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Citation: J. Chem. Phys. **135**, 141104 (2011); doi: 10.1063/1.3650700
View online: http://dx.doi.org/10.1063/1.3650700
View Table of Contents: http://jcp.aip.org/resource/1/JCPSA6/v135/i14
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Communication: Folding of glycosylated proteins under confinement

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(Received 31 May 2011; accepted 21 September 2011; published online 11 October 2011)

Conjugating flexible polymers (such as oligosaccharides) to proteins or confining a protein in a restricted volume often increases protein thermal stability. In this communication, we investigate the interplay between conjugation and confinement which is not trivial as the magnitude and the mechanism of stabilization are different in each instance. Using coarse-grained computational approach the folding biophysics is studied when the protein is placed in a sphere of variable radius and is conjugated to 0–6 mono- or penta-saccharides. We observe a synergistic effect on thermal stability when short oligosaccharides are attached and the modified protein is confined in a small cage. However, when large oligosaccharides are added, a conflict between confinement and glycosylation arises as the stabilizing effect of the cage is dramatically reduced and it is almost impossible to further stabilize the protein beyond the mild stabilization induced by the sugars. © *2011 American Institute of Physics*. [doi:10.1063/1.3650700]

Various external factors can modulate the information stored in the sequence of amino acids and, therefore, affect protein folding. This phenomenon is routinely used in the laboratory to study the thermodynamics and kinetics of folding by changing the chemical nature of the solvent or the temperature. Alteration of the biophysical characteristics of proteins is also exploited *in vivo* by the cell by various means. For example, confining a protein in a small biomolecular cage or changing the cellular crowding condition can affect its folding. Similarly, introducing post-translational modifications (PTMs), which are ubiquitous in the cell, may also change folding biophysics. In this study, we investigate the interplay between two effects: conjugating polymers to a protein and confining the protein in a small volume.

Conjugating moieties, either small chemical groups (e.g., phosphate, acyl, or methyl groups) or large polymers (e.g., oligosaccharides or poly-ubiquitin protein conjugates), to proteins are very common modifications and serve diverse biological functions in the cell. These PTMs often occur at specific locations on the protein to which the modification it attached covalently. In addition to the role of PTMs in regulating bioactivity, they may also affect the biophysical properties of proteins. For example, phosphorylation (the addition of a phosphate group) is often involved in conformational changes, myristoylation (the covalent linkage of a saturated C14 fatty acyl chain) can enhance folding kinetics,[1] and ubiquitination (conjugation of a poly-ubiquitin chain) can induce local unfolding that may assist protein proteasomal degradation.[2] In particular, glycosylation (conjugation of an oligosaccharide) is interesting since it was shown, both experimentally and computationally, that it can increase the thermodynamic stability of various proteins.[3–5] The primary effect of the solvent-exposed oligosaccharide moiety on protein thermodynamics arises not so much from the formation of new interactions between it and the protein[6] as from its bulkiness, which forces a less compact structure on the protein's unfolded state.[4]

Another well-known mechanism for altering the biophysical and structural features of proteins is confinement. In the cell, this is achieved for example by chaperonins, which are small cages in which the biophysical characteristics of proteins differ from those in the plasma environment of the cell and facilitate folding.[7–12] The influence of small cages on protein folding was already explored in several computational studies using various models.[13–19] In all these studies, the protein was stabilized by the confinement and the stabilization increased with increasing confinement.

In fact, the oligosaccharide (or other polar polymer conjugate) may be viewed as an intramolecular "crowding agent" that is permanently close to the protein. Glycosylation and confinement, thus, share similarities and it is intriguing to explore the interplay between them and the mutual influence of the combination of these mechanisms on the protein thermodynamics. While both glycosylation and confinement are suggested to enhance protein stability by affecting the dynamics of the unfolded state, the biophysical origin of the stabilization is not identical. Confinement results in a more compact unfolded state and, therefore, lower entropy than the unfolded state in the bulk, whereas glycosylation results in a more expanded unfolded state with loss of residual structure and thus higher enthalpy. Nevertheless, stabilization by confinement or glycosylation can have both enthalpic and entropic components and it is the goal of this study to examine the biophysical outcome when both perturbations are applied to the protein.

