Affinity transfer to a human protein by CDR3 grafting of camelid VHH

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Abstract: VHH is the binding domain of the IgG heavy chain. Some VHHs have an extremely long CDR3 that contributes to antigen binding. We studied the antigen binding ability of CDR3 by grafting a CDR3 from an antigen-binding VHH onto a nonbinding VHH. cAb-CA05-(1RI8), the CDR3-grafted VHH, had an antigen-binding ability. To find a human scaffold protein acceptable for VHH CDR3 grafting, we focused on the conserved structure of VHH, especially the N-terminal and C-terminal amino acid residues of the CDR3 loop and the Cys residue of CDR1. Human origin protein structures with the same orientation were searched in PDB and ubiquitin was selected. Ubi-(1RI8), the CDR3-grafted ubiquitin, had antigen-binding ability, though the affinity was relatively low compared to cAb-CA05-(1RI8). The thermodynamic parameters of Ubi-(1RI8) binding to HEWL were different from cAb-CA05-(1RI8). Hydrogen-deuterium exchange experiments showed decreased stability around the CDR3 grafting region of Ubi-(1RI8), which might explain the decreased antigen-binding ability and the differences in thermodynamic properties. We concluded that the orientation of the CDR3 sequence of Ubi-(1RI8) could not be reconstructed correctly.

Keywords: VHH; scaffold; CDR grafting; affinity transfer; thermodynamic parameter; protein stability; NMR analysis

Introduction

Antibodies are powerful tools for pharmaceutical research and medicine. Over 20 monoclonal antibodies are already on the market, with many antibodies in clinical-stage. Recently, many small and productive scaffold proteins have been studied,^{1,2} because antibodies, which are large (150 kDa), are considered to have limited capacity for tumor penetration.³ Most of the scaffold proteins are randomized in some regions of the protein,

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Abbreviations: CD, circular dichroism; CDR, complementarity-determining region; DSC, differential-scanning calorimetry; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FKBP, FK506 binding protein; HEWL, hen egg white lysozyme; IPTG, isopropylthiogalactoside; ITC, isothermal titration calorimeter; NCAM, neural cell adhesion molecule; NCS, neocarzinostatin; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PDB, protein data bank; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; T_m, thermal melting temperature; Tris, tris(hydroxymethyl) aminomethane; Ubi, ubiquitin; Ubi-(1RI8), engineered ubiquitin containing the CDR3 loop of anti-HEWL VHH D2-L19; VH, variable region of Ig heavy chain; VL, variable region of Ig light chain

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and phage or yeast display systems are used to screen for target binding. Therefore, it takes a lot of efforts to generate high-affinity scaffold proteins.

Conventional antibodies are composed of heavy and light chains, and the antigen-binding domain consists of VH and VL domains. Each domain has CDR1, CDR2, and CDR3 loops, so conventional antibodies bind antigen through six CDR loops. Camelids have functional heavy chain antibodies that lack light chains. The binding domain of a heavy chain antibody, which is called a VHH, is composed only of a single Ig domain, and binds antigen only through three CDR loops. VHHs have long CDR1 and CDR3 domains compared to classical antibodies.⁴ Moreover, some VHHs have extremely long CDR3s and an intramolecular disulfide bond between CDR1 and CDR3. This disulfide bond might contribute to maintenance of the long CDR3 structure and antigen binding.⁵ X-ray structure of VHH and antigen complexes showed that the solvent-accessible surface area of the VHH-antigen binding domain is dominated 60–80% by CDR3.⁶ Therefore, we propose that the CDR3 loops of camel VHHs have strong antigen affinity by themselves, and the disulfide bond between CDR1 and CDR3 is important for the affinity.

The anti-HEWL VHHs D2-L19 (PDB: 1RI8) and cAb-Lys2 (PDB: 1RJC) contact highly overlapping epitopes. These are located at the D-F carbohydrate substrate subsites of HEWL, and include the HEWL catalytic residues Glu-35^L and Asp-52^L. X-ray structures of 1RI8 and 1RJC show the solvent-accessible surface area of the VHH-antigen binding domain are dominated 78% and 80% by CDR3.⁷ Therefore, we hypothesized that these CDR3 loops have strong antigen affinities by themselves. CDR grafting technologies are a common and effective approach for humanizing mouse antibodies.⁸ To study the antigen-binding ability of the CDR3 loops, we selected cAb-CA05 (PDB: 1F2X), which binds carbonic anhydrase, as a scaffold VHH. The homology of the framework region between D2-L19 and cAb-CA05 is high, with only nine amino acid differences.

Nicaise *et al.*⁹ succeeded in grafting the camelid VHH CDR3 on neocarzinostatin (NCS). NCS is an Ig-like domain protein that inherently contains loops corresponding to antibody CDR3. However, the affinity of the CDR3 grafted onto NCS was very low. This might be because the grafted CDR3 did not form a disulfide bond between the framework of NCS and the Cys residue of the CDR3 sequence.

