Stabilization of an Immunoglobulin Fold Domain by an Engineered Disulfide Bond at the Buried Hydrophobic Region*

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We report for the first time the stabilization of an immunoglobulin fold domain by an engineered disulfide bond. In the llama single-domain antibody, which has human chorionic gonadotropin as its specific antigen, Ala⁴⁹ and Ile⁷⁰ are buried in the structure. A mutant with an artificial disulfide bond at this position showed a 10 °C higher midpoint temperature of thermal unfolding than that without the extra disulfide bond. The modified domains exhibited an antigen binding affinity comparable with that of the wild-type domain. Ala⁴⁹ and Ile⁷⁰ are conserved in camel and llama single-domain antibody frameworks. Therefore, domains against different antigens are expected to be stabilized by the engineered disulfide bond examined here. In addition to the effect of the loop constraints in the unfolded state, thermodynamic analysis indicated that internal interaction and hydration also control the stability of domains with disulfide bonds. The change in physical properties resulting from mutation often causes unpredictable and destabilizing effects on these interactions. The introduction of a hydrophobic cystine into the hydrophobic region maintains the hydrophobicity of the protein and is expected to minimize the unfavorable mutational effects.

One of the major objectives of antibody engineering is to stabilize the three-dimensional structure of the antibody. Disulfide bonds often significantly stabilize the structure of native proteins. Thus, the introduction of artificial disulfide bonds is recognized as a useful protein engineering technique to increase conformational stability. Although this technique has been applied to many proteins, there are no reports of engineered disulfide bonds in an immunoglobulin fold framework.

Cystine is hydrophobic, and thus, most of naturally occurring disulfide bonds are buried in the protein (1–4). Therefore, the introduction of an engineered disulfide bond into the hydrophobic core better maintains the biophysical properties of the target protein. There are several examples of artificial disulfide bonds that can replace a pair of buried hydrophobic residues, the accessible surface areas of which were <20% (5–11). The introduction of a disulfide bond into the buried hydrophobic core of human carbonic anhydrase (A23C/L203C) markedly stabilizes this enzyme; the midpoint temperature of thermal unfolding (Tm) of the mutant is 10 °C higher than that of the wild-type protein (6). The engineered disulfide bonds in alcalase protease AprP (G199C/F236C) (11), xylanase (V98C/A152C) (7), and manganese peroxidase (A48C/A63C) (10) mutants increase their tolerance against heat inactivation. On the other hand, subtilisin BPN’ mutants (V26C/A232C and A29C/M119C) exhibit similar or slightly lower stability to irreversible thermal inactivation (8). Tolerance against heat denaturation is not directly correlated with conformational stability. Only the mutational effect on human carbonic anhydrase II (6) was examined in a reversible system. Little information is available about the thermodynamic effects of artificial disulfide bonds in the buried hydrophobic region.

In a previous study (12), we screened the amino acid pairs substituting for the disulfide bonds of four different immunoglobulin fold domains by a method based on the cellular quality control system (13, 14). The intradomain disulfide bonds in the immunoglobulin fold domains are buried and are important for stability (15, 16). At least two pairs combining Ala, Val, or both, i.e. Ala/Ala, Ala/Val, Val/Ala, and Val/Val, were selected in all four domains in the screening, and they were stably folded at 30 °C. These mutants found in three domains exhibited similar or higher stability compared with the corresponding reduced wild-type domains. The pairs using Ala and Ile, Gly and Leu, Gly and Phe, and Gly and Val were also selected in more than two of the four domains. Among those, the stability of one of the Ala/Ile mutants was higher than that of the corresponding reduced domains. This indicates that specific pairs of hydrophobic amino acids can replace the buried disulfide bond with a minimum loss of stability. Conversely, for construction of an artificial disulfide bond, the proximal pair of buried hydrophobic amino acids as reported by a previous study (12) may be a good candidate for replacement with cystine.

