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Facial Control of Nanoparticle Binding to Cytochrome *c*

Halil Bayraktar, Chang-Cheng You, Vincent M. Rotello,* and Michael J. Knapp*

Department of Chemistry, University of Massachusetts, 710 North Pleasant Street, Amherst, Massachusetts 01003

Received October 19, 2006; E-mail: rotello@chem.umass.edu; mknapp@chem.umass.edu

Protein−protein interactions are crucial for complex cellular processes, providing a target for controlling biological events by protein surface binding.[1] The design of synthetic systems for protein surface recognition is a challenge, given the large binding interface required for specificity and affinity.[2] Nanomaterials are commensurate in size to proteins, and multivalent functionalization on their surfaces holds great promise for controlling biomolecular recognition.[3]

We have demonstrated previously that functionalized nanoparticles can (1) selectively recognize either cytochrome *c* (Cyt *c*) or Cyt *c* peroxidase, depending upon their surface charge characteristics and (2) consequently interrupt their mutual binding.[4] The particles used in that study, however, relied on simple electrostatic interactions, limiting their potential for facial selectivity. By employing anionic nanoparticles with variant side chains, we report here facial specificity in the binding of a nanoparticle to Cyt *c*. These studies demonstrate the possibility of targeting specific protein domains by tuning nanomaterial surface functionalities and by helping to determine the factors that dominate the interfaces between Cyt *c* and its partner proteins.

Cyt *c* binds a variety of proteins through its "front face", a domain near its exposed heme edge that consists of several lysine residues. While electrostatics dominate recognition between Cyt *c* and many of its partners, protein−protein interaction domains are typically enriched in aromatic amino acids.[5] We set out to test whether we could change the mode of nanoparticle binding to Cyt *c* through the incorporation of Phe, a typical interface residue, onto the surface of our nanoparticles.

Nanoparticles (**Au-TX**) functionalized with carboxylic acid (**X = COOH**), L-phenylalanine (**X = Phe**), and L-aspartic acid (**X = Asp**) were prepared by published methods.[4,6] Their binding affinities for horse heart Cyt *c* were determined by their ability to inhibit reduction by ascorbate. The reduction of ferri-Cyt *c* (Fe^{3+}) by ascorbate requires access to the heme, which can be inhibited by the binding of Cyt *c* oxidase, Cyt *c* peroxidase, or calix[4]arene receptors near the heme crevice.[7] Dose-dependent inhibition of the reduction of ferri-Cyt *c* by the three nanoparticles was fitted to a binding model in which the nanoparticle is assumed to have *n* equivalent binding sites (Figures 1 and S4).

To determine the binding interface of Cyt *c* with nanoparticles, amide hydrogen/deuterium exchange (HDX) was used to measure solvent accessibility of Cyt *c*. Amide HDX analyzed with MALDI-TOF is a powerful tool,[8] with utility in identifying changes in solvent accessibility due to conformational changes and macromolecular binding.[9]

In our experiments, Cyt *c* (20 μM in D_2O, 10 mM Tris, pH 7.4) was mixed with an excess nanoparticles to form supramolecular adducts. After 3 min, off-exchange was initiated by diluting the **Au-TX**:Cyt *c* adduct into buffered H_2O. Timepoints for HDX were quenched by acidifying the solution with trifluoroacetic acid, followed by proteolysis with immobilized pepsin on ice. The pepsin digest was separated by centrifugation, and the supernatant was frozen in liquid nitrogen for later analysis. The samples were quickly thawed, mixed with equal volumes of matrix solution, and spotted onto a pre-chilled MALDI target which was quickly transferred to the mass spectrometer for recording mass spectra.[8]

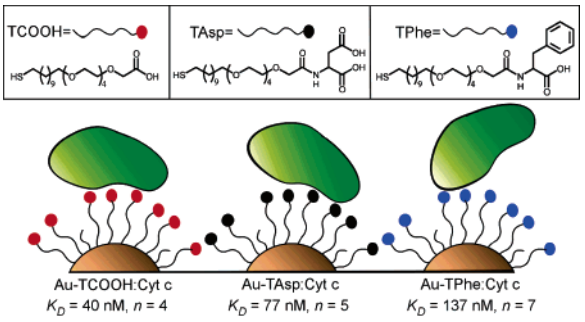

Figure 1. Chemical structures of the functionalized gold nanoparticles and schematic depiction of surface interactions with Cyt *c*.