In this study, we designed a CDR3 grafted onto a non-Ig-domain human protein containing a disulfide bridge between the framework of the human scaffold and the Cys residue of CDR3. And we succeeded in affinity transfer to human ubiquitin by CDR3 grafting of a camelid VHH.

Materials and Methods

CDR3 grafting design

The X-ray structure of nine camelid VHH sequences, four camel VHHs, and five llama VHHs were aligned using ClustalW [Fig. 1(A)] and superimposed [Fig. 1(B,C)]. We focused on the conserved VHH sequence and structure, especially the N-terminal and C-terminal amino acid residues of the CDR3 sequence and the Cys residue that forms a disulfide bond between CDR1 and CDR3. The distances between the C α positions of the N-terminal amino acid residues of the CDR3 loop (X, X+1) and the C-terminal amino acid residues of the CDR3 loop (Y, Y+1) were measured (Table I), along with the distance between the C α position of the Cys residue of CDR1 (Z) and the C α position of the N-terminal amino acid residues of CDR3 loop (X, X+1) or the C-terminal amino acid residues of CDR3 loop (Y) (Table I). Based on these information, the structure of human origin proteins in PDB were searched for loops with 6–30 amino acid residues, and for distances of X-Y, X-(X+1), (X+1)-Y, X-Z, (X+1)-Z, and Y-Z, within average plus or minus 10%.

Design of CDR3-grafted VHH

The CDR3 sequences of a HEWL binding VHH, D2-L19 (PDB:1RI8), and cAb-Lys2 (PDB:1RJC)⁷ were selected for grafting [Fig. 2(A)]. cAb-CA05 (PDB : 1F2X),¹⁰ which binds carbonic anhydrase, was selected as the scaffold VHH. The homology of the framework region between D2-L19 and cAb-CA05 has only nine amino acid difference. The homology of the CDR regions with D2-L19 and cAb-CA05 are 60% for CDR1, 56% for CDR2, and 37% for CDR3. These VHHs have two disulfide bridges at the same position and cAb-CA05 does not show a specific interaction with HEWL. The sequences of the designed CDR3-grafted VHH, cAb-CA05-(1RI8), which had the CDR3 sequence from D2-L19; and cAb-CA05-(1RJC), which had the CDR3 sequence from cAb-Lys2, are in Figure 2(A).

Expression and purification of VHHs

Synthetic genes encoding the VHHs, D2-L19, cAb-Lys2, cAb-CA05, cAb-CA05-(1RI8), and cAb-CA05-(1RJC), were digested with *Nde*I and *Who*I and ligated into appropriately digested plasmids pET22b (Takara Bio Inc.) as His-tag fusion form. The resulting plasmids were transformed into *Escherichia coli* strain BL21 (DE3) (Takara Bio Inc.). Recombinant *E. coli* clones were grown on LB medium containing ampicillin at 37°C until $A_{600} = 0.8$, followed by induction of protein expression with 1 mM IPTG for 3 h at 37°C. Cells were harvested by centrifugation at 1400g for 10 min, and the pellet was resuspended in 20 mM Tris-HCl (pH 8.0) containing 20 mM NaCl, and sonicated extensively in an ice-water

Figure 1. Design of CDR3 grafting. (A) Amino acid sequences of VHH. Nine camelid VHH sequences of known structure are aligned. Highly conserved amino acid residues are in red. Amino acids with similar properties are in blue. The N-terminal amino acid residues of the CDR3 loop are Ala-Ala (position X and position X+1), the C-terminal amino acid residues of the CDR3 loop are Tyr-Trp (position Y and position Y+1) and the Cys residue (position Z) of CDR1 is indicated by X, Y, and Z. (B) Superimposed 3D structures of known camelid VHHs. The N-terminal and the C-terminal amino acid residues of CDR3, and the X and Y and Cys residue (position Z) of CDR1 are superimposed in this figure. The green ribbon indicates CDR3 sequences of D2-L19 (1R18). (C) The CDR3 region. The red stick indicates the N-terminal amino acid residue (X) and the C-terminal amino acid residue (Y) of CDR3 and the green stick indicates the Cys residues (Z) of CDR1.

bath. D2-L19 was soluble and the others were expressed as inclusion bodies.

For preparation of D2-L19, the sonication mixture was centrifuged at 16,000g for 30 min and the supernatant was collected. The recombinant protein was purified by affinity purification using the His tag using Ni-NTA Superflow (Qiagen) according to

the manufacturer's instructions. The elution mixture was dialyzed against 20 mM AcONa (pH 5.0) and loaded on a column of RESOURCE S (1 mL, GE Healthcare). The recombinant protein was eluted with a gradient of 20 mM AcONa (pH 5.0) and 20 mM AcONa (pH 5.0) containing 1 M NaCl at a flow rate of 1 mL/min in 30 min.

