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Protein Encapsulation in Mesoporous Silicate: The Effects of Confinement on Protein Stability, Hydration, and Volumetric Properties

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In this work we were interested in testing the effects of excluded volume and confinement on protein un/refolding and stability, since these conditions may mimic the environment in which proteins evolve to fold. [1] On the basis of the predictions of statistical−thermodynamic models,[2] it is surmised that excluded volume effects due to the crowded nature within a cell may play a significant role in the stability, interaction, and function of biomacromolecules. Thus, one might question the completeness of any results obtained by the reductionist approach where biomolecules are characterized in dilute solutions only. Our approach was to encapsulate a model protein, ribonuclease A (RNase A), in a mesoporous silica, MCM-48, with glasslike wall structure and well-defined pores to create a confined microenvironment. There are only a few direct techniques which are able to probe the physicochemical properties of proteins in silicates. [3,4] To the best of our knowledge, this is the first report where pressure perturbation (PPC) and differential scanning (DSC) calorimetric techniques are employed to evaluate the stability, hydration, and volumetric properties of a protein confined in mesoporous silicate. Encapsulation of proteins in ordered mesoporous materials (OMM) made of silica has several advantages over its immobilization by other means: OMM possess large surface areas, highly ordered pore structures, very narrow pore size distributions, and variable pore diameters, which can be finely tuned (from about 15 to 100 Å) by changing the synthesis conditions, and hence are attractive candidates to host large molecules, including proteins. [4] They also hold promise for use as supports to immobilize enzymes and may find applications in molecules separation systems, biocatalysis, and biosensors.[3]

An average pore size of 25 Å siliceous MCM-48 material was synthesized using standard hydrothermal synthesis and calcination methods (pore size variation: 20−30 Å).[5] The dimensions are just comparable to the dimensions of RNase A (radius of gyration ≈ 15 Å)[6] to probe the maximum effect the confinement may exert on protein stability. RNase A is a single-domain protein, a pancreatic enzyme that catalyzes the cleavage of single-stranded RNA, which consists of 124 amino acid residues with a molecular mass of 13.7 kDa. RNase A was obtained from Sigma Chemicals (catalog no. R5500), Germany. In each experiment, 10 mg/mL of predried silicate was mixed with 0.7−4.0 mg/mL of RNase A solution in 10 mM potassium phosphate buffer at pH 5.5. The mixture was stirred for 4 h at 20 °C; within this time, solute incorporation reached equilibrium. The thermodynamic properties upon thermal unfolding, such as the temperature (T_m), the enthalpy change (ΔH), and volume change (ΔV) of unfolding as well as the apparent thermal expansion coefficient (α) of the protein, were measured by means of a high-precision VP DSC microcalorimeter equipped with a supplementary pressure perturbation calorimetric system (MicroCal, Northamption, MA). The working principle, data input, and sample injection procedure for conducting the PPC and DSC experiments as well as the data evaluation procedures are described elsewhere.[7]

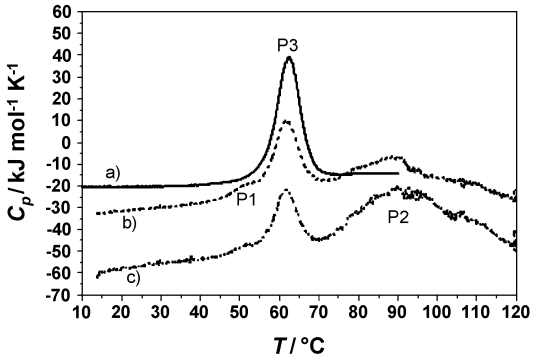

Figure 1. Representative DSC traces (background corrected, scan rate 40 °C/h) of RNase A in MCM-48 at variable concentrations in 10 mM phosphate buffer at pH 5.5. The thick line (a) corresponds to a 5 mg/mL RNase A solution in pure buffer solution. Dashed (b) and dash−dotted (c) lines represent protein concentrations of 4.5 and 2.2 mg/mL, respectively, dissolved in 10 mg/mL MCM-48.

Depending on the protein concentration, the protein can be adsorbed on the external surface (population represented as P1) and/or diffuse into the pores of the MCM-48 (P2), and there might be an excess protein fraction without any restricted mobility, i.e., with bulklike behavior of free protein (P3). Figure 1 exhibits the DSC traces of RNase A in MCM-48. The DSC data indicate the existence of adsorbed, encapsulated, and free states of the protein which are denoted as P1 ($T_m \approx 52$ °C), P2 ($T_m \approx 90$ °C), and P3 ($T_m \approx 62$ °C), respectively. Protein molecules adsorbed onto the silicate surface are susceptible to weak destabilization, and hence their unfolding temperature is shifted to slightly smaller values.[3] On the contrary, the encapsulated protein—owing to severe configurational restrictions[2]—is expected to show an enhanced stability. For example, in a rough statistical−mechanical calculation, assuming a random-flight chain model and neglecting intrachain excluded-volume interactions among the amino acid residues, the stability (Gibbs free energy) and temperature of unfolding for a confinement size which is about twice that of the size of the polymer increases already by about 10 k_BT and ∼20 °C, respectively.[2a]

As can clearly be seen in the DSC data, the silica-entrapped protein (P2) is in fact significantly more stable against temperature-induced unfolding compared to the protein in bulk solution. The DSC peak for the entrapped species exhibits a maximum around 90 °C ($\Delta T_m \approx 30$ °C), and the half width of the peak is at ∼20 °C, which probably largely reflects the variation in the pore size diameter in MCM-48, which varies from about 20 to 30 Å with a strong maximum around 25 Å.[5] At very low protein concentrations

