the activity of creatine kinase in solution dropped to about 50% of its original activity after only 10 days of storage at room temperature. These results are plotted in Figure 2 with the maximum activity normalized to 100%. The activity per unit mass of enzyme is different for the gels and solutions. The

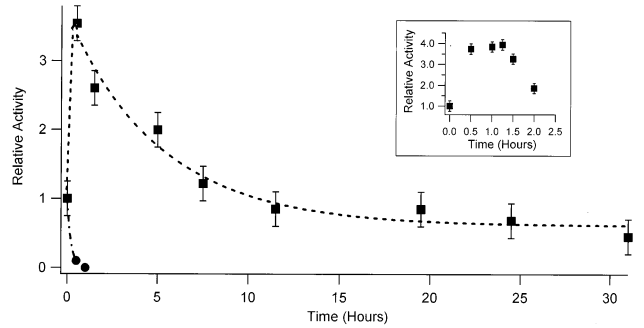


**Figure 3.** Plots of relative activity versus time of creatine kinase heated at 60 °C in solution (●) and in sol−gel monoliths (■). The activity of the monolith increases as it is heated for a short time and then starts to decrease (inset), but the solution activity decreases immediately.

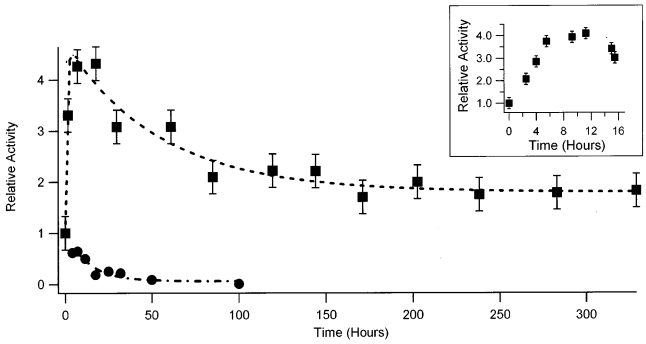


**Figure 4.** Plots of relative activity versus time of creatine kinase heated at 47 °C in solution (●) and in sol−gel monoliths (■). The activity of the monolith increases as it is heated for a short time and then starts to decrease (inset), but the solution activity decreases immediately.

mass of enzyme per mass of monolith is 22 mg of enzyme/g of monolith. The maximum activity per milligram of enzyme is $7 \times 10^{-5}$ for the gel and 0.2 for solution.

**Studies at Elevated Temperatures.** The CK monoliths and solutions were stored at 37, 47, and 60 °C for varying lengths of time to study the enzyme stability at elevated temperatures. At the elevated temperatures, the higher the temperature, the faster the loss of CK activity in the monoliths and in solutions. The maintenance of the immobilized CK activity was significantly longer than that in solution. At 60 °C (Figure 3), no activity was observed in the CK solution after an hour, whereas the immobilized enzyme decreased to 50% activity after 5 h of heating. At 47 °C, (Figure 4) the immobilized enzyme retained 50% of its activity after 5 days of heating, but in solution, it took less than a day of heating. After about 4 days in solution, the activity dropped to about 1%. At 37 °C, the CK monolith was about 20% active after 1 month of constant heating, but in solution, the activity dropped to 20% after 2 weeks

**Increase in Activity Following Heating.** CK monoliths that were heated at 37, 47, and 60 °C exhibited an initial increase in activity followed by the decrease described above (Figures 3 and 4 inset). Smaller time intervals were used to observe the time needed to reach maximum activity. Readings were taken every 2 h for the 37 and 47 °C samples and every half hour for the 60 °C samples. At 37 °C, the maximum activity was reached after heating for 20 h. At 47 °C, the maximum was reached after 10 h, and at 60 °C, it was reached in