Table I. Summary of Amino Acids Corresponding to Each VHH Sequence and Distances Between C α of Each Amino Acids

1RI8					1RJJC			1XFP		
Relationship		Amino acid			Amino acid			Amino acid		
A	B	A	B	Distance (Å)	A	B	Distance (Å)	A	B	Distance (Å)
X	Y	A96	Y114	5.57	A97	Y117	5.39	A97	S122	5.53
X	Y+1	A96	W115	4.64	A97	W118	4.28	A97	W123	4.53
X+1	Y	A97	Y114	5.22	A98	Y117	5.06	A98	S122	5.13
X	Z	A96	C33	8.09	A97	C33	8.36	A97	C33	8.11
X+1	Z	A97	C33	5.29	A98	C33	5.76	A98	C33	5.30
Y	Z	Y114	C33	10.20	Y117	C33	10.45	S122	C33	10.18

1OP9					1SHM			1G9E		
Relationship		Amino acid			Amino acid			Amino acid		
A	B	A	B	Distance (Å)	A	B	Distance (Å)	A	B	Distance (Å)
X	Y	A94	Y110	5.30	G97	W116	5.61	G97	S106	4.92
X	Y+1	A94	W111	4.39	G97	W117	4.46	G97	W107	3.86
X+1	Y	A95	Y110	4.92	A98	W116	5.29	A98	S106	4.65
X	Z	—	—	—	—	—	—	—	—	—
X+1	Z	—	—	—	—	—	—	—	—	—
Y	Z	—	—	—	—	—	—	—	—	—

2BSE					1U0Q			1I3V		
Relationship		Amino acid			Amino acid			Amino acid		
A	B	A	B	Distance (Å)	A	B	Distance (Å)	A	B	Distance (Å)
X	Y	A97	G112	5.51	A97	Y114	5.51	A102	Y118	5.49
X	Y+1	A97	W113	4.68	A97	W115	4.42	A102	W119	4.33
X+1	Y	A98	G112	4.89	V98	Y114	5.54	A103	Y118	5.11
X	Z	—	—	—	—	—	—	—	—	—
X+1	Z	—	—	—	—	—	—	—	—	—
Y	Z	—	—	—	—	—	—	—	—	—

For preparation of cAb-Lys2, cAb-CA05, cAb-CA05-(1RI8), and cAb-CA05-(1RJJC), the sonication mixture was centrifuged at 16,000g for 30 min and the precipitate was collected and solubilized in 0.1 M Tris-HCl (pH 8.0) containing 8 M urea and 100 mM DTT at room temperature. The resulting solution was centrifuged at 16,000g for 30 min, and the recombinant protein in the supernatant was purified by affinity purification using the His tag using Ni-NTA Superflow under denaturing conditions containing 8 M urea. Refolding of the recombinant protein was performed by multistep dialysis at 4°C: the first dialysis was performed for more than 12 h against 50 mM Tris-HCl (pH 8.0) containing 4 M urea and 1 mM EDTA; the second dialysis was performed against 50 mM Tris-HCl (pH 8.0) containing 4 M urea and 1 mM EDTA with glutathione redox mixture, 0.26 mM oxidized glutathione and 1.3 mM reduced glutathione for more than 12 h; the third dialysis was against 50 mM Tris-HCl (pH 8.0) containing 2 M urea, 10% glycerol and 1 mM EDTA without glutathione redox mixture; and the last dialysis was against 25 mM Tris-HCl (pH 8.0) containing 10% glycerol and 1 mM EDTA. Refolded recombinant proteins were dialyzed against 20 mM

AcONa (pH 5.0) and loaded on a column of RESOURCE S (1 mL, GE Healthcare). Recombinant protein was eluted with a gradient of 20 mM AcONa (pH 5.0) and 20 mM AcONa (pH 5.0) containing 1 M NaCl at a flow rate of 1 mL/min in 30 min.

Expression and purification of CDR3-grafted proteins

Synthetic genes encoding Ubi, Ubi-(1RI8), and Ubi-(1RJJC), were digested with *NdeI* and *WhoI* and ligated into appropriately digested pET28a (Takara Bio Inc.) with an additional “GSSHHHHHHSSGLVPRGSH” sequence at the N-terminal of the expressed proteins. The resulting plasmids were transformed and induced as above, except LB medium contained kanamycin. Target proteins were purified by a process similar to that used for D2-L19 after cell harvest.

SDS-PAGE analysis

SDS-PAGE was performed using Multi-Gel II mini 10/20 (Cosmo Bio) under nonreducing conditions and stained with Coomassie brilliant blue. Prestained SDS-PAGE standard (Low) (Bio-Rad) was used as a molecular marker.

Figure 2. Preparation of CDR3-grafted proteins. (A) Amino acid sequences of recombinant proteins. CDR3 sequences of D2-L19 (1R18) and cAb-Lys2 (1RJC) are in ochre. CDR3 sequence of cAb-CA05 replaced by CDR3 of D2-L19 or cAb-Lys2 is in pink. Grafted CDR3 sequences of cAb-CA05-(1R18) and cAb-CA05-(1RJC) are in blue. The Ubi loop replaced by CDR3 sequences is orange, and His68 of Ubi is green. Grafted CDR3 sequences of Ubi-(1R18) and Ubi-(1RJC) are cyan. H68C in Ubi-(1R18) and Ubi-(1RJC) is red. (B) Cation-exchange chromatography of refolded cAb-CA05-(1R18). (C) Cation-exchange chromatography of Ubi-(1R18). (D) SDS-PAGE of purified proteins. Molecular size by marker is indicated at right. (E) Reverse-phase HPLC of cAb-CA05-(1R18). (F) Reverse-phase HPLC of Ubi-(1R18).