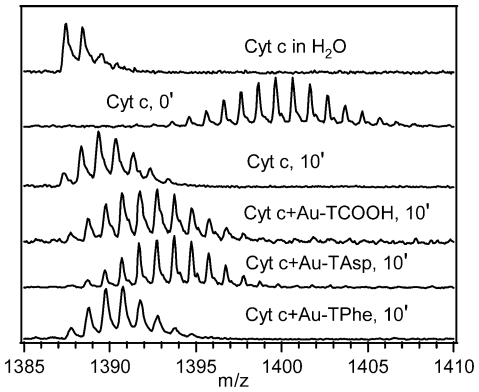

Figure 2. Hydrogen/deuterium exchange MALDI-TOF mass spectra of Ala^{83}-Leu^{94} in the absence and presence of functionalized nanoparticles (10 mM Tris, pH 7.4). Off-exchange times noted.

The Cyt *c* digest analyzed by MALDI-TOF displayed a reproducible digestion pattern. ESI-MS/MS sequencing was used to identify 12 peptides which covered ~85% of the Cyt *c* sequence. The peptide Ala^{83}-Leu^{94} exemplifies the changes in solvent accessibility due to binding different particles (Figure 2). In free Cyt *c* after 10 min of off-exchange, the centroid of mass for this peptide was 1390.93 *m*/*z* due to the 7% D content of water under the off-exchange conditions. In the complex with **Au-TCOOH** and **Au-TAsp**, the centroid was observed respectively at 1392.66 *m*/*z* and 1392.76 *m*/*z*. The ~2.3 retained deuterons indicate that Ala^{83}-Leu^{94} is protected from solvent when Cyt *c* binds to **Au-TCOOH** or **Au-TAsp**.

Significantly, the centroid of Ala^{83}-Leu^{94} appeared at 1390.96 *m*/*z* in the **Au-TPhe**:Cyt *c* complex. The reduced solvent protection for this peptide when Cyt *c* is bound to **Au-TPhe** relative to **Au-TCOOH** and **Au-TAsp** (~0.5 D vs ~2.3 D) indicates that this peptide is not at the binding interface with **Au-TPhe** and that **Au-TPhe** binds to Cyt *c* through an alternate binding site.

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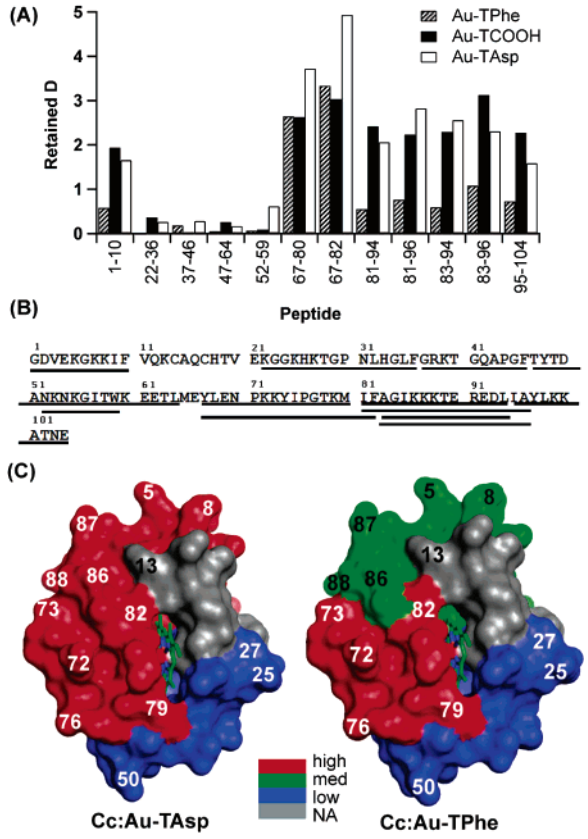

