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Published on Web 05/14/2005

Sol−Gel Materials as Efficient Enzyme Protectors: Preserving the Activity of Phosphatases under Extreme pH Conditions

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Abstract: By entrapment in (surfactant modified) silica sol−gel matrixes, alkaline phosphatase (AlP) − naturally with optimum activity at pH 9.5 − was kept functioning at extreme acidic environments as low as pH 0.9, and acid phosphatase (AcP) − naturally with optimum activity at pH 4.5 − was kept functioning at extreme alkaline environments, up to pH 13.0. Propositions are offered as to the origin of the ability of the matrixes to provide such highly efficient protection and as to the origin of the synergetic enhancing effect when both the silica and the surfactants are used as a combined entrapping environment. It was found that the protectability of the enzymes against harsh pH values is dependent on the nature of the surfactant.

Background

As the name of the enzyme alkaline phosphatase (AlP) implies, its catalytic activity (hydrolysis of phosphoesters to phosphate and to the corresponding alcohol or phenolate) is optimal at basic pH values (9−10[1,2]). Here, we show that by utilizing the protective features of sol−gel materials, one can keep this alkaline enzyme active under extreme *acidic* conditions, going down the pH scale to as low as pH 0.9!, and that when, for comparison purposes, the acidic enzyme acid phosphatase (AcP, optimal performance at pH 4.5−6.0[2]) is entrapped in these materials, it is kept active under extreme *alkaline* conditions, as high as pH 13. Silica-based sol−gel materials, with and without surfactant modification, were the key to these unusually large effects, which amount to practical alteration of the classical phosphatases' properties.

We recall that sol−gel materials have proven in the last two decades to be versatile carriers of active dopants.[3−5] Diverse reactive functionalities have been introduced into these materials by either direct physical doping[6−8] or covalent attachment.[9−11] Of the various families of functional sol−gel materials that have been developed, one, which has progressed particularly fast, has been the family of sol−gel materials with biochemical and biological activities.[12−16] Enhanced stability of entrapped biomolecules,[17] ease of their heterogenization,[18] compatibility with opposing reagents,[19] the convenience of tailoring the chemical and physical properties as needed for specific bioapplications,[17] improved endurance of the entrapped proteins to denaturing thermal conditions,[17] to long-term storage conditions,[20,21] and to organic solvents[22,23] are but some of the reasons for this fast growth. Here, we show an extreme pH-protectability of enzymes provided both by silica sol−gel matrixes and through *synergism* between matrix and surfactant interactions, thus utilizing yet another observation, namely, that the properties of dopants can be tailored and modified by the coentrapment of surfactants within sol−gel materials.[24,25] Finally, we note that the interaction of enzymes with surfactants in solution was studied in various contexts, such as providing enzymes with hydrophobic working environments[26,27] and shifting the optimal pH for activity.[28,29]

Results and Discussion

The activity of AlP entrapped in three types of sol−gel matrixes is shown in Figure 1A, and in Figure 1B, it is compared to the activity in solution.

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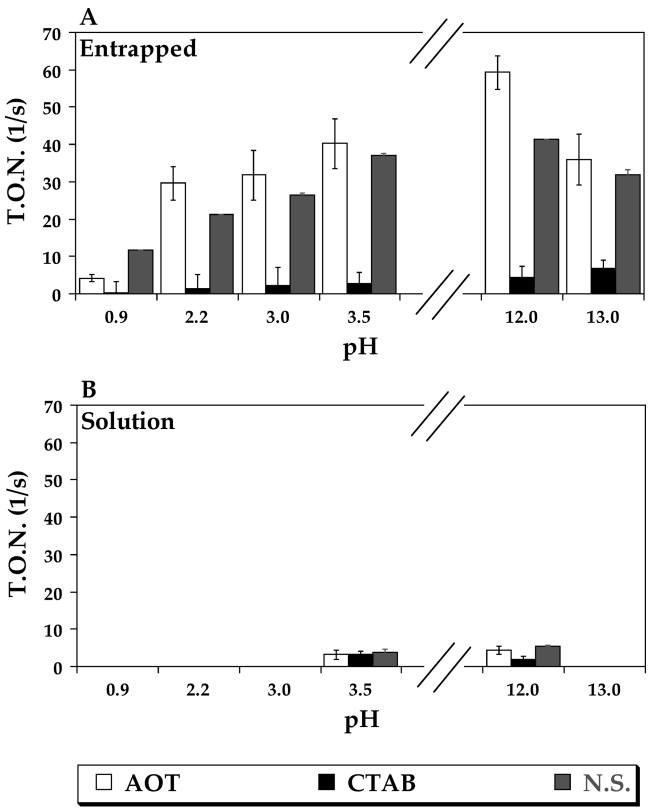