Reverse-phase HPLC analysis

Reverse-phase HPLC was performed using CAP-CELL PAK C1 (Shiseido). Recombinant protein was eluted with 5–100% gradient of 0.05% trifluoroacetic acid in H₂O and 0.05% trifluoroacetic acid in 30% 1-propanol, 70% CH₃CN at a flow rate of 1 mL/min in 30 min.

Detection of intramolecular disulfide bonds

Purified proteins were digested with trypsin (100 pmol proteins, 10 pmol trypsin, 0.1% rapigest, 37°C, 16 h), and the resulting peptides were analyzed by Voyager-DE STR (PerSeptive Biosystems).

Circular dichroism spectra analysis

Circular dichroism (CD) spectra were collected using a J-820 Spectropolarimeter (Jasco) in a 1-cm path-length quartz cell at 25°C. Purified Ubi and Ubi-(1R18) in 10 mM AcONa (pH 4.0) were measured at 30 µg/mL. Scan speed was 200 nm/min.

Thermal stability of purified Ubi and Ubi-(1R18)

The thermal stability of Ubi and Ubi-(1R18) was analyzed by scanning calorimetry on a VP-Capillary

DSC (MicroCal). All experiments were performed at a scanning rate of 1 K/min. Before measurements, all samples were dialyzed against 20 mM AcONa (pH 5.0) and adjusted to 250 µg/mL. The dialysis buffer was used as a reference solution.

Biacore analysis

SPR experiments were analyzed using a Biacore T100 instrument (GE Healthcare). HEWL was immobilized on a CM5 chip by standard amine coupling chemistry. For VHH binding experiments, five different concentrations from 1–16 nM of the D2-L19 and cAb-Lys2 were injected into the HEWL-coupled sensor chip for 2 min in HBS-EP⁺ buffer at a flow rate of 30 µL/min at 25°C. For CDR3-grafted VHH-binding experiments, five different concentrations from 5.4 nM to 4.0 µM of cAb-CA05, cAb-CA05-(1R18), and cAb-CA05-(1RJC) were injected. For CDR3-grafted scaffold protein-binding experiments, five different concentrations from 30 nM to 15 µM of Ubi, Ubi-(1R18), and Ubi-(1RJC) were injected. Kinetic constants were calculated from sensorgrams with Biacore T100 Evaluation Software, BIAevaluation. To calculate K_D values for D2-L19 and cAb-

Lys2, a 1:1 binding model for kinetic analysis in BIAevaluation was used. To calculate the KD values for cAb-CA05-(1RI8), cAb-CA05-(1RJc), Ubi-(1RI8), and Ubi-(1RJc), the affinity analysis of BIAevaluation was used.

Thermodynamic analysis of D2-L19, cAb-CA05-(1RI8), and Ubi-(1RI8) binding to HEWL was performed by the Biacore T100. Thermodynamic parameters were determined by fitting the following equations to KD values obtained at 10°C, 19°C, 25°C, 33°C, and 37°C:

$$\Delta G^\circ = RT \ln KD$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

$$\ln KD = \Delta H^\circ / RT - \Delta S^\circ / R$$

where ΔG° , ΔH° , and ΔS° represent free energy change (kJ/mol), enthalpy change (kJ/mol), entropy change (J/K*mol), respectively.

NMR analysis

Isotopically labeled Ubi and Ubi-(1RI8) were overexpressed in M9 minimal medium containing 1 g/L of $^{15}\text{NH}_4\text{Cl}$ (Shoko) and 4 g/L of glucose for the preparation of uniformly ^{15}N -labeled proteins, and 1 g/L of $^{15}\text{NH}_4\text{Cl}$ (Shoko) and 2 g/L of $[\text{U-}^{13}\text{C}_6]\text{glucose}$ (Cambridge Isotope Laboratories, Inc.) for the preparation of uniformly ^{15}N - and ^{13}C -labeled proteins.

For backbone chemical shift assignments of Ubi-(1RI8), two-dimensional ^1H - ^{15}N HSQC, and three-dimensional HNCACB, CBCA(CO)NH, HNCO, and HN(CA)CO experiments¹¹ were performed on uniformly ^{13}C , ^{15}N -labeled Ubi-(1RI8) in 95% H_2O , 5% $^2\text{H}_2\text{O}$ containing 25 mM sodium acetate (pH 5.5) and 100 mM NaCl. All spectra were processed by NMRPipe,¹² and analyzed with CARA.¹³ All NMR experiments were performed at 25°C on Bruker Avance 600- or 800-MHz spectrometers equipped with cryocooled triple-resonance probes.