Figure 3. (A) Solvent protection of Cyt *c* peptides (relative to unbound Cyt *c*). (B) Cyt c^{10} peptides observed by MALDI-TOF shown as lines. (C) Solvent protection for Cyt *c* in complex with **Au-TAsp** or **Au-TPhe**.

Binding surfaces were identified by analysis of solvent protection for all peptides (Figure 3). Intermediate solvent protection was observed in Gly^{1}-Phe^{10} and Ile^{95}-Glu^{104} for the **Au-TCOOH** and **Au-TAsp** particles. Those residues are located in close proximity in the folded protein, and appear to provide a second binding surface for **Au-TCOOH** and **Au-TAsp**. These peptides are more accessible in the **Au-TPhe** adduct, suggesting that this binding site is less favored by **Au-TPhe**. The two overlapping peptides Tyr^{67}-Met^{80} and Tyr^{67}-Phe^{82} were identified as a common binding site for all three nanoparticles (Figure 3A) with greater than two deuterons retained. This segment is located near the heme crevice and contains several aromatic residues, as well as $Lys,^{72}$ $Lys,^{73}$ and $Lys.^{79}$ As this is the only surface significantly solvent protected in the **Au-TPhe**:Cyt *c* complex, we assign this as the preferred binding site for **Au-TPhe**.

Our results show that Cyt *c* binds to these nanoparticles through surfaces which resemble functional binding sites for its redox partners.[11] For example, **Au-TCOOH** and **Au-TAsp** particles bind to a large surface spanning the front face of Cyt *c*, much as does Cyt *c* oxidase; the same front face has been implicated as the binding site to the surface of self-assembled monolayer films, implying that simple Coulombics may lead to diffuse binding.[12] In contrast, **Au-TPhe** binds to a smaller surface near Lys^{72}, which bears much resemblance to the surface recognized by Cyt *c* peroxidase.[11a]

Facial specificity for nanoparticle binding is apparently determined by a fine balance between electrostatics and hydrophobicity, an effect also shown by Cyt *c* point mutants.[13] For example, Crane and co-workers showed that mutation of Cyt *c* residue Phe^{82} to Ile or Tyr results in a rotation of Cyt *c* on the Cyt *c* peroxidase surface and a 25% decrease in the surface binding area.[13] The replacement of the carboxylate group in TAsp with a phenyl group in TPhe similarly induces a more localized binding interaction of the nanoparticle surface with Cyt *c*, which may be reasonably attributed to increased hydrophobic/stacking interactions with the aromatic residues in the Tyr^{67}-Phe^{82} peptide. Despite these changes in facial selectivity, the binding energy for **Au-TX**:Cyt *c* is nearly invariant (9.3−10.0 kcal/mol) based upon K_D values (Figure 1).

In summary, we have demonstrated facial specificity of synthetic nanoparticles binding to the surface of Cyt *c*. While the highly anionic surfaces of each nanoparticle led to tight binding ($K_D \approx 10^{-7}$ M) with the cationic Cyt *c*, selectivity was enhanced by the addition of the hydrophobic phenyl ring in **Au-TPhe**. These results suggest that a combination of hydrophobics and Coulombics would lead to better synthetic receptors for protein surfaces. Furthermore, this work establishes that amide HDX is a useful tool to probe binding interactions between proteins and nanostructures.

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Supporting Information Available: Experimental procedures for amide exchange and activity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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