To this end, we designed six glycosylated variants of the protein Src-SH3 domain with different degrees of glycosylation and confined each of them in a spherical cage of various dimensions. The folding of the conjugated proteins was studied using a coarse-grained native topology-based simulation model as described in detail in Ref. 3. Confinement was introduced by applying a confining potential around the protein

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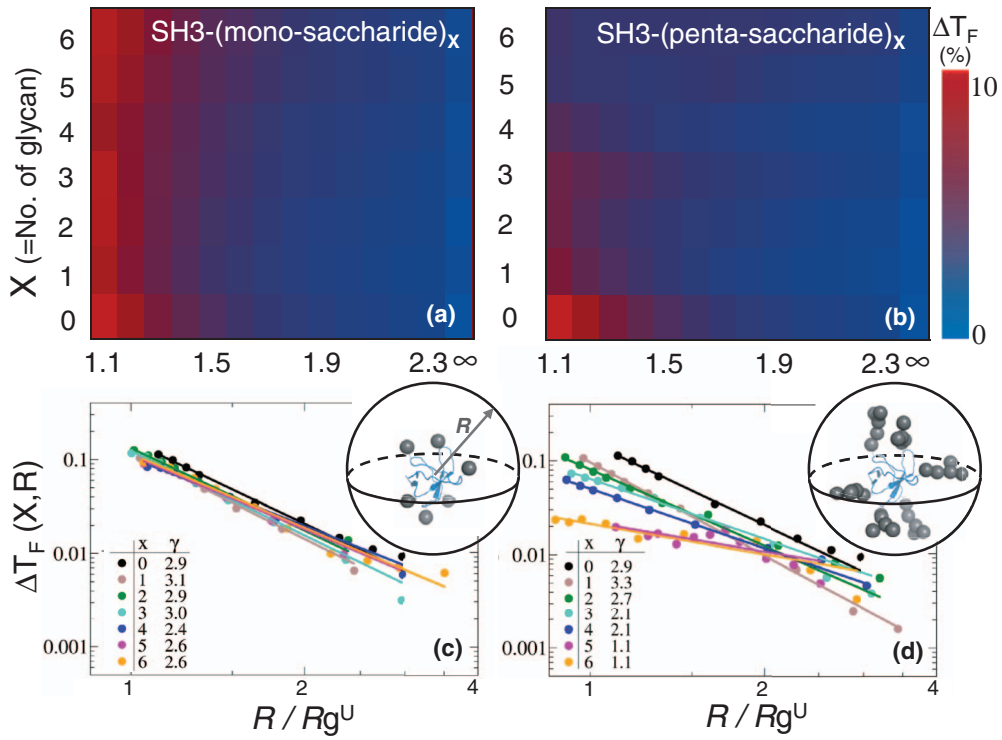

FIG. 1. The interplay between confinement and glycosylation on protein thermal stability. Changes in the thermal stability $[\Delta T_F(X, R') = (T_F{}^{X;R'} - T_F{}^{X=0;R'=\infty})/T_F{}^{X=0;R'=\infty}]$ of proteins conjugated to X = 1–6 oligosaccharides and placed in a sphere of radius R are shown. The scaling between the $\Delta T_F(X, R')$ and the normalized radius of the confined sphere (R/Rg^U) is measured by the slope γ. Two oligosaccharides were used: a mono-saccharide, GlcNAc (A and C) and a penta-saccharide, $Man_3GlcNAc_2$ (B and D). Illustrations of the protein with six conjugated mono- and penta-saccharide moieties confined in a sphere are shown. All glycosylated variants of SH3 with the mono-saccharide follow a similar scaling law to that of the unmodified protein, with γ of 2.6 to 3.1. The glycosylated variants with penta-saccharide conjugates show a larger range of γ values from 1.1 to 3.3.

of the form $U = K[(c/2r)^4 - 2(c/2r)^2 + 1]$ (Ref. 14) in which r is the distance between the ith residue and the ball, and is enforced when carbon α is within a distance of $C/2$ from the ball shell, where C was set to 4 Å and K was set to 100. We examined how the size of the cage and the number of conjugated sugars alter protein stability. We also explored the effect of the length of the conjugated sugars on the cross talk between glycosylation and confinement and thus on the degree of stabilization.

Typically, confining proteins in small spaces results in greater protein stability as reflected by an increase in the folding temperature, T_F (obtained from the peak of the specific heat curve and corresponding to the temperature at which protein stability is zero). While various proteins follow a similar scaling law for stabilization upon confinement, it is unclear whether this scaling law is valid for conjugated proteins. Proteins are linear polymers and the conjugation transforms them into branched polymers, which may restrict their compressibility. To examine the thermodynamics of branched proteins under confinement, we designed two sets of glycosylated variants of the SH3 domain with increasing numbers of glycans (between 1 and 6 oligosaccharide chains). One set of variants used up to six mono-saccharide GlcNAc units and the second set of variants incorporated up to six penta-oligosaccharide $Man_3GlcNAc_2$ units.