For hydrogen-deuterium (H-D) exchange experiment samples, ^{15}N -labeled protein in $^1\text{H}_2\text{O}$ buffer was lyophilized. H-D exchange was initiated by dissolving the sample in $^2\text{H}_2\text{O}$ and subsequently transferring the sample tube into the probe, which was pre-equilibrated at 25°C. Exchange reactions were followed by observing the intensities of amide proton cross-peaks in HSQC spectra. Six transients were acquired for each of t_1 (256 series) points. A series of HSQC spectra was recorded consecutively up to 26 h.

Results

Design of CDR3 grafting

The amino acid sequences of nine camelid VHHs (four camel VHHs and five llama VHHs) with known three-dimensional (3D) structures were aligned [Fig. 1(A)]. The results indicated that framework sequences were well conserved and N-terminal and C-termi-

Table II. Corresponding Amino Acid Residues Among D2-L19, cAb-Lys2, Ubi, NCAM, and FKBP

	1RI8	1RJc	1F9J	1QZ1	1A7X
Position	(D2-L19)	(cAb-Lys2)	(ubiquitin)	(NCAM)	(FKBP)
X	A96	A97	F4	V78	T27
X+1	A97	A98	V5	V79	G28
Y	Y114	Y117	I13	S87	D37
Y+1	W115	W118	T14	E88	S38
Z	C33	C33	H68	D32	V98

nal amino acid residues of CDR3 loop were composed of nearly the same amino acid residues. Figure 1(B,C) show superimposed 3D structures of these VHHs. The positions of the N-terminal and C-terminal amino acid residues of the CDR3 loop and the Cys residue in CDR1 were also conserved. The pairwise distances between the $\text{C}\alpha$ position of the N-terminal amino acid residues of the CDR3 loop (X, X+1) and the $\text{C}\alpha$ position of C-terminal amino acid residues of the CDR3 loop (Y, Y+1) were measured (Table I). Pairwise distances between the $\text{C}\alpha$ position of the Cys residue of CDR1 (Z) and the $\text{C}\alpha$ position of the N-terminal amino acid residues of the CDR3 loop (X, X+1) or the C-terminal amino acid residue of the CDR3 loop (Y) were also measured (Table I). As an example, the distance between the $\text{C}\alpha$ of X and Y was 5.57 Å in IRI8 (D2-L19). Individual distances were within plus or minus 10% of the averages.

Structures of human origin proteins in PDB were searched for CDR3 grafting candidates, yielding 4981 sites in 535 proteins. Secretory proteins, membrane proteins, and unstable proteins were removed from the candidate list. CDR3-grafted model structures of candidate proteins were built and steric hindrances assessed. Ubiquitin (Ubi),¹⁴ NCAM,¹⁵ and FKBP¹⁶ were selected as CDR3 grafting scaffolds. The corresponding amino acid residues for the X, X+1, Y, Y+1, and Z positions of D2-L19, cAb-Lys2, Ubi, NCAM, and FKBP are in Table II. To form a disulfide bond between Ubi and grafted CDR3, Ubi His68 was mutated to Cys. Figure 2(A) shows the sequences of VHHs, Ubi, and CDR3-grafted proteins.

Expression and purification of proteins

D2-L19 was expressed in soluble, folded form. On the other hand, the other VHH variants, cAb-Lys2, cAb-CA05, cAb-CA05-(1RI8), and cAb-CA05-(1RJc) were expressed insoluble, in inclusion bodies. Proteins were purified by affinity chromatography using Ni-NTA Superflow and cation-exchange chromatography. Figure 2(B) shows the result of cAb-CA05-(1RI8) cation-exchange chromatography. The purified VHH variants were homogenous as judged by SDS-PAGE [Fig. 2(D)] and reverse-phase HPLC [Fig. 2(E)]. CDR3-grafted NCAM and FKBP were expressed in *E.coli* BL21 (DE3) as inclusion bodies, and could not be prepared because they could not be properly refolded (data not shown).

Figure 3. CD spectra and thermal stabilities of Ubi and Ubi-(1RI8). (A) Comparison of the CD spectra of Ubi and Ubi-(1RI8). (B) Comparison of Ubi and Ubi-(1RI8) thermal stability by DSC.

Ubi and CDR3-grafted Ubi, Ubi-(1RI8), and Ubi-(1RJ), were expressed in *E. coli* BL21 (DE3) in a soluble, folded form. The soluble Ubi and CDR3-grafted Ubi were purified by affinity chromatography and cation-exchange chromatography. Cation-exchange chromatography of Ubi-(1RI8) is in Figure 2(C). Purified Ubi and CDR3-grafted Ubi were homogenous as judged by SDS-PAGE [Fig. 2(D)] and reverse-phase HPLC [Fig. 2(F)]. The overall yield of pure Ubi-(1RI8) and Ubi-(1RJ) were as high as 0.5 mg and 0.2 mg per liter, respectively.

