Cellular Quality Control Screening to Identify Amino Acid Pairs for Substituting the Disulfide Bonds in Immunoglobulin Fold Domains*

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We are interested in determining which amino acid pairs can be substituted for the disulfide (S–S) bonds in proteins without disrupting their native structures under physiological conditions. In this study, we focused on the intradomain S–S bonds in Ig fold domains and aimed to determine a simple rule for replacement of their S–S bonds. The cysteines of four different Ig fold domains were mutated randomly, and the amino acid pairs substituted for the S–S bonds were screened by the method utilizing a cellular quality control system. Among the 36 selected mutants, 31 were stably folded without S–S bonds, as judged from the cooperativity of thermal unfolding. In addition, the selected mutant llama heavy chain antibodies retained antigen-binding affinity. At least two of the pairs Ala:Ala, Ala:Val, Val:Ala, and Val:Val were found in the selected mutants for all four different Ig fold domains, and they were stably folded at 30 °C. This suggests that examination of these four pairs could be enough to obtain natively folded Ig fold domains without S–S bonds.

Native disulfide (S–S) bonds in proteins stabilize their functional structures, and the removal of an S–S bond results in marked destabilization of these proteins (1–8). It is of interest to determine which amino acid pairs can be substituted for the S–S bonds without disrupting their native structures under physiological conditions. Many S–S-bonded proteins are often regarded as targets for industrial and pharmaceutical uses. For practical applications, efficient production of these proteins with recombinant technology is essential, and the in vitro formation of correct S–S bonds is one of the most challenging problems for the preparation of functional recombinant proteins. Eliminating even one S–S bond critically decreases the possible S–S-bonding combinations, which results in higher yields of active proteins.

In this study, we focused on the replacement of intradomain S–S bonds in Ig fold domains. Four typical Ig fold domains were used, and amino acid pairs substituted for S–S bonds were selected by cellular quality control screening. Then, the selected mutants were examined for their ability to form the native structures using CD and functional assay. Two of the four Ig fold domains tested here were variable fragments, i.e. the variable region of the heavy chain of cameld heavy chain antibody (VHH) and an engineered mouse V_L domain (λ graft). The other two were β2-microglobulin (β2-m) and the constant domains of the human κ light chain (C_L fragment). The VHH used here recognizes human chorionic gonadotropin (hCG) as an antigen (9). The λ graft is based on the sequence of the humanized 4D5-derived V_L domain artificially designed to change the antigen specificity and interdomain interactions (10–12).

Our screening method, which utilizes a cellular quality control system for the secretory pathway in Saccharomyces cerevisiae and is referred to as “cellular quality control screening,” was developed to screen for sequences that can fold into the native structures under physiological conditions. This method is based on the efficiency of secretion of target sequences detected by an antibody directed against a generic tag (13, 14). In the secretory quality control system, native or correctly folded proteins are secreted, whereas misfolded proteins are retained and degraded within the cells; the efficiencies of secretion of proteins correlate with their structural statuses (14–18). Thus, cellular quality control screening does not require any prior knowledge of the target protein, such as its enzymatic activity. Taking advantage of this unique feature, we applied an identical screening system to the selection of amino acid pairs able to replace S–S bonds of four different Ig fold domains that do not share any functional properties.

EXPERIMENTAL PROCEDURES

Construction of Libraries of Mutant Ig Fold Domains—DNA fragments encoding a signal sequence, a pro-sequence, a multicloning site, and a FLAG tag sequence were all inserted sequentially into p415GALS (19) to create p415GSSE (14). Synthetic genes of VHH, C_L fragment, and λ graft were designed to optimize the codon frequencies for yeast using the peptide sequences of the Protein Data Bank accession number IG9E (from 1 to 117), the sequence published in Ref. 12 (from 1 to 111), and the Protein Data Bank accession number 1CLY (from 114 to 218), respectively. The plasmid containing the cDNA of human β2-m was a gift from Y. Goto (Osaka University) and H. Naiki (Pakui Medical University). Mutant Ig fold domains varying in the random mutations at the Cys positions were prepared by PCR using PuTu Turbo DNA polymerase (Stratagene) and oligo DNAs with random sequences at the Cys positions. In the case of β2-m, we used three different oligos to introduce the random mutations and to avoid the appearance of Cys at the mutated positions. In these oligos, the DNA sequences at Cys positions were VNN, NHN, and NNK, where V, N, H, and R indicate the mixed base pairs of ACG, A/C/G, A/C/T, A/G/C, and A/G, respectively. The prepared plasmids were transformed into Escherichia coli XL-1 Blue (Stratagene) by electroporation using a Gene Pulser II (Bio-Rad). The VHH, β2-m, C_L fragment, and λ graft libraries consisted of 13,000, 47,000, 26,000, and 480,000 colonies,
respectively. About 90% of the colonies contained successfully ligated plasmids. In the sequences of randomly picked clones, no clear preference for specific amino acids was observed.