The bare effect of glycosylation on protein stability was examined by studying the folding thermodynamics in the bulk (i.e., a confined sphere with a radius of ∞). In the bulk, glycosylation with the monosaccharide increases T_F by 0.6%–1.2% and glycosylation with the penta-saccharide increases T_F by 0.5%–1.9% compared to the T_F of the unmodified protein. This increase in T_F translates into an increase in the population of the folded state from 50% to 62% for SH3-$(GlcNAc)_6$ and to 70% for SH3-$(Man_3GlcNAc_2)_6$.

To investigate the dependence of stability on confinement, we calculated the T_F of the two sets of glycosylated variants in balls of different sizes (Fig. 1). Confining the SH3 variants conjugated to mono-saccharides in a small sphere (whose radius is similar to the Rg of each variant in the bulk) results in significant stabilization, with T_F increasing by about 10% (translates to a shift in the population of the folded state from 50% to 85%), regardless of the degree of glycosylation (Fig. 1(a)). The degree of stabilization upon confinement is considerably more limited for the variants with the larger oligosaccharides. For SH3 glycosylated with penta-saccharides, the stabilization by confinement decreases as the degree of glycosylation increases (Fig. 1(b)). For example, while the T_F of the variant with six GlcNAc increased by 9.7% when it was placed in a sphere of radius $1.1Rg^U$, the T_F of the variant with six $Man_3GlcNAc_2$ increased by only 3.6% when similarly confined, compared to unmodified SH3 in the bulk.

The dependence of the stability of mono-saccharide variants on the size of the ball is plotted in Figure 1(c) and is similar to that of the unmodified variants of SH3. The data are plotted on a logarithmic scale and fitted to a power-law function $\Delta T_F \propto (R/Rg^U)^{-\gamma}$. Takada *et al.*[14] have shown

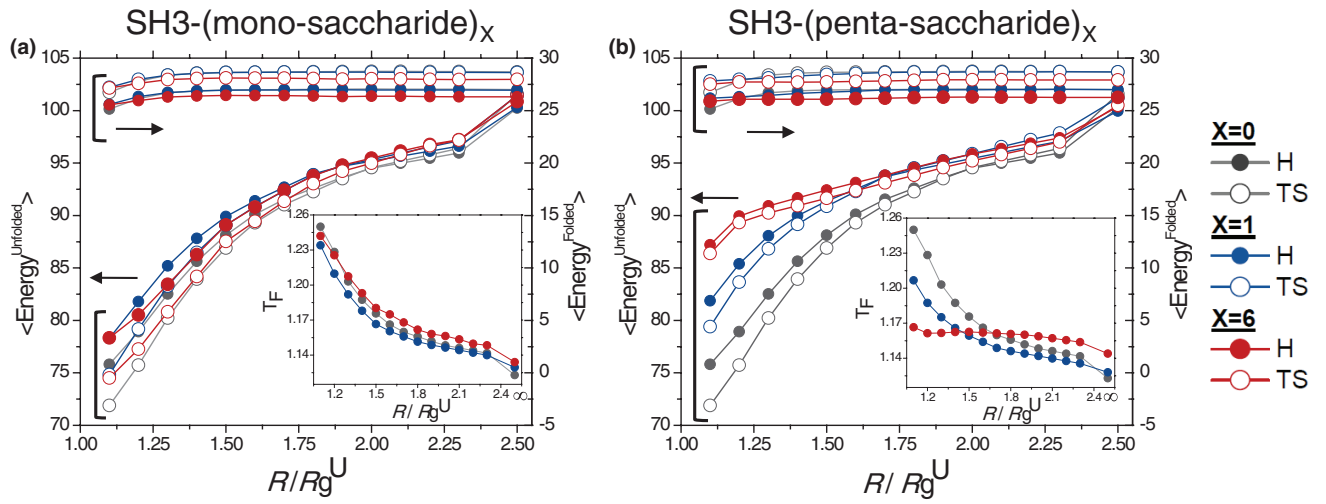

FIG. 2. Thermodynamic characterization of the conjugated protein with mono- or penta-saccharides. The enthalpy (H) and the entropy (TS) of the folded state (right y-axis) and the unfolded state (left y-axis) are shown for SH3 variants conjugated with X = 0 (gray), 1 (black), or 6 (red) mono-saccharides (a) or penta-saccharides (b). The T_F of the corresponding SH3 variants for different degrees of confinement is shown in the insets.