Figure 1. The activity of alkaline phosphatase (expressed in terms of turnover number (T.O.N.)) under extreme acidic and alkaline conditions. (A) Immobilized in three types of silica sol−gel matrixes (with the surfactants AOT or CTAB, or with no surfactant); and (B) comparison to solutions (with and with no surfactants).

It is seen that while in solution the enzyme loses all of its reactivity at pH 3.0, when entrapped in the sol−gel matrixes (silica and silica/AOT), this alkaline enzyme remains active at extreme pH values of 3.0, 2.2, and as low as 0.9, namely, a 30 billion times higher hydronium concentration compared to the optimal pH in solution (pH 9.5). At pH 3.5, where some activity is still detectable in solution, the entrapped enzyme activity is 4 times higher at that pH. Comparison of the activity at pH 2.4 to the activity at the optimal pH of 9.5 is provided in Table 1. Entrapment leads to increases in K_m and decreases in V_{max}, indicating trends which are consistent with diffusional limitations.

It is immediately evident from Table 1 that while pH 9.5 is still optimal for AlP@SG, moving *7 pH units* down the scale does take its toll, but that toll is not heavy, particularly when noticing that in solution, the enzyme is totally inactive at that pH. Interestingly, the matrix also offers efficient protectability at the very high alkaline pH range (Figure 1), with significant AlP activity at pH 12.0 and even at pH 13.0. The activities of AlP@SG at pH 12.0 and 13.0 are 2−3 and 40 times higher than those in solution, respectively; in fact, in solution, the enzyme is practically inactive at pH 13. It is also seen (Figure 1A) that while the anionic surfactant, AOT, improves the extreme pH durability of the entrapped enzyme (both at the low and high pH scale ends), the cationic CTAB spoils it (Figure 1A). In solution (Figure 1B), CTAB lowers the activity of AlP (Table 1) but does not quench it altogether. A nonionic surfactant, Brij 56, was tested as well, and while it did show some added protectability, the effect is much smaller (not shown), thus supporting, as we shall see, the ionic mechanism of the surfactant operation suggested below.

To see if this enhanced stability phenomenon is applicable beyond the specific case of AlP, we took acid phosphatase (AcP) not only because we wanted to see if one can "symmetrically" protect it against extreme alkalinity but also because the mechanism of operation of this enzyme, as well as the details of its active site, are completely different from those of AlP.[30] It was found that whereas this enzyme loses all of its activity in solution under high alkaline conditions (Figure 2B), it remains active within the sol−gel matrixes at pH values of 10.0, 11.0, 12.0, and as high as 13.0 (Figure 2A).

Unlike AlP, matrix isolation did not protect AcP activity below pH 3. As for the surfactants effect, a "mirror" behavior was observed. This time, CTAB was the surfactant that was found to enhance significantly the activity, compared to entrapment in pure silica (Figure 2A), while AOT poisoned the enzyme completely, both in the entrapped form and in solution (data not shown).

Another relevant observation, to be discussed below, is that the type or presence of the surfactant affected the amount of the entrapped enzyme (Table 2).

In brief, the main features of Table 2 are good entrapment with CTAB (somewhat better for AcP), somewhat lower entrapment efficiency without any surfactant, and about 50% lower AlP entrapment in the presence of AOT.

We shall now offer some interpretations for these striking phenomena, starting with the protectability provided by the pure silica sol−gel cage. The accumulated observations on the stabilization of enzymes within silica sol−gel matrixes have pointed to *physical* cage confinement as a major source of this stabilization. Thus, it has been proposed[31−35] that the rigidity of the ceramic cage does not allow the protein to undergo denaturing unfolding−refolding motions, and once the enzyme is held intact from that point of view, its activity is preserved. Here, we propose to consider a new possible explanation for the entrapment−protectability against steep pH gradients, which adds to the cage yet another role in the protection mechanism (Scheme 1).