**Screening of the Random Mutant Libraries—**S. cerevisiae strain RY810556-2B (MATa ura3-1 leu2-3, 112 his3-11, 15 can1-100) was a gift from G. Fink of the Whitehead Institute for Biomedical Research (Cambridge, MA). The expression and detection of secreted proteins were carried out as described previously (13, 14). After the transformation of libraries into yeast by electroporation, we obtained 22,000 colonies from the VHH library, 4,000 from the β2-m library, 9,000 from the Cβ fragment library, and 79,000 from the γ library. As a positive control, the transformants bearing the wild-type sequences were placed on separate areas of the plates for comparison. The secreted proteins were transferred to nitrocellulose membranes and then detected with an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) and chemiluminescence (Amersham Biosciences). On the initial on-plate screening, we selected 179, 32, 48, and 144 clones from transformants from the VHH, β2-m, Cβ fragment, and γ library, respectively. The candidate clones were cultured for 1 day in natural liquid medium containing 10 g/liter of yeast extract and 20 g/liter of peptone (YPEP) with 2% galactose as the carbon source (20). Each culture medium was subjected to SDS-PAGE under non-reducing conditions. The M2 antibody was used to detect proteins after blotting.

**Physicochemical Measurements of the Selected Mutants—**Isolated DNAs encoding the candidate mutants and the original Ig fold domains were cloned into an E. coli expression vector, pAED4 (21). In addition, several control mutants were generated by PCR-based site-directed mutagenesis. They were expressed in E. coli strain BL21 (DE3) pLysS (Stratagene), and the expressed proteins were accumulated in inclusion bodies. These inclusion bodies were dissolved with 6 M guanidine HCl or 8 M urea against 1% acetic acid and then purified by reversed-phase high performance liquid chromatography (HPLC). For VHIs and γ grafts, proteins in the denaturant were refolded by a 1/50 dilution with 10 mM sodium acetate (pH 5.3). A Resource S cation exchange column (Amersham Biosciences) equilibrated with 10 mM sodium acetate (pH 5.3) was used to purify crude VHIs and γ grafts. For the preparation of reduced proteins, the proteins were incubated with >20 mM dithiothreitol and 6 M guanidine HCl at pH 8.5. After overnight reaction, the midpoint temperatures of thermal unfolding (Tm) and enthalpy values of unfolding (ΔHf) were estimated on the basis of the two-state transition mechanism (Fig. 1). DNA sequencing of the candidates with intense signals revealed that all of these mutants had the Cys:Cys pair at the position of S–S bonds, found in 1, 1, and 232 nm cross-linking at 23 °C. (CL fragment), and 38 (β2-m) libraries from the VHH, β2-m, Cβ fragment, and γ library. (ASApaired(i)) contains only the S–S bonds in the native structure. ASAsingle(i) contains all single S–S bonds in the native structure. ASAmut(i) contains the S–S bond at position j and all single S–S bonds in the native structure. ASAsingle(i) contains all single S–S bonds in the native structure. ASAmut(i) contains the S–S bond at position j and all single S–S bonds in the native structure. ASAmut(i) contains the S–S bond at position j and all single S–S bonds in the native structure. ASAmut(i) contains the S–S bond at position j and all single S–S bonds in the native structure. ASAmut(i) contains the S–S bond at position j and all single S–S bonds in the native structure.