Here, we report the effects of an engineered disulfide bond in the hydrophobic core of the variable domain of the cameled heavy chain antibody (VHH), also known as the single-domain

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² The abbreviations used are: VHH, variable domain of the cameled heavy chain antibody; hCG, human chorionic gonadotropin; DSC, differential scanning calorimetry; SPR, surface plasmon resonance; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
Ig Fold Domain Stabilization by an Engineered Disulfide Bond

antibody. In the three-dimensional structure of llama VHH raised against the α-subunit of human chorionic gonadotropin (hCG) (17), Ala\(^{49}\) and Ile\(^{70}\) are buried in the domain, and the distance between β-carbons of these amino acids is within a normal distance of naturally occurring β-carbons of cystines. We replaced these two amino acids with Cys in the wild-type and mutant domains, substituting the native disulfide bond with the Trp/Ala amino acid pair. Then, the stability and antigen binding ability of the mutants created were studied by CD, differential scanning calorimetry (DSC), pulldown assay, and surface plasmon resonance (SPR) spectrometry.

EXPERIMENTAL PROCEDURES

Preparation of VHH Mutants—Construction of the expression vector containing wild-type VHH and the mutant lacking a disulfide bond was described previously (12). The mutations were introduced by PCR-based site-directed mutagenesis. The mutation vector containing wild-type VHH and the mutant lacking a disulfide bond was described previously (12). The mutations were introduced by PCR-based site-directed mutagenesis. The mutation vector containing wild-type VHH and the mutant lacking a disulfide bond was cloned into pAED4 (18), and mutant proteins were expressed in Escherichia coli strain BL21(DE3) pLysS (Stratagene, La Jolla, CA), where they accumulated in inclusion bodies. These inclusion bodies were dissolved with 6 M guanidine hydrochloride or 8 M urea, followed by overnight air oxidation at 4 °C. Proteins in the denaturant were refolded by 1:50 dilution with 10 mM sodium acetate (pH 5.3 or 4.7). A Resource S cation-exchange column (GE Healthcare) equilibrated with 10 mM sodium acetate (pH 5.3 or 4.7) was used to purify crude VHHs.

The VHH gene with two disulfide bonds was cloned into pPIC1, which is a derivative of pPIC9 (Invitrogen) with the replication origin of the pUC vector. The constructed vector was digested with AatI (Stul) and transformed into Pichia pastoris GS115. The most efficient transformant was selected on the basis of expression efficiency in small-scale test tube culture. High cell density fermentation of the selected strain was carried out in a 2-liter fermentor (Mitsuwa Scientific Corp., Oswego, OR). The formation of disulfide bonds was examined by the method of Ellman (20) in 4 M guanidine hydrochloride, 0.1 M Tris-HCl (pH 8.5), and 1 mM 5,5′-dithiobis(nitrobenzoic acid) at a protein concentration of 20 µM, in which the fully reduced samples were expected to exhibit \(A_{412\text{ nm}}\) 0.5–1.0 with a UV-2500PC spectrophotometer (Shimadzu Corp., Kyoto, Japan). Free thiols of VHHs with one disulfide bond were undetectable under the experimental conditions used.

MALDI-TOF mass spectrometry was then used to confirm that the molecular weights of the purified proteins were identical to the expected values calculated from their amino acid sequences (with an error of ±0.025%). The concentration of protein in the stock solution was determined by measuring the absorbance at 280 nm (21).

Thermodynamic Analysis of VHH Unfolding—Thermal unfolding was monitored as the change in ellipticity at 235 nm at a protein concentration of 4 µM using a J-720 spectropolarimeter (Jasco, Tokyo, Japan) and a 10-µm cell. The buffers used for the experiments contained 20 mM sodium acetate (pH 4–5), glycine HCl (pH 2–4), or potassium phosphate (pH 7.1). In unfolding experiments using CD, >70% of the CD signals at 235 nm were retained at 10 °C after heat denaturation, except for the unfolding of 0-SSVHH at pH 7.1, for which ~40% of ellipticity at 235 nm was observed. We thus did not use the data of 0-SSVHH at neutral pH to calculate the thermodynamic parameters. DSC measurements of VHHs were made using a VP-DSC MicroCalorimeter (MicroCal, Northampton, MA) in buffer containing 20 mM sodium acetate (pH 4) or glycine HCl (pH 2–4). For both CD and DSC measurements, the heating rate was 1 °C/min. We examined the reversibility of the DSC measurements in each VHH in at least two different pH environments. More than 80% of the heat absorption was observed in the second run after the first heat denaturation. The CD and DSC results were analyzed on the basis of the two-state transition mechanism using IGOR Pro 4 (WaveMetrics, Inc., Lake Oswego, OR).