To begin with, we note that the relatively free rotation of sol−gel entrapped enzymes[31,32] implies that the entrapping cage still has some free space. That free space, the space between the outer surface of the protein and the silica surface of the cage, is composed of very few, perhaps as low as one or two, water molecule layers. Let us focus then on this layer and evaluate what do large pH changes actually mean in such very small local environments? Suppose, for the sake of demonstrating the point, that the water layer is a small reservoir of 100 water molecules surrounding the protein as a blanket against the silica cage wall, and that the external pH is very acidic, say pH 0, and that the hydronium ions penetrate that reservoir until

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Table 1. Effects of Surfactants on the Activity of Entrapped Alkaline Phosphatase at pH 9.5 and 2.4

	Entrapped Enzyme						Enzyme in Solution					
	pH 9.5			pH 2.4			pH 9.5			pH 2.4		
surfactant	V_{max} (μM/s)	K_m[a] (mM)	T.O.N.[b] (s^{-1})	V_{max} (μM/s)	K_m (mM)	T.O.N. (s^{-1})	V_{max} (μM/s)	K_m (mM)	T.O.N. (s^{-1})	V_{max} (μM/s)	K_m (mM)	T.O.N. (s^{-1})
none	0.12	0.167	23.5	0.04	0.119	7.8	0.45	0.059	81.8			
AOT	0.15	0.331	58.8	0.04	0.267	15.7	0.37	0.048	67.3	no activity under any condition		
CTAB	0.04	0.216	7.5	no activity			0.13	0.0004	23.6			

[a] Michaelis−Menten constant. [b] Calculated by dividing the rate of reaction at substrate saturation by the concentration of the entrapped enzyme. We refer to enzyme concentration within the gels as the amount used for entrapment minus the various washings.

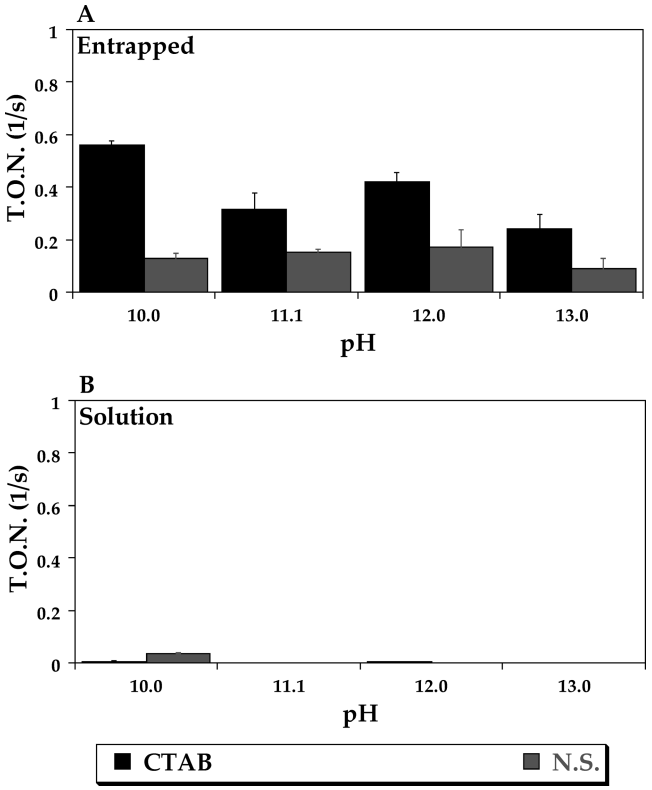

Figure 2. The activity of acid phosphatase under extreme alkaline conditions. (A) Immobilized in two types of silica sol−gel matrixes (with or with no CTAB); and (B) comparison to solutions (with and with no CTAB).

Table 2. Amount of Enzyme Entrapped in the Matrixes

surfactant	enzyme	entrapment (%)
none	AlP	93.5
	AcP	94.6
AOT	AlP	46.7
	AcP	*a*
CTAB	AlP	96.4
	AcP	98.7

[a] Since AcP is not active when exposed to AOT in solution, it is not possible to determine leaching by measuring the enzymatic activity of the washings (as done for all other entries in the table).