**RESULTS**

**Screening of Stable Ig Fold Mutants without the S–S Bond—**In the first on-plate screening step, we selected 179, 32, 48, and 144 clones from the VHH, β2-m, Cβ fragment, and γ library, based on the secretion levels of target sequences. As a positive control, we placed the transformants with the wild-type sequences on separate areas of the plates. To prevent the false positives on screening, we introduced multi-step selection, in which the first on-plate screening is followed by secondary liquid culture secretion screening (13, 14). In the subsequent liquid culture secretion screening, the supernatants of cultures of the candidates were subjected to SDS-PAGE and immunoblotting. We selected 64 (VHH), 24 (β2-m), 24 (Cβ fragment), and 38 (γ graft) clones that produced bands of substantial intensity (Fig. 1). DNA sequencing of the candidates revealed 18 (VHH), 3 (β2-m), 8 (Cβ fragment), and 11 (γ graft) different amino acid sequences (Fig. 1 and Table I). Those of VHH, Cβ fragment, and γ graft included revertants, which had Cys:Cys at the positions of S–S bonds, found in 1, 1, and 11 clones, respectively. In screening of the Cβ fragment library, the Cys:Val mutation was found in seven clones. As Cys:Val contains cysteine, we did not carry out further analysis of this mutant. In the case of β2-m, we used three different oligo DNAs to introduce random mutations and to avoid the appearance of Cys at the mutated positions. It should be noted that the levels of secretion of the β2-m mutants were higher than in liquid medium, and the false positives were low. In another 20% of the experiments with relatively low reversibility, at least 40% of the CD signals were retained after high temperature. Binding Assay of VHH with Antigen hCG—VHH mutants were immobilized on cyanogen bromide-activated Sepharose (Amersham Biosciences) using the standard protocol supplied by the manufacturer. Aliquots of immobilized beads (20 μl), to which about 10 μg of VHIs had been immobilized, were incubated with 10 μg of hCG (Sigma-Aldrich) in a 20 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl at room temperature for 90 min. After washing the beads three times with the same buffer, samples were boiled for 15 min at 95 °C with standard SDS-PAGE sample buffer containing β-mercaptoethanol and then applied to the 10–20% gradient SDS-polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue.
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The wild-type VHH domain provides a valuable framework for engineering.

**FIG. 1.** Secretion of VHH (A), β₂-m (B), C_L fragment (C), and λ graft mutants (D) into liquid medium. The panels showing the western blots of VHH, C_L fragment, and λ graft mutants were prepared for comparison using selected candidates and false positive clones, which were selected on the first screening but did not show a considerable level of secretion on the second screening. The panel for β₂-m was used to select candidates. The clones with mutants and wild-type sequences were cultured for 1 day in YEP medium with 2% galactose as the carbon source. Each culture medium was subjected to SDS-PAGE under non-reducing conditions. The name of the mutant pair is shown above each lane. CC denotes the wild-type sequence. The secretion levels of the λ graft mutants were very low, thus the supernatant of the wild-type λ graft culture was diluted at 1/5 before electrophoresis.

![Western Blot Images](image)

| Mutant Description | Secretion Levels |
|--------------------|------------------|
| VHH                | High             |
| β₂-m               | Low              |
| C_L fragment       | Low              |
| λ graft            | Very Low         |

**Thermal Unfolding of the Obtained Mutants**—Almost 90% of the selected mutants were unfolded in a cooperative manner, as judged from the thermal transition detected by means of CD, indicating that they retained the ability to form the native structures (Fig. 2). The Trp:Ile G17D mutant of λ graft was not soluble after refolding, and thus, we examined 35 mutants. Among them, 31 showed cooperative thermal unfolding curves. Because ΔH_{U,λ} reflects the steepness of the thermal transition, this value is a good index of the cooperativity of unfolding. The ΔH_{U,λ} values of these mutants were 240–390 kJ/mol for VHH, 240–310 kJ/mol for β₂-m, 180–230 kJ/mol for C_L fragment, and 180–260 kJ/mol for λ graft. The ΔH_{U,λ} values of oxidized wild-type VHH, β₂-m, C_L fragment, and λ graft at their T_m values were 380, 260, 270, and 290 kJ/mol, respectively. Considering the difficulty in estimating their T_m values were 380, 260, 270, and 290 kJ/mol, respectively. Considering the difficulty in estimating their T_m values, the ΔH_{U,λ} values of the mutant and wild-type proteins were similar. In addition, the ΔH_{U,λ} values of mutants were reasonable values as compared with those of typical globular proteins. In the case of λ graft, the observed T_m was about 14 °C higher than the predicted value. This suggests that reduction of the S–S bond in λ graft triggers new and preferable effects, such as hydrogen bond formation or the relaxation of main chain distortion caused by the S–S bond.