In the analysis of the unfolding transitions detected by CD, the free energy change of unfolding at a given pH at temperature \(T\) was as shown in Equation 1,

\[
\Delta G_{U}^{\text{pH}}(T) = (T_{m}^{pH} - T)T_{m}^{pH-1}(\Delta H_{U45} - \Delta C_{p,U}(318.15 - T_{m}^{pH})) - (T_{m}^{pH} - T)\Delta C_{p,U} + T \Delta C_{p,U} \ln(T_{m}^{pH}T^{-1}) \quad (\text{Eq. 1})
\]

where \(T_{m}^{pH}\) and \(\Delta H_{U45}\) represent the \(T_{m}\) at a given pH and the enthalpy change from the native state to the unfolded state (\(\Delta H_{U-N}\)) at 45 °C, respectively. In this analysis, we assumed that the difference in heat capacity between the folded and unfolded states (\(\Delta C_{p,U}\)) was constant. Because \(\Delta H_{U45}\) and \(\Delta C_{p,U}\) are independent of pH, multiple CD unfolding curves at different pH values could be analyzed by global fitting using the same \(\Delta H_{U45}\) and \(\Delta C_{p,U}\) values.

For DSC analysis, \(\Delta C_{p,U}\) was estimated to be the difference between the base-line values for the unfolded (\(BL_{U}\)) and native (\(BL_{N}\)) states. \(BL_{U}\) and \(BL_{N}\) are represented by \(BL_{U} = BL_{U1} + BL_{U2}\) and \(BL_{N} = BL_{N1} + BL_{N2}\), respectively. Thus, the \(\Delta C_{p,U}\) at temperature \(T\) is given by Equation 2.

\[
\Delta C_{p,U}(T) = (BL_{U1} - BL_{N1}) + (BL_{U2} - BL_{N2}) \quad (\text{Eq. 2})
\]

Therefore, the \(\Delta H_{U}\) and entropy changes of unfolding (\(\Delta S_{U}\)) at temperature \(T\) are described by Equations 3 and 4, respectively.

\[
\Delta H_{U}(T) = \Delta H_{U}(T_{m}) - (0.5(BL_{U1} - BL_{N1})(T_{m}^{2} - T^{2}) + (BL_{U2} - BL_{N2})(T_{m} - T) \quad (\text{Eq. 3})
\]
stained with Coomassie Brilliant Blue. The kinetics of binding of VHHs to antigen was examined by SPR spectroscopy. Antigen hCG (0.05 mg/ml) was coupled to a CM5 sensor chip (GE Healthcare) in 10 mM sodium acetate buffer (pH 6) by amine coupling according to the manufacturer’s instructions. Analysis was performed on a Biacore 2000 instrument (GE Healthcare) in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 at 20 °C. Five serial dilutions (250 to 15 nm) of VHHs were injected into the cuvette, followed by regeneration with 20 mM HCl. As a control, binding of β2-microglobulin (500 to 125 nm) to hCG was also examined.

