Communication

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Stabilization of Yeast Cytochrome *c* Covalently Immobilized on Fused Silica Surfaces

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Understanding the conformational change(s) of a protein upon adsorption to a substrate is central to the development and application of modern protein chip technology.[1,2] The generally accepted concept is that the protein undergoes partial unfolding or "spreading" of its structure on surface,[3−6] caused by protein−surface interactions that lower the energy of the partially unfolded state relative to the energy of the corresponding native conformation in solution. However, it has been predicted by Dill and co-workers[7] that, in some cases, the surface actually could enhance the stability of the protein conformation to a great extent. Spatial confinement such as that occurring on surfaces may raise the barriers of all dynamic processes in the protein, eliminate some extended configurations, and shift the equilibrium from the unfolded state toward the native state. For proteins confined within small cavities[7] or in crowded environments,[8] the theories have been tested by experiments conducted for proteins entrapped in silica cages produced by sol−gel processes.[9,10]

Spectroscopic studies of proteins tethered on a flat surface provide the most stringent test for the theory. The macroscopically large flat surface, preferably from that of a single crystal, serves as a platform for protein adsorption (either covalent or noncovalent attachment) under well-defined conditions. The study, however, is hampered by the low content of the proteins in a monolayer (typically 1×10^{13} molecules cm^{-2}) subject to the interrogation. To increase the effective sample thickness, we have recently developed an attenuated total reflection (ATR) technique and successfully probed the adsorption, orientation, and conformational changes of cytochrome *c* on fused silica surfaces.[11] Cytochrome *c* is a globular water-soluble protein that is ubiquitous in eukaryotes and functions as a member of the mitochondrial electron transport chain. As such, the protein typically resides in the intermembrane space of the mitochondrion, where it can reversibly bind to cytochrome *c* reductase and cytochrome *c* oxidase complexes as well as to the phospholipid bilayer by electrostatic forces.[12] Spectroscopically, cytochrome *c* is well suited for the present investigation because it exhibits a strong absorption at 409 nm (the Soret band of the heme prosthetic group), an intrinsic probe to the protein's conformational change. A wealth of information concerning the conformational change resulting from acid- and alcohol-induced denaturation is available in the literature.[13−17]

A prior experiment[11] showed that horse cytochrome *c* (HCC) partially unfolds when *noncovalently* attached to the fused silica surface. In this communication, we report our study on yeast cytochrome *c* (YCC) *covalently* attached to the surface to elucidate further the nature of this surface effect. The wild-type YCC is chosen because it possesses a cysteine at residue 102, which is not present in HCC. This cysteine residue resides at the solvent-exposed surface in the native conformation of YCC and can be employed to our advantage in this work to covalently tether the protein on the fused silica surface by use of heterobifunctional cross-linkers.[18−20] Since the amount of YCC tethered on the surface is fixed, direct comparison of the absorption profile (both peak position and intensity) of the Soret band with those of free proteins in solution can be made with high precision.

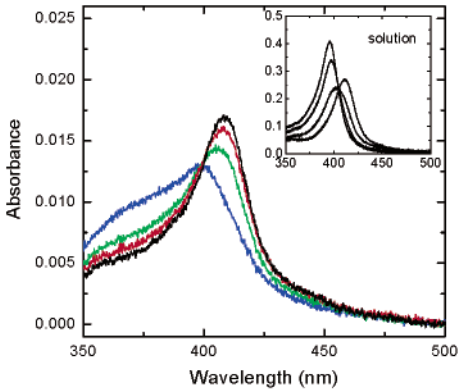

Figure 1. Absorption spectra of YCC immobilized on fused silica at pH 6.9, 3.4, 2.7, and 1.9 (from right to left). Inset: Spectra of free YCC in solution at pH 6.9, 3.2, 2.9, and 1.9 (from right to left).

Standard protocols[21] were adopted to derivatize the front face (λ/10 flatness) of a UV-graded fused silica prism (CVI) with the heterobifunctional linker, *N*-[γ-maleimidobutyryloxy]sulfosuccinimide ester (sGMBS). This was done by dipping the front face of the prism in 2% (v/v) (3-aminopropyl)-trimethoxylsilane (Sigma) in dry toluene, followed by exposure of the amino-terminated surface to 5 mM sGMBS (Pierce) in pH 7.0 phosphate buffer. A modified viton O-ring, sandwiched between the surface-derivatized prism and a glass plate, formed a cell to hold the reactant solutions.[11] Protein immobilization was established by adding to the cell a freshly prepared solution of 20 μM *Saccharomyces cerevisiae* iso-1 cytochrome *c* (Sigma) in 7 mM phosphate buffer at pH 7.5. Noncovalently bound proteins were removed by extensively rinsing the substrate with 1 M KCl and deionized water.[22] Unfolding of the immobilized proteins by either acids or alcohols was monitored in situ using a UV−Vis spectrophotometer (Hitachi U-3200) with the prism arranged in a single-pass ATR configuration.[11]

