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Selection of a platinum-binding sequence in a loop of a four-helix bundle protein

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Protein–metal hybrids are functional materials with various industrial applications. For example, a redox enzyme immobilized on a platinum electrode is a key component of some biofuel cells and biosensors. To create these hybrid materials, protein molecules are bound to metal surfaces. Here, we report the selection of a novel platinum-binding sequence in a loop of a four-helix bundle protein, the Lac repressor four-helix protein (LARFH), an artificial protein in which four identical α-helices are connected via three identical loops. We created a genetic library in which the Ser-Gly-Gln-Gly-Gly-Ser sequence within the first inter-helical loop of LARFH was semi-randomly mutated. The library was then subjected to selection for platinum-binding affinity by using the T7 phage display method. The majority of the selected variants contained the Tyr-Lys-Arg-Gly-Tyr-Lys (YKRGYK) sequence in their randomized segment. We characterized the platinum-binding properties of mutant LARFH by using quartz crystal microbalance analysis. Mutant LARFH seemed to interact with platinum through its loop containing the YKRGYK sequence, as judged by the estimated exclusive area occupied by a single molecule. Furthermore, a 10-residue peptide containing the YKRGYK sequence bound to platinum with reasonably high affinity and basic side chains in the peptide were crucial in mediating this interaction. In conclusion, we have identified an amino acid sequence, YKRGYK, in the loop of a helix-loop-helix motif that shows high platinum-binding affinity. This sequence could be grafted into loops of other polypeptides as an approach to immobilize proteins on platinum electrodes for use as biosensors among other applications.

[**Keywords:** Alanine scanning; Atomic force microscopy; Flow-quartz crystal microbalance; Four-helix bundle; Phage display; Metal-binding sequence]

The hybridization of metals and biomolecules, such as nucleic acids and proteins, can generate functional materials with applications in various fields. For example, a biosensor can be created by conjugating a redox enzyme to an electrode to convert a biological response into an electric signal. This idea was first proposed by Clark and Lyons in 1962 (1). A critical step in the creation of such functional hybrid materials is the immobilization of biomolecules on metal surfaces. In 1967, Updike and Hicks (2) produced an enzyme sensor in which glucose oxidase molecules were immobilized on the surface of an electrode with polyacrylamide gel. Homotripeptides have also been used as N-terminal tags for the immobilization of proteins on gold nanoparticles (3): for example, tri-histidine, tri-tryptophan, tri-lysine, tri-methionine and tri-tyrosine were reported to strongly interact with gold in this order, suggesting that aromatic rings and positively charged groups preferentially interact with the metal (3). Gold-binding peptides (GBPs) have also been selected from a random-sequence peptide library by using the cell-surface display method (4). Many other studies have similarly selected metal-binding peptides from peptide libraries containing randomized sequences by using the phage display method (5–9).

The binding affinity of a peptide for a metal is not generally high due to its flexible backbone (10). In addition, the affinity of a peptide for a metal is thought to be inversely correlated with the extent of the increase in translational and rotational entropy of the peptide upon detaching from the metal (11,12). A binding sequence will therefore show higher affinity for a metal if it is part of a rigidly folded protein. Umetsu and colleagues previously selected peptides that bind to zinc oxide by using the M13 phage display system (13). Subsequently, they grafted the zinc oxide-binding peptides into the complementarity-determining region of an antibody, and thus developed antibody fragments that interacted with zinc oxide with improved affinity relative to other peptides (14). Via the same approach, an antibody that interacts with gold particles has also been developed (15).

As well as the strand-loop-strand motif present in the complementary-determining region of an antibody, a helix-loop-helix motif is often observed in naturally occurring folded proteins. On the basis of several functional proteins, therefore, it may be beneficial to create a metal-binding sequence in the loop that forms part of this helix-loop-helix motif for the development of a high-affinity metal binder. Recently, we designed an artificial four-helix bundle protein, termed LARFH (Lac repressor four-helix protein) (16). This artificial protein consists of four tandemly fused copies of the *Escherichia coli lac* repressor C-terminal α-helix, which are connected by three identical loops. The protein has a stable and

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well-folded structure with a typical antiparallel four-helix bundle and a relatively small molecular mass (10 kDa) that is comparable to those of smaller antibody fragments (17). These structural features make LARFH both suitable for display on phage particles and representative of a folded protein containing a helix-loop-helix motif. In the present study, to create a novel high-affinity platinum-binding sequence in the helix-loop-helix region, we engineered a sequence on a loop connecting two of the α-helices in LARFH. Platinum was selected as the target metal, because it is a valuable metal that is often used in electrodes, particularly in biofuel cells and biosensors (18,19).