Because Val:Ala was not selected on the screening of C_L fragment mutants, although this pair was found on the other three screenings, we prepared a Val:Ala mutant of C_L fragment and compared it with the selected mutants. This comparison revealed a preference, or polarity, for Ala:Val and Val:Ala mutations in C_L fragment. The differences in T_m between the Ala:Val and Val:Ala mutants of VHH, β₂-m, and λ graft were 4, 1, and 1 °C, respectively. On the other hand, the T_m difference between the Ala:Val and Val:Ala mutants for C_L fragment was 8 °C. The occurrence of polarity implies that the pairs substituted for the S–S bonds were determined not only by the structural similarity to the Cys pair but also by the environment around the positions of the replaced Cys residues. For comparison, and because Ala:Ala has often been used to replace S–S bonds (5, 91, 32), we made Ala:Ala mutants for β₂-m, C_L fragment, and λ graft. In the case of C_L fragment, the T_m was >10 °C lower than that of the most stable selected mutant, Ala:Val, indicating that Ala:Ala is not always an appropriate pair to replace an S–S bond. Although Val:Val was selected from β₂-m, C_L fragment, and λ graft libraries, we could not find this pair in the selected VHH mutants. We prepared a Val:Val mutant as a control and compared the stability. The Val:Val mutant of VHH was 2–13 °C less stable than the selected pairs and thus was not selected on the screening.

**Antigen Binding of the Obtained VHH Mutants**—The wild-type VHH used here was the variable domain of a llama heavy chain antibody raised against the α-subunit of hCG. To determine whether S–S bond substitution affects the antigen-binding ability, Sepharose-immobilized VHH variants were prepared and incubated with hCG at room temperature (Fig. 3). The amounts of hCG bound to immobilized Ala:Ala, Ala:Ser, Gly:Val, Ala:Gly:Val, Ala:Val, Trp:Pro G10D, Trp:Ala, Trp:Gly, and Ser:Ser were similar to those of wild-type VHH, indicating that these mutants retain antigen-binding affinity. Ala:Leu, Ala:Phe, Val: Ala, Gly:Gly:Ser:Ala G97V, Gly:Phe, Gly:Ala, and Gly:Leu also bound hCG; however, the amounts of bound hCG were smaller.
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**TABLE I**

Comparison of the thermal stabilities of the obtained mutants and control proteins

| Ig fold domain | Amino acid pair for replacing S–S bond | $T_m$ °C |
|----------------|---------------------------------------|--------|
| VHH            | C/C (oxidized)                        | 61     |
|                | C/C (reduced)                         | 41     |
|                | V/V (ctrl)                            | 32     |
|                | W/A                                  | 45     |
|                | A/A                                  | 42     |
|                | W/P G10D b                           | 42     |
|                | AV                                   | 41     |
|                | W/G                                  | 40     |
|                | S/A G97V                              | 39     |
|                | AI                                   | 39     |
|                | AF                                   | 39     |
|                | GV                                   | 38     |
|                | GL                                   | 38     |
|                | VA                                   | 37     |
|                | AS                                   | 37     |
|                | G/F                                  | 36     |
|                | SS                                   | 36     |
|                | GI                                   | 35     |
|                | GA                                   | 34     |
|                | AL                                   | 34     |
|                | C/L fragment                          |        |
|                | C/C (oxidized)                        | 57     |
|                | C/C (reduced)                         | 32     |
|                | V/A (ctrl)                            | 29     |
|                | A/A (ctrl)                            | 23     |
|                | AV                                   | 37     |
|                | BV                                   | 35     |
|                | VY                                   | 34     |
|                | G/F                                  | 29     |
|                | GL                                   | 26     |
|                | GV                                   | 24     |
|                | C/λ graft                            |        |
|                | C/C (oxidized)                        | 54     |
|                | C/C (reduced)                         | 45     |
|                | A/A (ctrl)                            | 39     |
|                | A/V                                  | 44     |
|                | AV                                   | 40     |
|                | LG                                   | 40     |
|                | V/A                                  | 39     |
|                | F/L W94R                             | 32     |
|                | V/V                                  | Not cooperative |
|                | I/V                                  | Not cooperative |
|                | F/Y                                  | Not cooperative |
|                | W/V                                  | Not cooperative |
|                | W/I V17D Insoluble                    | Insoluble |