that for many small proteins (having different topologies), the dependence of stability on degree of confinement in a cylinder follows the same scaling exponent of $\gamma = 3.2$. In our simulations, we observed $\gamma = 2.9$ for the unmodified SH3 in accordance with the observations of Takada *et al*. A scaling exponent $\gamma = 2.5$ for SH3 was also found in simulations using a ball with a hard repulsive cavity (similar to the potential used here).[20] In that work, however, the scaling exponent parameter differed significantly with the nature of the repulsive potential and with the topology of the protein. The scaling exponent parameters for the six glycosylated variants of SH3 involving mono-saccharides are between 2.4 and 3.1 (Fig. 1(c)). This implies that conjugation of a small glycan does not change the nature of the protein although the excluded volume of a glycan ring is larger than that of an amino acid. When the larger conjugates were attached to the protein, it was observed (Fig. 1(d)) that the variant with few penta-saccharides follow the scaling law that was described for short glycans, but variants with a larger number of long glycans deviate significantly from that rule (e.g., SH3 with five or six $Man_3GlcNAc_2$ have $\gamma \approx 1$). This implies that when a significant part of the protein is glycosylated with bulky glycans (i.e., branched chains with a large excluded volume), the change in stability upon confinement is smaller. To understand the discrepancy between the effect of short and long glycans on folding stability upon confinement, we thoroughly analyzed the folding thermodynamics of four representative variants with low and high degrees of glycosylation (i.e., with a single or six conjugated sugar moieties of each type).

Figure 2 shows the enthalpic and entropic components of both the folded and unfolded states of the four glycosylated variants of SH3 as well as of the unmodified protein. For SH3 conjugated with mono-saccharides, the folded state is hardly affected by the conjugation or the confinement (Fig. 2(a)). The unfolded state, however, is strongly affected by the confinement as both the enthalpy and the entropy are reduced (Fig. 2(a)). Since the entropy of the unfolded state is reduced more than its enthalpy, there is an overall thermodynamic stabilization of the protein, as reflected by the increase in T_F when folding takes place in small cavities. The relative effect of the degree of glycosylation on the enthalpy and entropy of the unfolded state is quite small, compared to the effect of confinement.

A stronger decrease in entropy than in enthalpy is observed also for the unfolded state of SH3 when it is conjugated with penta-saccharides (Fig. 2(b)). As for the unmodified protein or the protein with the short glycan, the reduction in entropy is not compensated by the reduction in enthalpy and so results in a higher T_F. However, in the case of the long glycan moieties, the decrease in entropy is smaller than that which occurs for the corresponding variant with the shorter glycan. The T_F of the SH3 variant glycosylated with six penta-saccharides remains almost unaffected upon reducing the radius, R, of the confined space. The enthalpy and entropy of this variant decreases similarly as the sphere contracts (Fig. 2(b)) and, therefore, the free energy of the unfolded state in a small sphere is similar to that in the bulk and the overall thermal stability is not affected by the confinement.

To understand the dependence of the stabilizing effect of confinement on the length of the oligosaccharide chain, we followed two protein structural properties, namely, the average radius of gyration of the protein, $\langle Rg \rangle$, and the average number of native contacts, $\langle Q \rangle$, in the folded (F) and unfolded (U) states during folding reactions simulated in confined spaces of different sizes. The change in the $\langle Rg^U \rangle$ of four variants of SH3 upon confining in increasingly small spheres is nearly the same (~3.5 Å), indicating that the degree of compaction of the unfolded state is constant irrespective of the type or number of glycans attached to the protein (Fig. 3(a)). The changes in $\langle Q^U \rangle$, however, strongly depend on the type and number of attached glycans (Fig. 3(b)). When small glycans are attached, a substantial number of native contacts (~30 contacts) are formed on average in the unfolded state upon confinement. This is similar to the number formed in the unfolded state of the unmodified protein in a confined space. When a larger glycan is attached, however, a smaller