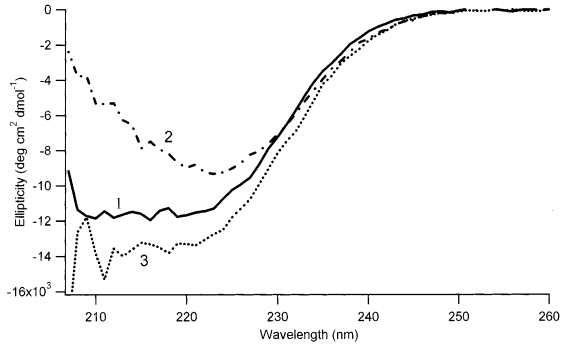


**Figure 5.** Circular dichroism spectra of CK in (1) a freshly made solution (—), (2) an unheated monolith (—•—•), and (3) a monolith heated for 3 h at 47 °C (- - -). In the unheated monolith, the enzyme is in a confined non-native conformation that is different from that in solution. After heating the monolith for 3 h, the enzyme confirmation becomes more like that in solution.

1 h. The apparent activation energy for the increase is 2.6 ± 0.6 kJ/mol. The maximum activity typically increased about 4-fold as a result of heat treatment. In contrast, heating the solution did not cause an increase in the activity, but instead caused an immediate decrease.

**Matrix Modifications.** Some known stabilizers and modifiers were added to the gel mixture before polymerization to investigate their effects on CK activity. The matrix was modified by incorporating methyltrimethyl orthosilicate to make the pores larger and mercaptopropyltrimethoxysilane to incorporate a reducing agent for CK in the sol−gel. Stabilizers for CK that were added included dithiolthreitol, *N*-acetylcysteine, and poly(ethyleneimine). Room-temperature activity measurements were taken as described above for about a month and the monoliths were heated at 47 °C for a week. In all cases, the addition of stabilizers or modifiers did not significantly affect the activity of CK in the sol−gel.

**Pore Size Measurements.** The pore sizes of the monoliths were determined by using the nitrogen adsorption desorption isotherms. The monoliths were heated at 37, 47, or 60 °C for 3 h. The average pore size, pore volume, and surface area of the monolith at room temperature are 83 ± 3 Å, 2.0 cm$^3$/g, and 900 m$^2$/g, respectively. At 37 °C, the average pore size, pore volume, and surface area are 88 ± 2 Å, 1.9 cm$^3$/g, and 780 m$^2$/g, respectively. At 47 °C, the average pore size, pore volume, and surface area are 93 ± 3 Å, 2.2 cm$^3$/g, and 800 m$^2$/g, respectively. At 60 °C, the average pore size, pore volume, and surface area are 109 ± 3 Å, 2.2 cm$^3$/g, and 730 m$^2$/g, respectively. The data show a relatively narrow pore size distribution in which 80% of the pore volume is within ±10% of the average pore size. As the heat treatment temperature increased, the pore sizes also increased.

**CD Spectra.** The CD spectra of creatine kinase in both solution and monoliths were obtained. The CD spectra of the enzyme in solution exhibited a minimum at 220 nm, but the spectral minimum for the enzyme encapsulated (after 3 days) in the monolith was at 225 nm (Figure 5). Upon heating the monoliths for 3 h at 37, 47, and 60 °C, the CD minima shifted to 220 nm, similar to the spectral position of the dissolved enzyme. The spectra of the enzyme in the monoliths at 37 and

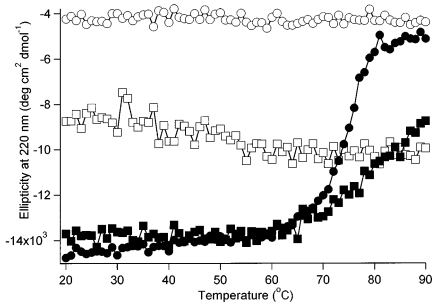


**Figure 6.** Thermal unfolding transition of creatine kinase in monoliths and in solution monitored by the ellipticity at 220 nm. The sample was heated at 2 °C/min from 20 to 90 °C and then cooled from 90 to 20 °C. Solution during heating (●); solution during cooling (○); monolith during heating (■); monolith during cooling (□).

47 °C were similar to those of solution, but the minimum at 60 °C increased compared to the monoliths heated at 37 and 47 °C.