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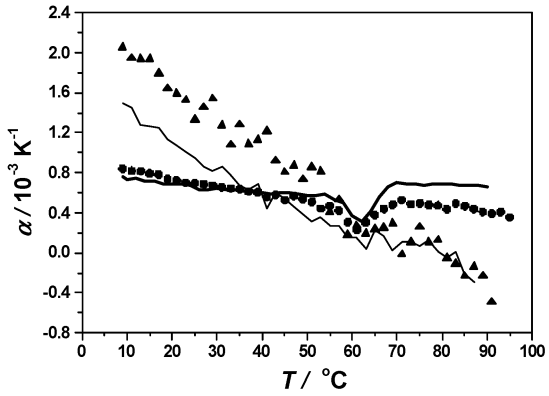

Figure 2. Temperature dependence of the apparent thermal expansion coefficient α (background corrected, appromixate scan rate 40 °C/h) of RNase A in MCM-48 at variable concentrations in 10 mM phosphate buffer at pH 5.5. Circles, thin line, and triangles, represent 4, 1.2, and 0.7 mg/mL concentrations, respectively, of RNase A dissolved in 10 mg/mL MCM-48. The thick line corresponds to a 5 mg/mL RNase A solution in pure 10 mM phosphate buffer solution at pH 5.5.

(< ~1 mg/mL), the noise of the DSC traces is increasing significantly, and C_p no longer exhibits a well-pronounced peak structure but rather increases more or less steadily up to the highest temperatures measured (data not shown).

The isoelectric point of RNase A is 9.6, making it positively charged at pH 5.5. The silanol groups of the silicate are negatively charged at this pH. As a result, protein migration into the silica pores is enhanced by this electrostatic potential difference. The interaction between the positively charged protein and negatively charged silica surface is still weak and does not lead to strong adsorption and destabilization of the protein, however.

The aim of our work was also to explore if the hydration properties of the protein change upon entrapping in the MCM, which—in addition to the entropic confinement effect—might play a role in protein stabilization as well. It has been shown recently, that PPC measurements are able to yield valuable information on protein hydration and compactness as well as accurate volume changes in the course of protein unfolding.[7]

Figure 2 reveals the PPC data (apparent thermal expansion coefficient, α) of RNase A in pure buffer solution and when confined in MCM-48 at different concentrations. Our previous results on cosolvent effects on protein PPC data revealed that the level of hydration contributes significantly to protein stability as can be revealed from the absolute value and the magnitude of the negative slope of the apparent thermal expansion coefficient, $d\alpha/dT$, of the protein.[7] Surprisingly, Figure 2 shows that the α and $d\alpha/dT$ values measured for a concentration where almost all protein is incorporated in the MCM-48 are drastically enhanced. For example, at a concentration of 0.7 mg mL^{-1} protein, α (10 °C) and $d\alpha/dT$ values of 2.1×10^{-3} K^{-1} and -3.3×10^{-5} K^{-2} are obtained, compared to corresponding values of 0.85×10^{-3} K^{-1} and -4×10^{-6} K^{-2} for RNase A in pure buffer solution. The higher α and $d\alpha/dT$ values of the encapsulated protein indicate that the protein is more strongly hydrated in the narrow silica pores. As a consequence, this is also expected to increase its thermal stability (preferential hydration effect).[7] It is generally believed that Hofmeister ions, and hence also the close-by silanol groups at the silica surface, influence the protein structure indirectly through changes in the hydrogen bonding properties of water, which might lead to an increased hydration strength of the embedded protein. Part of this effect could also be due to a decrease of the rotational and translational dynamics of the confined system.

The PPC curves between 50 and 70 °C reflect the unfolding of free RNase A in buffer solution. The relative volume change upon unfolding, $\Delta V/V$, for free RNase A is negative and amounts −0.27%, which is in good agreement with literature data (−0.29%).[7] With regard to the embedded protein, α is continuously decreasing with temperature, though with decreasing slope at high temperatures. This is in contrast to RNase A in pure buffer solution, whose posttransitional α values are lying above the pretransition baseline, indicating an increase of the expansivity in the unfolded state. A well-resolved free protein denaturation transition curve is observed at high protein loadings, only. With regard to the confined protein, the data indicate that inside the silica pores significant unfolding is no longer feasible and hence must be incomplete. No volume change can be determined even with this sensitive PPC method applied, which allows measuring of ΔV values as small as ~0.1%.

Several important corollaries may be inferred from this study: The stability of the protein RNase A confined in the mesoporous silicate system MCM-48 is drastically increased ($\Delta T_m \approx 30$ °C). A similar effect is expected to occur in crowded systems of high protein concentrations, although the effect may be less pronounced as these are soft-matter systems. In dense protein solutions, irreversible protein aggregation often leads to spurious effects, however. No significant volume change upon unfolding of the confined protein is observed even up to temperatures as high as 120 °C. It is intriguing that the protein penetrates into the mesopore network despite the fact that the pore size is similar to that of the protein. The increase in stability is probably not only due to a restriction in conformational space (excluded volume effect), but may—at least partially— also be due to an increased strength of hydration of the protein in these narrow silica pores. The latter effect is expected to depend on the surface chemistry of the OMM and may be induced by the particular water-structuring properties of the silanol groups at the silica surface, which is in close proximity to the protein surface in our case.

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