Detection of an intramolecular disulfide bond in CDR3-grafted Ubi, Ubi-(1RI8)

Ubi-(1RI8) was digested by trypsin and peptides were analyzed by mass spectrometry. A peak in the mass spectrum of one of the trypsin-digested Ubi-(1RI8) peptides was detected at $m/z = 3154.63$ in non-reduced conditions, and at $m/z = 2124.08$ in reduced conditions. Ubi-(1RI8) was designed to form a disulfide bond between the mutated Cys68 of Ubi and the Cys of the grafted CDR3 peptide. If the disulfide bond is formed in Ubi-(1RI8), the theoretical mass peak of the trypsin-digested peptide is $m/z = 3154.52$ in non-reduced conditions and $m/z = 2123.98$ and 1033.57 in reduced conditions. In this experiment, the $m/z = 1033.57$ fragment could not be detected. Additionally,

regarding Ubi-(1RI8), the thiol oxidation state of Cys residues and their participation in the disulfide bond were assessed by using $^{13}\text{C}\beta$ chemical shift values of Cys 13 (39.28 ppm) and Cys 77 (41.61 ppm).¹¹ These results indicated that intended intramolecular disulfide bond was formed in Ubi-(1RI8). Ubi-(1RJ) also formed intramolecular disulfide bond, as judged by the same analysis (data not shown).

Circular dichroism spectra analysis of CDR3-grafted Ubi and Ubi

CD analyses of purified Ubi and Ubi-(1RI8) were performed, yielding similar spectra [Fig. 3(A)]. This indicated that Ubi-(1RI8), the CDR3-grafted Ubi mainly maintained the original structure of Ubi.

Stability of CDR3-grafted Ubi and Ubi

The thermal denaturation-induced unfolding transition of Ubi and Ubi-(1RI8) was measured. The thermal melting temperature (T_m) of Ubi was 95.5°C at pH 5.0 [Fig. 3(B)], the T_m of the CDR3-grafted protein Ubi-(1RI8) was 84.4°C at pH 5.0.

Antigen-binding abilities of CDR3-grafted proteins

Antigen-binding of CDR3-grafted proteins were analyzed using a Biacore T100 instrument (Fig. 4). The

Figure 4. Binding analysis of recombinant proteins to HEWL by Biacore T100. (A) D2-L19, (B) cAb-CA05-(1R18), (C) Ubi-(1R18), and (D) Ubi. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

interaction between cAb-CA05 and HEWL was very weak, and the K_D value could not be determined. The K_D values for HEWL were $7.3 \times 10^{-6} M$ for cAb-CA05-(1R18) and $2.2 \times 10^{-6} M$ for cAb-CA05-(1RJC) (Table III). The interaction between the human scaffold protein, Ubi, and HEWL was also very weak [Fig. 4(D)], and the K_D value could not be determined. The affinity for HEWL was $2.4 \times 10^{-5} M$ for Ubi-(1R18) and $2.3 \times 10^{-4} M$ for Ubi-(1RJC) (Table III).

Thermodynamic analysis of antigen binding

Thermodynamic analysis of D2-L19, cAb-CA05-(1R18), and Ubi-(1R18) binding to HEWL was performed. Table IV summarizes the K_D values of these recombinant proteins against HEWL in different conditions. Thermodynamic parameters, free energy change (ΔG°), enthalpy change (ΔH°) and entropy change ($-T\Delta S^\circ$) of binding were calculated according to the K_D values (Table IV). The results indicated that D2-L19 interacted with HEWL mainly in an enthalpy-driven manner. cAb-CA05-(1R18) interacted with HEWL equally in an enthalpy- and an entropy-driven manner. Ubi-(1R18) interacted with HEWL mainly in an entropy-driven manner.

NMR analysis

Hydrogen-deuterium (H-D) exchange experiments of Ubi and Ubi-(1R18) were performed (Fig. 5). The H-D exchange rates of the N-terminal and C-terminal amino acid residues of the CDR3-grafted sequence in Ubi-(1R18), which were -3, -1, +1, +3, +4, +5, +6

amino acids, were obviously faster Ubi [Fig. 5(A-C)]. In contrast, the H-D exchange rates of the amino acid residues involved in the structural formation of Ubi were not significantly changed by CDR3 grafting [Fig. 5(D)].

Discussion

To design CDR3-grafted scaffold proteins, we focused on the conserved sequence and structure of VHH, particularly the N-terminal and C-terminal amino acid residues of the CDR3 loop and the Cys residue of the disulfide bond between the CDR1 and CDR3 of VHH [Fig. 1(A,B)]. Based on this information, the

Table III. K_D Values of VHH and CDR3-grafted Proteins for HEWL

	$K_D (M)$
D2-L19	4.7×10^{-9}
cAb-Lys2	6.0×10^{-10}
cAb-CA05	ND
cAb-CA05-(1R18)	7.3×10^{-6}
cAb-CA05-(1RJC)	2.2×10^{-6}
Ubi	ND
Ubi-(1R18)	2.4×10^{-5}
Ubi-(1RJC)	2.3×10^{-4}

K_D for each protein toward HEWL was analyzed with a Biacore T100 instrument. To calculate the K_D of D2-L19 and cAb-Lys2, the 1:1 binding model of kinetics analysis in BIA evaluation was used. To calculate the K_D of cAb-CA05-(1R18), cAb-CA05-(1RJC), Ubi-(1R18), and Ubi-(1RJC), affinity analysis in BIA evaluation was used. Binding affinities of cAb-CA05 and Ubi were too weak for K_D values to be determined.