The thermal transitions of CK in solution and in the sol−gel matrix were examined by monitoring both the change in ellipticity at 220 nm and the change in the spectra at temperatures ranging from 20 to 90 °C (Figure 6). The spectra of the solution and the monolith were taken at 20 °C before heating. Then the temperature was ramped to 90 °C and maintained for 10 min. During the ramping, the change in ellipticity was monitored at 220 nm. The thermal denaturation was not reversible in solution or within the silica matrix. However, the encapsulated enzyme unfolded to a lesser extent than the enzyme in solution as measured by the change in ellipticity at 220 nm. The spectra were then taken at 90 °C, and the temperature was reversed back to 20 °C, while the ellipticity was monitored at 220 nm. The spectra of both the monolith and solution were taken again at 20 °C after heating (data not shown). For the sol−gel-encapsulated CK, the ellipticity increased by only about 30% of the enzyme in solution. In the sol−gel, CK did not unfold to the same extent as that in solution.

## Discussion

**1. Stabilization of CK in the Sol−Gel Matrix.** This study shows that CK activity is stabilized significantly in the sol−gel monoliths. The enhanced stabilization can be accounted for by the interplay between the sol−gel matrix and the biomolecule. The stabilizing effect of the monolith on the enzyme may be a result of both the interactions of the pore surface with the enzyme surface and the influence of the pore structure on the enzyme structure. The surface interactions are probably primarily an electrostatic attraction between positively charged patches on the enzyme surface and negatively charged pore walls. The structural effects mainly involve the confinement of the enzyme in the pore that prevents dimer dissociation and also inhibits unfolding.

Creatine kinase is a key enzyme in cellular energetics and has a molecular weight of 82 000 Da.[42] It catalyzes the reversible phosphorylation of creatine by ATP. It rapidly regenerates ATP in the cells when demand is high. Muscle creatine kinase exists as a dimer held together by hydrogen bonds, and the crystal structure has been determined for rabbit CK.[43,44] It has been suggested that the enzyme must exist as a dimer to be active,[43,45] although this suggestion is still not conclusive.[46,47] It is clear, however, that the dimeric state preserves the stability of the enzyme and its activity by stabilizing the secondary and tertiary structure, thus slowing deactivation due to enzyme unfolding.[48,49]

The reaction that is catalyzed is

$$\text{creatine phosphate} + \text{ADP} \xrightleftharpoons{\text{CK}} \text{creatine} + \text{ATP} \quad (1)$$

The rate of the reaction (enzymatic activity) is monitored via the generation of ATP that in turn is measured by a coupled enzyme reaction involving the enzymes hexokinase and glucose-6-phosphate dehydrogenase:

$$\text{ATP} + \text{glucose} \xrightleftharpoons{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP} \quad (2)$$

$$\text{glucose-6-phosphate} + \text{NADP}^+ \xrightleftharpoons[\text{dehydrogenase}]{\text{G-6-P}} \text{gluconate-6-phosphate} + \text{NADPH} \quad (3)$$

The rate of formation of NADPH is linearly related to the rate of formation of ATP and thus to the creatine phosphate concentration. The concentration of NADPH is measured by its absorbance at 340 nm. CK is the only component immobilized in the sol−gel matrix; the reagents for measuring CK activity contain glucose, ADP, $NADP^+$, hexokinase, glucose-6-phosphate dehydrogenase, and a thiol activator, *N*-acetylcysteine.

In all of the experiments, the activity of both the monoliths and solutions is normalized to the initial rate, and the activities are measured at identical temperatures. The encapsulated enzyme activity is considerably larger than that of the reference solution. For example, the activity is almost constant inside the monoliths for over 5 months at room temperature, whereas the solution activity decreases to 50% after 10 days (Figure 2). To test the thermal stability of the enzyme, the monoliths and solutions were stored at 37, 47, and 60 °C and the activity of the heated sample was measured at ambient temperatures. The activity of the encapsulated CK is retained longer than that in solution at all temperatures. Heating the solution at 60 °C for 1 h causes loss of activity in solution, whereas the enzyme is active for over 24 h in the monoliths (Figure 3).