*We have represented the amino acid pairs replacing the S–S bonds as one-letter amino acid code combinations, such as AV. In this representation, the first letter indicates the mutated amino acid at the position of the first Cys, which is Cys-22 (VHH), Cys-25 (C/L fragment), or Cys-23 (λ graft). The second amino acid corresponds to the mutation at the position of the second Cys, which is Cys-96 (VHH), Cys-80 (C/λ fragment), or Cys-91 (λ graft). bThe third mutation, G10D, did destabilize the protein. W/P without the third mutation showed a higher $T_m$ (44 °C) than W/P G10D.

DISCUSSION

It is notable that at least two of the pairs Ala:Ala, Ala:Val, Val:Ala, and Val:Val were found in the selected mutants for all four different Ig fold domains and that they were stably folded at 30 °C (Table I and Fig. 2). This suggests that examination of only four mutations, i.e. Ala:Ala, Ala:Val, Val:Ala, and Val:Val, could be enough to obtain natively folded Ig fold domains without S–S bonds. The number of pairs using Ala and/or Val is far smaller than those using all 20 amino acids, reducing the efforts necessary to identify amino acid pairs to replace S–S bonds without disrupting the native structures under physiological conditions. Therefore, our finding makes it easy and practical to replace the S–S bonds of preexisting antibodies.

Removing the S–S bonds from antibodies simplifies their in vitro folding and thus enables their production at low cost using a bacterial expression system. In addition, reducing the number of S–S bonds of antibodies is a key issue for extending their applications, such as the broad use of engineered multivalent antibodies and “intrabodies,” another form of engineered antibody that is a recombinant antibody used to block the function of a target protein within the cell (33–41). A single chain variable fragment of an antibody (scFv), which consists of variable domains of heavy and light chains, comprises a small antibody fragment with antigen-binding ability. scFvs with different antigen specificities can be fused to produce high affinity multivalent antibodies. Although in principle any number of scFvs can be connected, the difficulty in the correct formation of many S–S bonds limits the number of scFvs that can be fused. A known problem of intrabodies is improper folding in the cell caused by the reductive environment of the cytosol, in which the S–S bonds of antibodies are hardly formed (42).

As an S–S bond in an Ig fold domain is critical for its structure and stability (2, 7, 8), replacement of the S–S bond was considered to be challenging. So far, only two artificial pairs (Val:Ala and Ala:Tyr) have been found to be functional replacements for the S–S bonds in Ig fold domains (3, 40, 43, 44). It has also been reported that one of the Cys residues involved in an S–S bond can be replaced by Ala or Tyr without disrupting the native folding curve (45). Thus, it is notable that at least two of the pairs Ala:Ala, Ala:Val, Val:Ala, and Val:Val were found in the selected mutants for all four different Ig fold domains and that they were stably folded at 30 °C, indicating that more than half of the molecules are unfolded at the temperature for yeast culture. The occurrence of these false positives suggests that the level of secretion is determined not only by the structural status and stability but also by other unknown factors.

False Positives on the Cellular Quality Control Screening—

There were some examples that did not satisfy the basic assumption behind our screening method based on a cellular quality control system. For λ graft, we could not observe the cooperative transition for the Val:Val, Ile:Val, Phe:Tyr, and Trp:Val mutants, and Trp:Ile V17D mutant was insoluble after refolding, suggesting that these mutants have non-native or aggregated structures. The Gly:Phe, Gly:Leu, and Gly:Val pairs of C/λ fragment exhibited significant levels of secretion in the second screening (Fig. 1C, lanes 8, 11, and 16) and showed cooperative thermal unfolding curves. However, their $T_m$ values were <30 °C, indicating that more than half of the molecules are unfolded at the temperature for yeast culture. The occurrence of these false positives suggests that the level of secretion is determined not only by the structural status and stability but also by other unknown factors.
The most surprising mutant was Trp:Pro G10D of VHH. Proline is a strong β-sheet breaker (47–49), and Cys-96 of VHH is at the center of the eighth strand. Thus, we first thought that Trp:Pro G10D was a false positive in the screening. However, this mutant bound to the antigen hCG, indicating that the introduction of proline at position 96 did not cause unfolding of VHH. Furthermore, the Tm of this mutant was the second highest among the VHH mutants. Because the third mutation, G10D, would contribute to the stability of this mutant, we prepared a Trp:Pro mutant without the third mutation and found that the G10D mutation rather decreased the Tm by about 2 °C. Thus, the Trp:Pro pair alone is a suitable pair for replacing the S–S bond in VHH.