RESULTS

Introduction of an Artificial Disulfide Bond into VHH—We found that llama VHH has a potential candidate for the introduction of the artificial disulfide bond in its hydrophobic amino acid pair: Ala[49] and Ile[70] (Fig. 1, A and B). Amino acid pairs matching the following criteria were searched for in the three-dimensional structure of VHH (Protein Data Bank code 1HCV) (17), for which the antigen is hCG. First, we selected pairs containing combinations of Ala, Val, and Ala and Ile, which were reported previously to be stable at 30 °C in more than two different immunoglobulin fold domains (12). We excluded pairs with Gly because it is hard to predict the orientation of the side chain of Cys substituted for Gly. Second, the solvent-exposed surface area of each amino acid needed to be <10 Å², which corresponds to 5% of the total surface of Ala, because most naturally occurring disulfide bonds are buried (1–3). The solvent-exposed surface area was calculated by MOLMOL (22) considering a solvent radius of 1.4 Å. Usually, the distance between two β-carbons of cystine is ~3.5–4.5 Å (5). Thus, a third criterion was that the distance between the β-carbons of the pair of amino acids needed to be <4.5 Å.

The Ala[49]/Ile[70] pair matched all of the above-mentioned criteria. The accessible surface areas of Ala[49] and Ile[70] are 3.4 and 7.5 Å², respectively. These correspond to 1.6% (Ala[49]) and 2.7% (Ile[70]) of the total accessible surface areas of these amino acids alone. The distance between the β-carbons of Ala[49] and Ile[70] is 4.1 Å. We prepared two mutants in which we replaced these two amino acids with Cys (Fig. 1C). One was with a wild-type disulfide bond between Cys[22] and Cys[56], named 2-SSVHH. The other, 1-SSVHH, was constructed on the non-disulfide-bonded
framework, where the wild-type disulfide bond was substituted with the Trp/Ala pair. In addition to these mutants with engineered disulfide bonds, we used wild-type VHH and a non-disulfide-bonded mutant (0-SSVHH) for comparison. Although the Trp/Ala pair was selected only once in a previous study, 0-SSVHH, in which the native disulfide bond was replaced with this pair, showed the highest thermal stability of the mutants that were found in the screening (12). Thus, we selected a mutant with Trp/Ala as the non-disulfide-bonded framework. It should be noted that the VHH mutants with Ala/Ala and Ala/Val exhibited the second and fourth highest $T_m$ values of the selected mutants, respectively (12).

The oxidative formation of the disulfide bond in the 1-SSVHH and wild-type domains can be completed by air oxidation in denaturant. However, in 2-SSVHH, the reproducibility of the renaturation of two disulfide bonds was low when we used E. coli as a host organism to express 2-SSVHH (data not shown). Thus, we selected the methylotrophic yeast P. pastoris to produce 2-SSVHH. After the expressed 2-SSVHH mutant was heated to complete the formation of disulfide bonds, the amount of free thiol was <5% of the amount of total thiol. Because the remaining free thiol may influence the thermodynamic parameters for the stability of the mutants, we analyzed 2-SSVHH in terms of $T_m$ and antigen binding affinity, which are not sensitive to minor contamination with free thiols. For comparison among 1-SSVHH, 0-SSVHH, and wild-type domains, we precisely analyzed the thermodynamic effects of the engineered disulfide bond on the stability of VHH.

**Stability of VHH Mutants with an Artificial Disulfide Bond**—The engineered disulfide bond significantly stabilized the VHVs. We measured the thermal unfolding curves of the mutant and wild-type VHVs at pH 7.1 following the change in ellipticity at 235 nm (Fig. 2). The $T_m$ values of the 2-SSVHH, 1-SSVHH, 0-SSVHH, and wild-type domains were 74, 56, 46, and 64 °C, respectively. The most stable mutant, 2-SSVHH, was unfolded at a 10 °C higher $T_m$ compared with the wild-type domain. The difference in $T_m$ between 1-SSVHH and 0-SSVHH was 10 °C and was identical to that between 2-SSVHH and wild-type VHH.

The thermal unfolding curves of 1-SSVHH, 0-SSVHH, and the wild-type domains at different pH values were measured by CD and DSC to further characterize the effects of the artificial disulfide bond (Fig. 3). The data from CD were analyzed by global fitting. We analyzed individually the heat absorption curves derived from the DSC measurements. Because we could measure the unfolding curves with $T_m$ values of ~45 °C in all three VHVs, we decided to compare the thermodynamic parameters at 45 °C.