equilibrium is reached, and a nominal "pH 0" is obtained there, too. What does this mean from the point of view of the protein? It means that the protein gets protonated by only *two* protons! (Recall that "pH 0" means ~2 moles H_3O^+ for each 100 moles water.) So, on one hand, two protons are enough to compensate for the extreme pH gradient, but on the other hand, for the protein itself, these two protons pose no stress at all. Perhaps two basic amino acid residues are protonated, and that is all. In other words, whereas in solution pH 0 means constant bombardment with hydronium ions, promoting efficient denaturing reactions, the confinement within a small cage containing a small reservoir of water molecules shunts this process and renders a local nominal value of high acidity benign. A similar proposed argument holds for the protectability at the alkaline side of the pH scale. Finally, a word of caution is in order regarding the terminology used for this molecular level description; it is, of course, not possible to use terms which belong to the realm of very large (thermodynamic relevant) assemblies, such as pH. Therefore, the picture of what happens inside a small pore should indeed be described in terms of the small numbers involved, that is, 2 protons in an environment of 100 molecules, in our example; yet, as we have shown here, the shift from thermodynamic concepts to very small numbers is an eye opener.

For AlP, this effect, as mentioned above, is moderately enhanced by the use of coentrapped AOT but quenched when CTAB is used (Figure 1). We also mention again the "mirror" behavior found for AcP, namely, that CTAB enhanced significantly the activity (by a factor of 130 at pH 10) and that AOT poisons the enzyme completely both in solution and in the entrapped form. It is thus evident that the nature of the surfactant plays a crucial rule in affecting the reactivity of the entrapped enzymes. We recall that in solution, surfactant binding onto a protein surface is a mixed event. It usually starts with electrostatic interactions between the polar head of the surfactant and polar moieties on the protein surface, and when these become saturated, the interaction reverses and the hydrophobic chains of the surfactant bind by van der Waals interactions with hydrophobic moieties on the protein.[36] However, when this system is confined within a solid matrix, the interactions are further complicated by competing with the binding sites of the solid substrate.[37,38] In the case of the sol−gel matrixes, the AOT and CTAB differ in their modes of interaction both with the surfaces of the two proteins (although their pK_i values are quite similar, 5.0 for AcP[39] and 5.7 for AlP[1]) and with the two entrapping silica surfaces. This is so because the entrapment procedures, which take place at two different pH values, charge the silica surface and the proteins surfaces differently. Thus, AlP, which is entrapped at pH 9.5, becomes rich in negatively charged moieties, and these sites adsorb strongly the cationic CTAB to the level that this tight adsorption blocks the ability of pNPP to penetrate the active site. CTAB also adsorbs strongly to the negatively charged silica cage surface (SiO^- at this pH), adding to the closed packing around the enzyme in a (disor-

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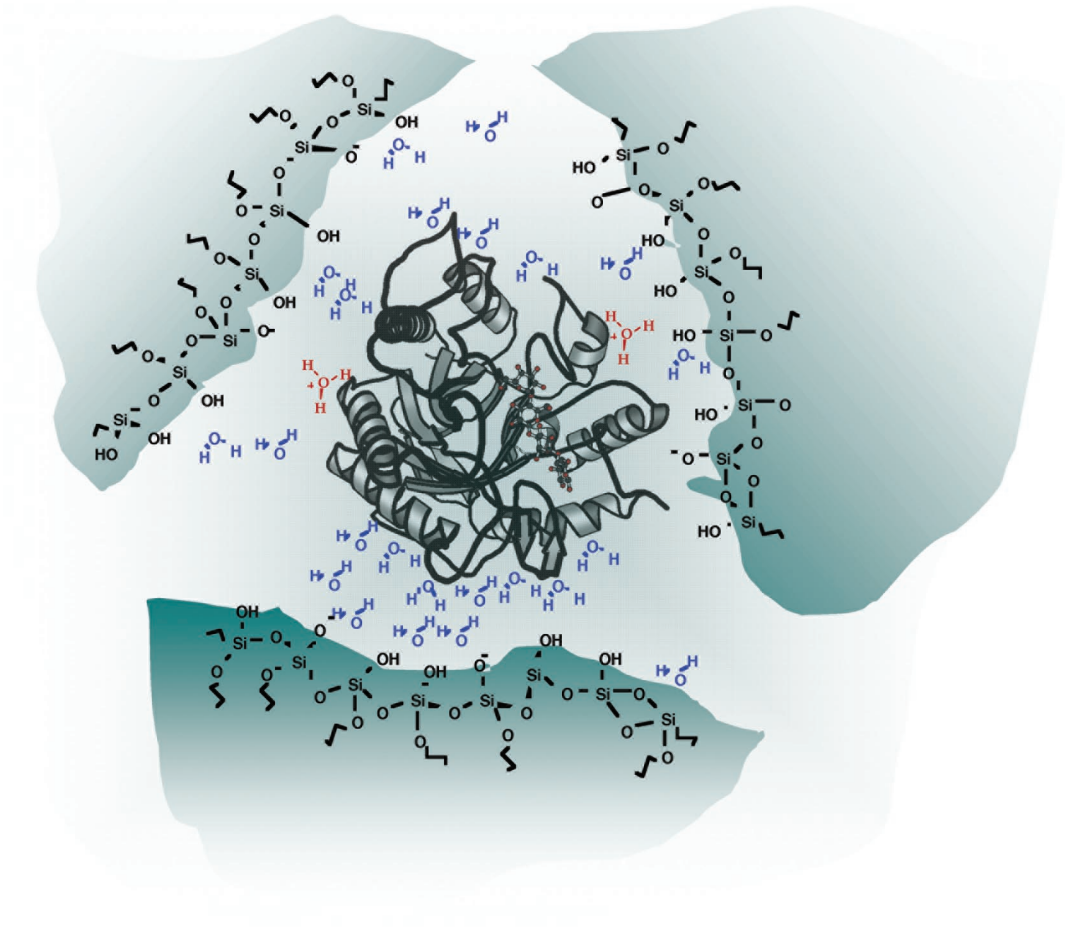
Scheme 1. Schematic View of the Entrapped Enzyme with a Few Water Molecules Inside a Pore, Two of which are Protonated: The Nominal ″pH″ is Very Low