We first genetically randomized five residues in a loop of LARFH and then selected mutants that strongly interacted with platinum particles by using the T7 phage display method. Most of the mutants contained the sequence Tyr-Lys-Arg-Gly-Tyr-Lys (YKRGYK) within the loop. We characterized the interaction between platinum and the mutants, as well as the one between platinum and a 10-residue peptide containing the YKRGYK sequence, by using quartz crystal microbalance analysis. Alanine scanning experiments were also performed to identify the residues responsible for the interaction with platinum. Our data suggest that basic side chains are the major contributors to this interaction, but two tyrosine residues also play a role in binding to the metal.

MATERIALS AND METHODS

Construction of the phage display LARFH library To create an LARFH gene library, amino acid residues at positions 21, 22, 23, 25, and 26 were subjected to semi-random mutagenesis (Fig. 1). Each of their codons was replaced with the NVS codon (N = A + T + G + C, V = A + G + C, S = G + C). Genes were then amplified by using two different primers so that the *Eco*RI and *Hin*dIII recognition sites could be added at the 5′ and 3′ termini, respectively. Amplified DNA fragments were digested with *Eco*RI and *Hin*dIII and then ligated to T7 Select Vector Arms (Merck Millipore, Germany). For *in vitro* packaging, ligated DNA fragments were added to T7 phage packaging extracts (Merck Millipore) and the mixtures were incubated at 25 °C for 2 h.

The plaque assay to evaluate the number of gene variants generated and phage amplification were performed according to the manufacturer's instructions (Merck Millipore).

Biopanning selection Platinum beads (0.1 mg, powder 0.5−1.2 μm diameter, Sigma Aldrich, MO, USA) were washed with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.3% Triton X-100, and then mixed with $2.0–15 \times 10^8$ T7 phage particles. Unattached and weakly attached phages were removed by washing the beads as follows. First, beads were washed three times with 500 μL of PBS containing 0.2% BSA and 0.3% Triton X-100. Next, beads were incubated at room temperature for 10 min with 25 μL of T7 elution buffer (Merck Millipore) containing 2 M urea and then sonicated for 10 min. Subsequently, 100 μL of 5 M NaCl and 375 μL of PBS containing 0.2% BSA and 0.3% Triton X-100 were added to the mixture. The second step was repeated six times. For amplification of bound phages, the washed platinum beads were mixed with an *E. coli* BLT5403 culture. Amplified phages were used for the subsequent bio-panning round. After three rounds of bio-panning, LARFH genes were individually amplified from 97 plaques according to the manufacturer's instructions (Merck Millipore). The nucleotide sequences of amplified DNAs were determined using a Big-Dye Terminator v3.1 kit and an Applied Biosystems 3130 Capillary Sequencer.

Expression and purification of LARFH protein The construction of an expression vector for the selected LARFH mutant, which carries a YKRGYK amino acid sequence, was performed as described previously (16). After protein expression in *E. coli* M15 cells (pREp4), LARFH protein was purified as previously described (16).