The pairs able to replace the S–S bonds might be simply explained by the extent to which the cavity caused by removing the cysteines is filled. To test this possibility, we estimated the areas of contact between the mutated residues and the amino acids around the S–S bond (Fig. 4). In addition, we calculated...
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the areas of internal contact between pairs (Fig. 4B). Structural minimization of the introduced pair and the shell formed by the amino acids around the S–S bond was carried out as described under “Experimental Procedures.” As the van der Waals contact is supposed to stabilize the protein structure, an increase in the contact between amino acids should result in an increase in stability. However, there was no clear correlation between stability and the contact surface area. In a cavity filling experiment on T4 lysozyme, introduction of a bulky amino acid into the hydrophobic core caused bond angle distortion and unfavorable van der Waals contacts (50). These unfavorable strains could explain why the amount of the contact area did not correlate with the stability. The lack of correlation observed here indicates that empirical testing is required to identify the suitable pairs for replacing the S–S bonds in Ig fold domains.

Systematic replacement of the S–S bonds of other types of folds has been reported. Pairs that can be substituted for the S–S bond between Cys-14 and Cys-38 of bovine trypsin inhibitor (BPTI) have been searched for using cellular quality control screening (13). In this experiment, nine pairs were obtained, and Gly:Val and Gly:Met A27V showed the highest $T_m$ values. Although Val:Val was also selected on screening, its $T_m$ was about 5 °C lower than those of Gly:Val and Gly:Met A27V.

In the present study, the amino acid pairs using Gly were also selected on screening (Table I). However, in contrast to the S–S bond between Cys-14 and Cys-38 of BPTI, the $T_m$ values of these mutants were 4–13 °C lower than those of the most stable mutants selected. This inconsistency would be a result of the solvent accessibility of the S–S bond replaced, because the S–S bond between Cys-14 and Cys-38 of BPTI is solvent-accessible, and the S–S bonds examined here are buried in the hydrophobic core. Indeed, for the buried S–S bond in BPTI, the $T_m$ values were 4–13 °C higher than those of the other six pairs studied, i.e. Gly:Ala, Thr:Ala, Ser:Ala, Ala:Ser, Ser:Ser, and Gly:Met. Thus, it is possible that four pairs of Ala and/or Val could be substituted for the buried S–S bonds in proteins other than Ig fold domains.

We have demonstrated that cellular quality control screening allows the identification of amino acid pairs for replacing S–S bonds in Ig fold domains. The screening showed a low frequency of false positives; the selected mutants exhibited cooperative thermal unfolding and were folded well at 30 °C, except for the several false positives noted under “Results.” Moreover, the selected VHH mutants showed antigen-binding affinity under our experimental conditions. Our screening system is independent of the functions of proteins and thus can be applied to other proteins with different folds. Therefore, this method is especially useful for comprehensive research on the ability of many different sequences to fold into the native structures.

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Fig. 4. Plots of external (A) or internal (B) contacts against $\Delta T_m$. $\Delta T_m$ is defined as the difference in $T_m$ values between the mutants and the corresponding reduced wild-type protein. Green, red, and blue characters indicate VHH, $\beta_{\text{m}}$, and C$_\text{ll}$ fragment mutants, respectively. In this figure, CC indicates the reduced wild-type protein. External contact indicates the area of contact between mutated amino acids and the residues around them, which was calculated by summation of the $\Delta AS_{\text{mut}}(i,j)$ values for the mutated amino acid pairs as described under “Experimental Procedures.” The internal contact between the mutated pairs is defined as $\Delta AS_{\text{mutpair}}(i,j)$, as described under “Experimental Procedures.” As Ser: Ala 97/1V of VHH had a third mutation, we did not include this mutant in this analysis. The linear correlation coefficients between $\Delta T_m$ of all proteins and contact areas were 0.43 (external contact) and 0.36 (internal contact).
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