**2. Matrix−Enzyme Surface Interactions.** When the CK is encapsulated within the sol−gel matrix, there are favorable interactions between the enzyme and the silica matrix and the enzyme may act as a pore template during the sol−gel process. Gelation around the enzyme keeps it from unfolding and denaturing.

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The outer surface of CK contains more positively charged patches than negative patches as shown by electron density calculations.[50] Within the pores, the silica walls are negatively charged at pH 6.8 because the isoelectric point of silica is 2.1.[26] Therefore, the silica matrix is attracted to the enzyme surface as it is formed and stabilizes the structure due to the electrostatic interactions.

Although the outer surface of CK interacts significantly with the matrix, the active site of the enzyme is well embedded in the active site cleft and is not affected deleteriously by the interactions. Although the exact structure and amino acid sequence at the active site is not known, it has been determined that Cys 283 is essential for enzyme activity.[51−53] Cys 283 is not directly involved in catalysis, but plays an important role in substrate binding. Upon immobilization in the sol−gel matrix, the active site is not blocked and the enzyme remains active. The combination of exterior structural stabilization caused by surface−surface (enzyme−pore wall) electrostatic interactions and the absence of significant perturbations of the active site in a cleft spatially separated from the surface results in a stabilized and active biomaterial.

Electrostatic interactions are known to affect the stability and activity of enzymes.[15,54] Glucose oxidase is stabilized when encapsulated in a sol−gel matrix.[15] Two positively charged arginine residues line the active channel, but the charges are balanced by negatively charged residues in close proximity, thereby keeping the active site neutral.

It has been hypothesized that sol−gel-encapsulated proteins may be stabilized by the excluded volume of the silica matrix.[22,55] Excluded volume effects on protein structure may be important in living cells where a large fraction of the available volume is occupied by macromolecules.[56]

Additives that are present in the sol−gel may help stabilize the encapsulated biomolecules.[4,38,41] For example, the addition of methyltrimethoxysilane to tetramethyl orthosilicate enhances the thermal stability of lipase.[41] In another study, poly(ethylene glycol) is added to tetraethyl orthosilicate with methyltriethoxysilane- or dimethyldimethoxysilane-derived samples to provide a concentration-dependence enhancement of lipase activity.[38] Poly(ethylene glycol) is also added with trypsin and acid phosphatase to improve the half-life of these enzymes.[4]

We incorporated methyltrimethyl orthosilicate and mercaptopropyltrimethoxysiliane with tetramethyl orthosilicate to alter the silica pores and also added known CK stabilizers, dithiolthreitol, *N*-acetylcysteine, and poly(ethyleneimine). Dithiolthreitol destabilized the enzyme, but the other additives did not affect the stabilization of the enzyme in the sol−gel under the conditions tested. For this reason, the effects of additives were not studied further.

**3. Heat-Treatment-Induced Activity Increases.** The second aspect of the material's stability involves the interplay between the pore structure and the enzyme structure. Heating the monoliths produces an unexpected result: the enzymatic activity *increases* after a short period of heating (Figures 3 and 4 inset). In solution, the activity decreases immediately due to unfolding and/or aggregation of the enzyme, but the monolith-encapsulated enzyme shows a 4-fold increase in activity followed by the expected decrease. The higher the temperature, the shorter the heating time needed to cause the increase. To investigate the reason for the increase of the activity in the monoliths, the structure of the enzyme in the silica and the structure of the matrix material are investigated.

*Heat-Induced Changes in the Enzyme Structure.* To observe the structural changes and maintenance of the structural integrity of CK, circular dichroism spectra were monitored and interpreted. CD utilizes circularly polarized light to probe the secondary structure ($\alpha$ helixes and $\beta$ pleated sheets) of the enzyme. The CD spectrum is sensitive to the conformational changes and is a common spectroscopic method for studying enzyme structure.[57] The CD measurements are used to follow the course of the conformational changes of the CK in monoliths during and after heating and are compared to the spectra obtained from the enzyme solutions.