Determination of dissociation constants The interaction of LARFH or 10-residue peptides with a platinum surface was monitored and quantified by using a hand-made flow-quartz crystal microbalance system (f-QCM) with a custom-made acryl flow cell equipped with 9-MHz Pt or Au quartz crystals (Kyocera, Japan). Oligo-peptides were purchased from Eurofins genomics (Japan). To remove organic contaminants on the surface of the crystals, the crystals were cleaned by sonication in piranha solution (H_2O_2:H_2SO_4, 3:1 v/v) for 15 min and then pure distilled water, followed by O_2 plasma etching using the soft plasma etching equipment SEDE-GE (Meiwafosis, Japan) for 10 s. Additionally, crystals were rinsed with running buffer (20 mM Tris−HCl, pH 8.0, 100 mM NaCl, 2 M urea) and subsequently with pure water to remove electrolytes. The resulting crystals were applied to the f-QCM system. The cell was washed with running buffer at a flow rate of 100 μL/min until the oscillation frequency reached a plateau. Protein (1 μM) or peptide (30 μM) solution diluted with running buffer was applied to the quartz crystals for 300 or 600 s to monitor the association process, followed by running buffer to monitor the dissociation process. The change in frequency of the quartz crystal was recorded at 25 °C. The observed resonance frequency data were corrected by subtracting the background drift. According to the Sauerbrey equation, there is a linear relationship between the measured frequency change (ΔF) and the adsorbed mass (Δm) (20):

$$\Delta m = -C\Delta F \quad (1)$$

where C is a constant based on the physical properties of the quartz crystal ($C = 1.07$ ng/cm^2 Hz).

To estimate kinetic parameters, the observed binding curves were fitted to a 1:1 Langmuir binding model with drifting baseline by using BIA evaluation software (GE Healthcare, UK). If the curve did not fit the 1:1 model, a 1:2 binding model was used. The binding of LARFH proteins and oligo-peptides to platinum was measured with triplicate and duplicate samples, respectively.

Observation by atomic force microscopy Approximately 3−5 μL of 10 nM solutions of LARFH protein was absorbed for 5 min onto gold-coated mica, which was then washed with buffer (20 mM Tris−HCl, pH 8.0, 100 mM NaCl, 2 M urea) and milliQ water, and dried overnight at room temperature. The mica was pre-treated by flame annealing in order to create a flat gold surface. Atomic force microscopy (AFM) images were acquired in dynamic mode using an SPM-9600 microscope (Shimadzu, Japan), with a 240-μm long micro-cantilever (spring constant of 2 N/m) (Olympus, Japan).

RESULTS

Selection of mutant LARFH that interacts with platinum Fig. 1A shows the amino acid sequence of LARFH, with rectangles representing segments that are likely to form α-helices. We inserted a platinum-binding sequence in the loop connecting the first and second α-helices. When LARFH is displayed on phage particles, the first loop may be positioned at the opposite face of the phage and may be well exposed to solvent (Fig. 1B). Therefore, we considered that platinum and other metal particles might access this loop more easily. The residues Ser21, Gly22, Gln23, Gly25 and Ser26 within the loop were semi-randomly mutagenized. Gly24 was not mutated because the high flexibility of the residue was thought to be important for the local conformation of the loop. The codons corresponding to the five residues described above were replaced with the NVS codon, where N indicates any base, V indicates A, C or G, and S indicates C or G. The NVS codon can encode a subset of 20 amino acids, but not the hydrophobic amino acids phenylalanine, leucine, isoleucine, methionine and valine. The assumption behind the use of the NVS codon was that hydrophobic residues do not contain any metal-binding groups. In contrast, most of the other amino acids contain groups that are likely to contribute to the binding to platinum.

Substitution of these five codons with the NVS codon can theoretically generate 8.0×10^6 independent sequences. The randomized LARFH genes were ligated with T7 phage DNA arms so that the mutated LARFH proteins could be fused to the C-terminus of the capsid protein gp10B. After phage packaging, a T7 phage library containing 7.4×10^6 independent clones was constructed. The resulting phage library was used to screen for platinum binding by three rounds of bio-panning (Fig. 1B). In each panning step, a mixture of phage particles and platinum beads was sonicated in the presence of 2 M urea to remove phages that weakly interacted with the beads. The phages that remained bound to the beads were directly used for infection of *E. coli*, and the amplified phages were subjected to a second round of panning. At the end of the third round of panning, we arbitrarily selected 97 plaques, from which T7 DNAs were extracted and sequenced. The sequences that were present in two or more phage clones are shown in Table 1. Among them, 50 DNAs displayed the same sequence within the randomized segment: namely, 21-Ser-Gly-Gln-Gly-Gly-Ser-26 was replaced by 21-Tyr-Lys-Arg-Gly-Tyr-Lys-26 (YKRGYK). The bio-

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