The $\Delta H_U$ of 0-SSVHH was significantly higher than that of the 1-SSVHH and wild-type domains (Fig. 4 and Table 1). Thus, the introduction of a disulfide bond, both native and artificial, is enthalpically unfavorable for the stability of VHH. The linear extrapolation of the individual results of the DSC analysis to 45 °C showed that the $\Delta H_U$ value of 0-SSVHH was 30 and 50 kJ/mol higher than those of the 1-SSVHH and wild-type domains, respectively. The analysis of the CD measurements showed the difference in $\Delta H_U$ at 45 °C between 0-SSVHH and
Ig Fold Domain Stabilization by an Engineered Disulfide Bond

1-SSVHH to be ~50 kJ/mol and that between 0-SSVHH and wild-type VHH to be ~80 kJ/mol.

ΔC_p,U was estimated using both CD and DSC data (Table 1). With both techniques, estimated ΔC_p,U values were identical within experimental errors, which indicates that the effect of disulfide introduction on ΔC_p,U was negligible. ΔC_p,U is considered to be related to the difference in the amount of hydration in the folded and unfolded states. Our data suggest that the introduction of a disulfide bond does not alter the extent of hydration upon unfolding. However, the error in estimated ΔC_p,U was relatively high (~10%), and uncertainty exists about the hydration effect based on this parameter.

Both native and engineered disulfide bonds decreased ΔS_U compared with 0-SSVHH (Table 1). We estimated the average difference in ΔS_U at 45 °C between the 0-SSVHH and the domains with one disulfide bond at the same pH values. The ΔS_U values of 0-SSVHH were 205 (CD) and 133 (DSC) J/mol larger than those of 1-SSVHH and 328 (CD) and 203 (DSC) J/mol larger than those of the wild-type domain. The effect of disulfide formation on ΔS_U (ΔS_U,loop) can be calculated using Equation 6,

\[ \Delta S_{U,loop} = -R\left(3/2 \ln N + A\right) \]  

(6)

where N is the number of residues in the loop-forming disulfide bond. Constant A differs in the literature. Poland and Scheraga (23) and Pace et al. (24) assumed that constant A is 3.47 and 1.06, respectively. The calculated ΔS_U,loop values using both values were −67 and −47 J/mol for 1-SSVHH and −83 and −63 J/mol for wild-type VHH. The differences in experimental ΔS_U values of 1-SSVHH and wild-type VHH compared with 0-SSVHH deviated significantly from ΔS_U,loop.

Antigen Binding of VHH Mutants—All prepared VHH mutants bound to the antigen, the α-subunit of hCG. First, we carried out pulldown antigen experiments using immobilized 0-SSVHH, 1-SSVHH, 2-SSVHH, and wild-type domains (Fig. 5). Sepharose-immobilized VHHs were prepared and incubated with hCG at room temperature. After the residual proteins were washed, bound antigen was eluted by SDS. Then, each eluent was subjected to SDS-PAGE. The amounts of hCG bound to the immobilized mutants were similar compared with wild-type VHH. hCG did not interact with Sepharose alone (Fig. 5, lane 3).

![Figure 4: Enthalpy of unfolding (ΔH_U) of wild-type VHH (black), 0-SSVHH (red), and 1-SSVHH (blue). ΔH_U values from individual analysis of DSC curves are indicated by open circles (wild-type VHH), open squares (0-SSVHH), and closed circles (1-SSVHH). Solid and dashed lines were obtained by linear fitting of individual ΔH_U values from DSC and by global fitting of CD data, respectively.]