Figure 1 shows the typical absorption spectra of the surface-bound ferric-YCC as a function of solution pH. The Soret band peaks at 409 ± 1 nm at neutral pH, with the absorption intensity varying between 0.014 and 0.017, depending on the sample preparation. Photometry analysis[11] of the intensity suggests a packing density of $1.5 \pm 0.2 \times 10^{13}$ molecules cm^{-2}, denoting that a full monolayer of the proteins forms on the surface. Upon acid denaturation of the proteins, the Soret band shifts to the blue. Comparison of the spectra between the surface-bound YCC and the free YCC (inset in Figure 1)[23] reveals two significant differ-

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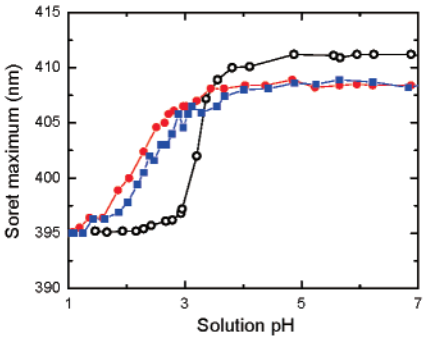

Figure 2. Shifts of the Soret band maximum of free YCC (○) and surface-bound YCC (●, ■) as a function of solution pH. The band shifts marked with ● and ■ are derived from the change of solution pH from 7 to 1 and from 1 to 7, respectively. The differences between these two sets of data are essentially within the limit of our experimental errors (±1 nm), showing the reversibility of the transition for the surface-bound protein.

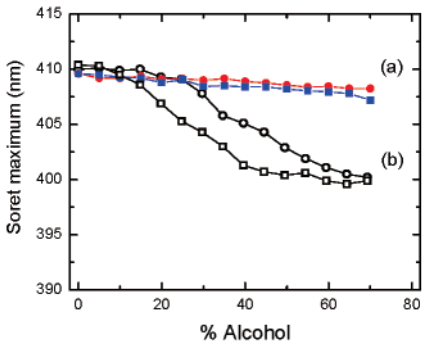

Figure 3. Shifts of the Soret band maximum of (a) surface-bound YCC and (b) free YCC as a function of methanol (●,○) and 1-propanol (■,□) concentrations at pH 4.0.

ences. First, the surface-bound YCC shows only a gradual shift of the Soret band from 408 nm at pH 7 to 396 nm at pH 2 (cf. Figure 2). The transition is less cooperative than that of free YCC, with the midpoint residing at lower pH (2.3 vs 3.2). It stands as an interesting contrast to the acid-induced conformational changes of HCC physisorbed on the bare silica surface, where the transition midpoint shifts to a slightly higher value.[11] Second, the Soret band intensity of the surface-bound YCC shows only weak pH dependence. The total intensity of this band stays nearly the same over a wide pH range (1−7), distinct from the marked intensity increase for free YCC when the solution pH decreases to pass across the transition point at pH = 3.2. These two pronounced differences are in line with the hypothesis[7] that spatial confinement stabilizes a protein in its native conformation, given that the protein (such as cytochrome *c*) is in its native state when initially immobilized on the surface.[24]

Experiments on alcohol denaturation also verify the surface stabilization effect for the spatially confined YCC. It is known that addition of alcohol to aqueous proteins stabilizes the helical structure but destabilizes the tertiary structure of a protein. The order of effectiveness toward destabilization of cytochrome *c* is propanol > ethanol > methanol.[16,17] Figure 3 shows the folding/unfolding transitions observed for methanol and 1-propanol denaturation. As the methanol content increases from 0 to 70% (v/v) at pH 4.0, the Soret band of the free YCC is seen to blue-shift smoothly from 410 to 400 nm, whereas the bands belonging to the surface-bound YCC stay essentially at the same position (λ_{max} = 409 ± 1 nm). Similar to the result of methanol denaturation, there is no dramatic shift in the Soret maximum in 1-propanol denaturation other than an increase in the band intensity by about 25%.

It should be noted that stabilization of a protein covalently tethered on a surface is not a phenomenon specific to yeast cytochrome *c* but has been found for myoglobin (Mb) as well. Using a genetically engineered Mb, Sligar and co-workers[22] observed enhanced stability of the proteins covalently immobilized on glass surfaces upon denaturation by acid, alcohol, and urea. Although the possibility of the surface-enhanced stabilization effect has been addressed by the authors, the Mb result is complicated by the irreversible loss of the heme from the protein packet in the course of the folding/unfolding transition. The conformational change of the surface-bound YCC, in contrast, is a reversible process (cf. Figure 2).

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Supporting Information Available: Figures showing UV−vis spectra for free and surface-bound YCC in alcohol solutions and control experiments for removal of noncovalently bound YCC by KCl/DI rinse (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) For larger or less stable proteins, such a stabilization effect may not be seen because the proteins are in their denatured states already when initially immobilized.

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