dered) bilayer arrangement. The anionic AOT, on the other hand, "suffers" from an unfriendly environment of negative charges on the surfaces of both the enzyme and the silica cage. AlP is thus held quite loose by this surfactant blanket, as indeed evident both by the fact that about 50% is washed away during entrapment (compared to 5% in the case of CTAB; see Table 2) and by the very fact that it is free enough to remain reactive. The added stability against a steep hydronium gradient is probably provided by the negative head of AOT, acting as a hydronium sponge,[18,40] and by the difficulty of the hydronium ions to penetrate the hydrophobic portions of the protecting blanket. The added protectability against hydroxyls can be attributed to the negative charge repulsion of the whole system: protein, surfactant, and silica.

Similar arguments, although in "symmetrically" reverse charge picture, hold for AcP. Since the entrapment was carried out at pH 4, CTAB molecules adsorb on the silica through the positive nitrogen (CTAB interacts strongly with either SiO^- or SiOH[41]) but to a much lesser degree on the mostly positively charged protein surface, thus coating it loosely with the hydrophobic chains of the surfactant molecules that allow the penetration of pNPP. As already shown by Rottman et al.,[40] entrapped micellar structures exhibit enhanced hydrophobicity, and this may account for the significant synergetic effect of both the silica cage and surfactant in protecting the AcP against extreme alkaline pH values (Figure 2). In fact, this effect is much stronger here than for AlP@AOT/silica, and we attribute this difference to the different pH values at which the proteins were entrapped (9.5 for AlP and 4.5 for AcP), resulting in the known more spacious network and cages for the latter.[42] This interpretation is corroborated by the lower entrapment of AlP compared to that of AcP (Table 2), by its higher T.O.N. values (Table 1), and also by the inability of the matrix to protect AcP activity below pH 3. Also in support for the ionic mechanism involved with the surfactants in both cases is the observation mentioned above, that is, the nonionic surfactant Brij 56 provides only minimal added value.

In conclusion, we have revealed an unusually high level of protection of phosphatases against harsh pH conditions by entrapment in silica sol−gel matrixes and have shown the added value of coentrapment of surfactants. The main objective of this research has been to answer the need for having enzymes capable of operating under unorthodox environments as may be required for sensors and for biocatalysts operating under non-native conditions. We believe that this objective has been achieved by showing its feasibility in the case of an alkaline enzyme operating at pH 0.9 and of an acidic enzyme operating at pH 13.0.

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Acknowledgment. Various parts of this research project were supported by the Ministry of Science, the Eshkol Scholarship (to H.F.-M.), the Infrastructure Project (Tashtiot), and the French−Israeli AFIRST Program.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0507719