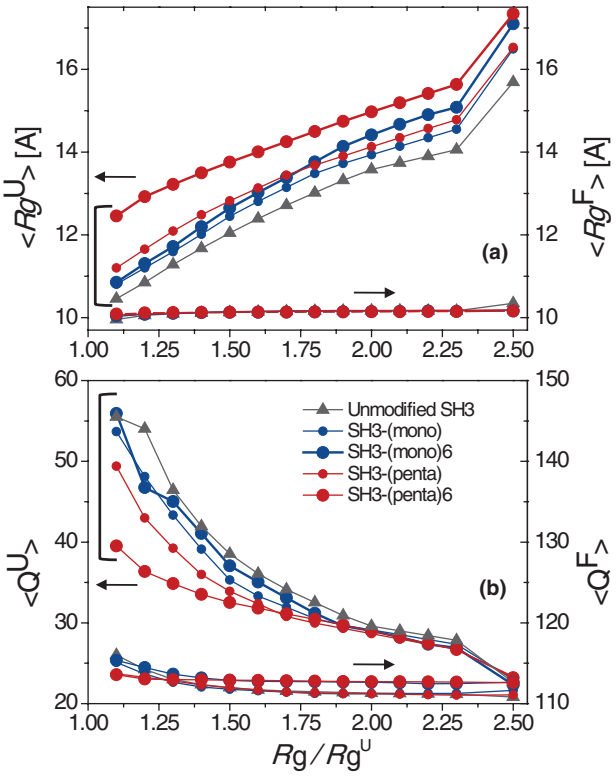

FIG. 3. Structural characterization of conjugated proteins upon confinement. The average radius of gyration (a) and of the number of native contacts (b) in the folded state ($\langle Rg^F \rangle$ and $\langle Q^F \rangle$, respectively) (right y-axis) and in the unfolded state ($\langle Rg^U \rangle$ and $\langle Q^U \rangle$, respectively) (left y-axis) are shown for a protein conjugated with X = 0 (gray triangles), 1 (black circles), and 6 (red circles) mono- and penta-saccharide chains.

number of native contacts (~23 contacts) are formed upon confinement. The number of contacts in the unfolded state in a highly confined space is even more limited when six of the longer glycans are conjugated to the protein ($\langle Q^U \rangle$ increases by only 13 contacts upon confinement).

The folded state is hardly affected by confinement or by the glycosylation. The values of $\langle Rg^F \rangle$ and $\langle Q^F \rangle$ are almost identical for all variants and all confinement conditions (Fig. 3) and this is supported by the constant enthalpy and entropy of the folded state for all the variants (Fig. 2). The increase in $\langle Q^U \rangle$ upon compressing the unfolded state is compensated for by the decrease in its enthalpy. The decrease in the entropy of the unfolded state upon compaction (as reflected by its $\langle Rg^U \rangle$, Fig. 3) is larger than the decrease in its enthalpy, with the overall result being the observed increase in thermodynamic stability with increasing confinement.

Glycosylation in the bulk results in the loss of some native contacts in the unfolded state and this is accompanied by an expansion of the protein's dimensions (i.e., larger $\langle Rg^U \rangle$). The stabilization upon glycosylation stems from the unwinding of some residual interactions in the unfolded state and is thus enthalpic in origin.[3,4] Confining the protein in a small cage, by contrast, is characterized by a sharp increase in the residual structure of the unfolded state together with its compaction (i.e., smaller $\langle Rg^U \rangle$). The origin of the stabilization upon confinement is the large decrease in the entropy of the unfolded state, which is not compensated for by the decrease in its enthalpy. For large glycans, the compensation between the entropy and the enthalpy of the unfolded state under confinement conditions is more balanced and the net stabilization of the protein is close to zero.

To summarize, this communication discusses the interplay between the effects of glycosylation and confinement on protein stability. While both glycosylation and confinement can enhance protein thermodynamic stability, the magnitude and the mechanism of stabilization are different in each instance. Confining the protein in a small spherical cage can result in much larger stabilization, which is dominated by the reduced entropy of the unfolded state. The loss of entropy that follows restricting the allowed space is accompanied by a gain in residual structure (namely, a lower enthalpy for the unfolded state). Glycosylation, however, is characterized by a loss of residual structure in the unfolded state and an expansion of the unfolded state and the stabilization, which is typically ~5 fold smaller than the maximal stabilization that can be achieved by confinement, is enthalpic in origin. Confining proteins that are conjugated to short oligosaccharides results in a similar stabilization effect as achieved for the unmodified protein. A conflict between confinement and glycosylation arises when longer oligosaccharides are used. In particular, when the protein is highly glycosylated with bulky oligosaccharides, the opportunity for further compaction of the unfolded state and further increases in its residual structure is limited. In this case, the ability of confinement to enhance stability is negligible.

Y.L. acknowledges support from the Kimmelman Center for Macromolecular Assemblies and the Israel Science Foundation.

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