![Figure 5: Binding of antigen hCG to immobilized VHHs. Wild-type VHH (lane 4), 0-SSVHH (lane 5), 1-SSVHH (lane 7), and 2-SSVHH (lane 8) were immobilized on cyanogen bromide-activated Sepharose, and hCG was incubated with VHH-Sepharose at room temperature for 90 min. After washing and elution, bound hCG was applied to 10–20% gradient SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue. The antigen binding ability of non-disulfide-bonded VHH, in which the native disulfide bond was replaced with Ala/Ala (A/A*), is also shown for reference (lane 6).]

| VHH/method | ΔH_U at 45 °C | ΔC_p,U | Average difference in ΔS_U at 45 °C |
|------------|---------------|--------|-----------------------------------|
| 0-SSVHH    |               |        |                                   |
| CD         | 360 ± 7       | 5.4 ± 0.7 |                                 |
| DSC        | 342 ± 5       | 4.8 ± 0.4 |                                 |
| Wild-type VHH |            |        |                                   |
| CD         | 275 ± 6       | 5.3 ± 0.5 | 328                               |
| DSC        | 295 ± 5       | 4.8 ± 0.3 | 203                               |
| 1-SSVHH    |               |        |                                   |
| CD         | 309 ± 3       | 5.3 ± 0.7 | 205                               |
| DSC        | 312 ± 5       | 5.2 ± 0.4 | 133                               |

* ΔH_U and ΔC_p,U, were calculated by global fitting of CD data and linear fitting of ΔH_U values obtained from individual DSC data, respectively.  
** The ΔS_U values of wild-type and 1-SSVHH were subtracted from the ΔS_U values of 0-SSVHH at the same pH values, and these values were averaged.
Ig Fold Domain Stabilization by an Engineered Disulfide Bond

SPR measurements were performed to further confirm the binding of VHH mutants to hCG. SPR spectra were similar for all prepared mutants (Fig. 6A). The SPR spectra obtained could not be analyzed by a simple Langmuir model, and a heterogeneous ligand-parallel fit model gave relatively well fitted theoretical curves (Fig. 6B). It is unclear why there are two phases in binding and dissociation. In SPR experiments, hCG was immobilized by a rather nonspecific amine coupling method, followed by a harsh condition (50 mM NaOH) to wash out residual hCG. Thus, we speculate that some fraction of hCG may expose its epitope, resulting in high affinity binding to VHVs. The $K_d$ values calculated from major phases (slow association and fast dissociation) were 25, 38, 43, and 23 nM for 0-SSVHH, 1-SSVHH, 2-SSVHH, and wild-type domains, respectively. The minor phases (fast association and slow dissociation) were <20% of the calculated maximum resonance. The $K_d$ values of the minor phases were <10 pm, and it was impossible to determine the precise values in the range of protein concentration examined here. The $K_d$ values of mutants with engineered disulfide bonds were less than twice those of wild-type VHH in major phases. Hence, we conclude that the artificial disulfide bond only slightly interfered with antigen binding of VHH. The affinity of this VHH was reported to be 300–400 nM (25). However, the precise experimental conditions, such as temperature and pH, were not described in that work, and thus, it is difficult to compare those data with our results.

DISCUSSION

The introduction of an artificial disulfide bond at Ala$^{49}$ and Ile$^{70}$ significantly stabilizes the native structure of llama VHH. This is the first report of an immunoglobulin fold domain being stabilized by an engineered disulfide bond. In addition, the antigen binding affinities of the mutants were comparable with that of wild-type VHH. Camelid VHH is famous for its high thermodynamic stability (25–27). Unique applications are proposed to utilize this high stability of VHH, such as detection of caffeine in hot beverages (28) and prevention of dandruff by VHH mixed with shampoo containing a high concentration of surfactant (29). Stabilization by the engineered disulfide bond reinforces the utility of VHVs.

The amino acids corresponding to Ala$^{49}$ and Ile$^{70}$ in VHH are conserved in the framework region of camelid VHVs, although there are different combinations other than Ala/Ile, such as Ser/Ile, Gly/Ile, Ala/Val, and Val/Ile. We searched ~30 three-dimensional structures of llama and camel VHVs. In more than one-half of the sequences, Ala/Ile pairs were found at the position equivalent to the Ala$^{49}$ and Ile$^{70}$ residues of VHH used here. Also, all of the Ala/Ile pairs are buried inside the domains, and the distance between the $\beta$-carbons ranges from 3.8 to 4.3 Å, which matched our criteria for the introduction of an artificial disulfide bond. Thus, we expect that other VHVs with proximal Ala and Ile residues that match the Ala$^{49}$ and Ile$^{70}$ residues in this study can be stabilized by the introduction of an engineered disulfide bond.