Table IV. K_D values at different temperature and thermodynamic parameters for D2-L19, cAb-CA05-(1RI8), and Ubi-(1RI8) binding toward HEWL

K_D (M) values at different temperature			
Temperature	D2-L19	cAb-CA05-(1RI8)	Ubi-(1RI8)
10°C	1.3×10^{-9}	5.4×10^{-6}	3.7×10^{-5}
19°C	3.0×10^{-9}	7.0×10^{-6}	3.1×10^{-5}
25°C	4.7×10^{-9}	7.3×10^{-6}	2.4×10^{-5}
33°C	8.5×10^{-9}	8.4×10^{-6}	1.8×10^{-5}
37°C	1.2×10^{-8}	1.1×10^{-5}	1.7×10^{-5}
Thermodynamic parameters			
Parameter Name	D2-L19	cAb-CA05-(1RI8)	Ubi-(1RI8)
ΔH° [kJ/mol]	−58	−16	23
ΔS° [J/(K·mol)]	−33	43	160
$-T\Delta S^\circ$ [kJ/mol]	9.8	−13	−49
ΔG° [kJ/mol]	−48	−29	−26

ΔH° , ΔS° , and ΔG° representing enthalpy change, entropy change, and free energy change.

structure of human proteins was searched in PDB and Ubi,¹⁴ NCAM,¹⁵ and FKBP¹⁶ were selected as CDR3-grafting scaffolds. The CDR3 sequences of D2-L19 and cAb-Lys2 were grafted onto Ubi, NCAM, and FKBP. CDR3-grafted NCAM and the CDR3-grafted FKBP could not purified because they could not be refolded. These CDR3-grafted proteins might be unstable. Normally, the stability of proteins with a long peptide insertion decreases.⁹ We were able to prepare CDR3-grafted Ubi, Ubi-(1RI8), and Ubi-(1RJ). The Ubi was mutated from His68 to Cys to

form a disulfide bond with the Cys residue of the grafted CDR3. The C α position of His68 corresponded to the C α position of the Cys residue of CDR1 that forms the disulfide bond in VHH. The results of mass spectrometry and $^{13}\text{C}\beta$ chemical shift values of Cys residues indicated that Ubi-(1RI8) formed a disulfide bond between the mutated Cys68 of Ubi and the Cys residue of the grafted CDR3. Moreover, the results of SDS-PAGE analysis [Fig. 2(D)] and reverse-phase HPLC analysis [Fig. 2(F)] indicated that prepared Ubi-(1RI8) was homogeneous. Therefore, we concluded that all Ubi-(1RI8) molecules formed the disulfide bond. The secondary structure of Ubi-(1RI8) was similar to Ubi [Fig. 3(A)]. Ubi-(1RI8) was unstable compared to Ubi [Fig. 3(B)], however, it was still considerably more stable than other proteins. For example, VHH is a so-called very stable protein,^{17,18} but the T_m of VHH is around 60–80°C.¹⁹ These results indicated that Ubi-(1RI8) mostly maintained the Ubi structure. However, the yield of Ubi-(1RJ) was low compared to Ubi-(1RI8). This indicated a difference in the protein-folding efficiency because of the grafted CDR3 sequences.

To evaluate the antigen-binding ability of the CDR3 sequence, the binding affinity of the CDR3-grafted VHs was analyzed (Table III, Fig. 4). These results indicated that the CDR3 sequence of D2-L19 and cAb-Lys2 had affinity toward HEWL by itself. K_D values of the CDR3 sequence against HEWL were about 10^{-6} M when they were grafted on cAb-CA05. However, these K_D values were relatively low

Figure 5. H-D exchange with Ubi and Ubi-(1RI8). Plots of three amino acid residues around the CDR3 grafted root −3 to +6: (A)5V; CDR3-1, (B)13I; CDR3+1, (C)16E; CDR3+4. (D)29K; exist in α -helix that is a portion of the scaffold. Ubi (●), Ubi-(1RI8) (▲). Peak intensity (%) is the relative peak intensity ratio for each measurement compared with the corresponding peak intensity of the initial measurement.

compared to the original VHH, D2-L19, or cAb-Lys2 (Table III, Fig. 4). Furthermore, the thermodynamic parameters of D2-L19 and cAb-CA05-(1RI8) binding to HEWL differed (Table IV). The interaction between D2-L19 and HEWL was mainly in an enthalpy-driven manner, which would be associated with a formation of hydrogen bond and/or of van der Waals interaction. The CDR3-grafted VHH cAb-CA05-(1RI8) interacted with HEWL in an enthalpy-entropy-driven manner. These results suggested that CDR1 and CDR2 affected not only antigen-binding affinity but also the thermodynamic property of antigen recognition. To reconstruct the affinity and binding pattern entirely by CDR3 grafting, the functions of CDR1 and CDR2 must be provided to the scaffold protein.