In a first series of experiments, the CD spectrum of the solution enzyme is compared with those of the heated and unheated immobilized enzyme (Figure 5). The CD spectrum of the unheated solution exhibits a minimum at 220 nm that represents the unconfined enzyme in its native state. The spectrum of an unheated CK monolith shows a shifted minimum at 225 nm, indicating that a large fraction of the enzyme is in a different, non-native, conformational state. Upon short-time heating of the monoliths, the minimum shifts to 220 nm, which indicates that heating causes the secondary structure of the enzyme to become more like that of the protein in solution. This change in structure may explain the observed increase in activity of the confined CK.

In the second series of experiments, the thermal transitions of CK are observed by monitoring changes in the ellipticity at a characteristic wavelength (Figure 6). Upon heating the monolith and solution to 90 °C and monitoring the $\alpha$-helical content at 220 nm, the encapsulated enzyme does not fully denature like the enzyme in solution. Protein unfolding is not a reversible process for the enzyme in solution or in the matrix, but the extent of unfolding is much greater in solution. The CD spectra taken before and after heating confirm that the solution is denatured whereas the enzymes in the monoliths still retain structure. The $T_m$, the midpoint temperature of the unfolding transition, is 75 °C for the CK solution. In contrast, it is not possible to determine the $T_m$ for the monoliths because the enzyme has not unfolded completely.

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*Heat-Induced Changes in the Silica Matrix.* The pore sizes were determined using the nitrogen desorption−adsorption experiments. The average pore size increased as the monoliths were heated for a set period of time; the higher the temperature, the larger the pore sizes. The increase in pore size with temperature offers a materials-based explanation for the increased rate of the enzyme reaction in the heat-treated samples. Upon heating the monoliths, the enzyme is able to rearrange to a more desirable conformation in the larger pores. Concurrently, increased heating denatures the enzyme, resulting in a decrease in activity. These opposing effects explain the initial activity increase with short time heating followed by a decrease at longer times.

Hydrolysis and polycondensation to form the gel occur on the order of a minute. During the process of sol−gel formation, the enzyme becomes trapped inside the pores. The encapsulation process may not trap all the enzyme molecules in their native state. Encapsulation of hemoglobin shows that conformational changes are greatly slowed or even stopped.[23] The starting conformational distribution is likely to be the factor controlling the encapsulated structure. In our study, we find that although initial heating of the monoliths may cause the enzyme to partially unfold in the pore, it apparently refolds to its optimum native state upon cooling. This effect, in combination with improved molecular accessibility through the somewhat larger diameter pores, accounts for the short-term increase of activity.

## Summary

Creatine kinase is stabilized by encapsulation in sol−gel silica monoliths. Its activity is retained for significantly longer periods of time at both room temperature and elevated temperatures compared to that in buffer solution under the same conditions. For example, at 47 °C the activity of the enzyme in a monolith requires more than 125 h to decrease to 50% of its maximum value compared to 13 h in solution. Encapsulation of the enzyme in a pore of the sol−gel material helps to prevent unfolding. Unexpectedly, heating causes a 4-fold *increase* in activity before it begins to decrease. In the example of the material heat-treated at 47 °C, the activity increases to its maximum after about 10 h; even after 350 h, the activity does not decrease to that of the initially prepared monolith. Both the stabilization and the activity increase are a result of interactions between the surface of the enzyme and the pore surface. Circular dichroism spectroscopy shows that the initially encapsulated enzyme has a conformation different from that of the enzyme in solution. In this conformation, the activity is not at its maximum. Heating for short periods of time allows the conformation of the enzyme within the pores to change to one more closely resembling that in solution, resulting in increased activity. Heating the monoliths also causes the pore size in the silica to increase slightly, which in turn may allow the enzyme to rearrange, but unfolding is still inhibited. The optimum heating time and temperature for maximizing encapsulated enzyme activity depend on a delicate balance between the interactions and will need to be determined for each gel formulation and specific enzyme. This discovery offers a new method of improving the performance of sol−gel biomaterials.

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