The naturally occurring second disulfide bonds are often found in the complementarity-determining region loops of camel wild-type VHVs (30) and are called interloop disulfide bonds. These disulfide bonds are buried in a region distant to the corresponding position of the Ala$^{49}$ and Ile$^{70}$ residues examined here (31, 32). Lipovsek et al. (33) carried out the selection and affinity maturation of an antibody mimic based on a fibronectin type III domain, which belongs to the immunoglobulin fold superfamily. They found that the highest affinity clones had an interloop disulfide bond, which is structurally analogous to that of VHH. Although the effects of interloop disulfide bonds on the structural stability remain unclear, their results indicate that the interloop disulfide bond in the fibronectin type III-domain-based antibody mimic is critical for the affinity against its antigen. As the VHH studied here lacks an interloop disulfide bond, its addition may enhance the antigen binding ability of 2-SSVHH.

Cystine has been shown to be fairly hydrophobic, and thus, most disulfide bonds in the native protein are buried in the protein structure (1–4). To suppress the modification of the hydrophobicity of the protein, it would be better to mutate the buried hydrophobic amino acids. On the basis of previous study (12), we hypothesized that the combinations of Ala, Val, Ala and Val, and Ala and Ile are suitable for replacement. Indeed, the engineered disulfide bond at Ala$^{49}$ and Ile$^{70}$, which are buried in the VHH structure, stabilized VHH. However, restoration of the wild-type disulfide bond, which was replaced with Trp/Ala in 0-SSVHH, caused similar thermodynamic effects in response to the introduction of the engineered disulfide bond. Thus, it is plausible that...
suitable combinations of hydrophobic amino acids for the engineered disulfide bond are not limited to those examined here.

Both native and engineered disulfide bonds in the hydrophobic region of VHH caused considerable decreases in $\Delta H_U$ and $\Delta S_U$, indicating that loop entropy is not the only factor determining the stability of VHH with disulfide bonds. The same phenomenon was observed in studies using cytochrome c (34), Cucurbita maxima trypsin inhibitor V (35), and barnase (36). Restriction of chain conformation in the unfolded state by the disulfide bond is considered to affect only the entropy term, and thus, the decrease in enthalpy should originate from other factors. In addition, the calculated $\Delta S_{U,\text{loop}}$ value is $>2$ times smaller than the experimental entropy difference. These findings indicate that the change in stability induced by the formation of a disulfide bond was controlled by factors other than strain of chain configuration. These factors are considered to be internal interactions, such as hydrogen bonding and van der Waals interactions and hydration of the native and unfolded states (37). In general, internal interaction causes positive $\Delta H_U$ and $\Delta S_U$ value. On the other hand, the hydration effect results in negative $\Delta H_U$ and $\Delta S_U$ values. The absolute values of $\Delta H_U$ and $\Delta S_U$ came from both factors, are large, and compensate each other. Slight modification of either the native or unfolded state induced by mutations would significantly change the internal interaction and hydration effect.

The thermodynamic characterization indicates that loop entropy alone cannot account for the stabilization resulting from the introduction of a disulfide bond into wild-type VHH and 1-SS-VHH compared with 0-SSVHH. The replacement of an amino acid pair with other amino acids, which was Cys in this study, alters VHH compared with 0-SSVHH. The replacement of an amino acid pair with other amino acids, which was Cys in this study, alters the hydrophobicity of the native and unfolded states is maintained. This strategy can be pursued by introducing an engineered disulfide bond, this strategy can be pursued by introducing a disulfide bond into wild-type VHH and 1-SS-VHH.

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