Ubi have a very weak interaction with HEWL, but response values of interaction in association phase were very low and response values of interaction in dissociation phase completely returned to 0 [Fig. 4(D)]. On the other hand, Ubi-(1RI8) and Ubi-(1RJ) interacted with HEWL significantly, and the response values of interactions were still remaining in dissociation phase [Fig. 4(C)]. These results indicated that the affinity toward HEWL was transferred by CDR3 grafting. Additionally, we also analyzed the interaction between Ubi-(1RI8) and carbonic anhydrase, antigen of cAb-CA05, and the result was same as the interaction between Ubi and HEWL (Sup. 1). In summary, the response values of interaction between Ubi-(1RI8) and carbonic anhydrase completely returned to 0 in dissociation phase. These results indicated that the specificity toward HEWL was also transferred by CDR3 grafting. However, the K_D values of CDR3-grafted Ubi-(1RI8) and Ubi-(1RJ) were relatively low against HEWL compared to the CDR3-grafted VHHs, cAb-CA05-(1RI8) and cAb-CA05-(1RJ) (Table III). Additionally, the thermodynamic parameters of Ubi-(1RI8) binding toward HEWL were different from those of D2-L19 and cAb-CA05-(1RI8) (Table IV). Ubi-(1RI8) interacted with HEWL mainly in an entropy-driven manner, which was based on hydrophobic interaction. cAb-CA05-(1RI8) and Ubi-(1RI8) have the same CDR3 sequence and different scaffolds. These results indicated that recreating the disulfide bond between CDR3 and Ubi succeeded, but was not enough to restructure the CDR3 in Ubi to be the same as the VHH structure.

To examine the low affinity of Ubi-(1RI8) to HEWL, hydrogen-deuterium (H-D) exchange with Ubi and Ubi-(1RI8) was performed (Fig. 5). Moreover, the ^{15}N spin-spin relaxation time (T_2) analysis showed that T_2 values of the area around the grafted CDR3 sequences of Ubi-(1RI8) were notably shorter than for Ubi (data not shown). These results showed that the area around the grafted CDR3 sequences of Ubi-(1RI8) was destabilized by CDR3 grafting. This might be one cause for the affinity decrease of Ubi-(1RI8). It might also be responsible

for the differences in thermodynamic properties. We hypothesized that the orientation of the CDR3 sequence of Ubi-(1RI8), which would be important to form hydrogen bonds and/or van der Waals interactions with HEWL, could not be reconstructed correctly and Ubi-(1RI8) interacted with HEWL mainly in an entropy-driven manner rather than an enthalpy-driven manner.

CDR grafting on nonimmunoglobulin proteins has been reported,^{9,20} and Nicaise *et al.* created CDR3-grafted NCS. In ITC experiments, the affinity of the CDR3-grafted NCS against antigen could be measured at 15°C but not at 25°C because of aggregation. The CDR3-grafted NCS did not form a disulfide bond between framework of the NCS and Cys residues of the CDR3 sequence. We propose that the grafted CDR3 structure of the protein is unstable and this low stability might be one of the reasons for aggregation at 25°C at high protein concentrations. On the other hand, the CDR3-grafted Ubi-(1RI8) was stable and formed a disulfide bond between the mutated Cys68 of Ubi and the Cys of the grafted CDR3 sequence, as designed. As far as we know, this is the first report of CDR3 grafting with a recreated disulfide bond, and we expect that Ubi-(1RI8) could have affinity toward the antigen at 25°C or higher temperatures. As expected, the K_D values of Ubi-(1RI8) could be measured at 10, 19, 25, 33, and 37°C (Table IV). We hypothesize that the disulfide bond between the mutated Cys68 of Ubi and the Cys residue of the grafted CDR3 sequence contributed to the stability of the grafted CDR3 structure. However, the affinity against HEWL of Ubi-(1RI8) was relatively low compared to cAb-CA05-(1RI8) and the thermodynamic properties of Ubi-(1RI8) were different from cAb-CA05-(1RI8). These might be caused by the low stability of the area around the CDR3-grafted sequences of Ubi-(1RI8).

In conclusion, we succeeded in affinity transfer to human Ubi by CDR3 grafting of a camelid VHH. The CDR3-grafted Ubi was stable as a protein and formed a disulfide bond between the mutated Cys68 of Ubi and the Cys residue of the grafted CDR3 sequence. This CDR3 grafting method could be applied to a number of Ig and non-Ig domain proteins by grafting the CDR3 loop of VHH. A design upgrade and searches for new scaffolds for grafting CDR3 will be the subject of future studies.

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