SUPPORTING INFORMATION

Removal of a Conserved Disulfide Bond Does Not Compromise Mechanical Stability of a VHH Antibody Complex

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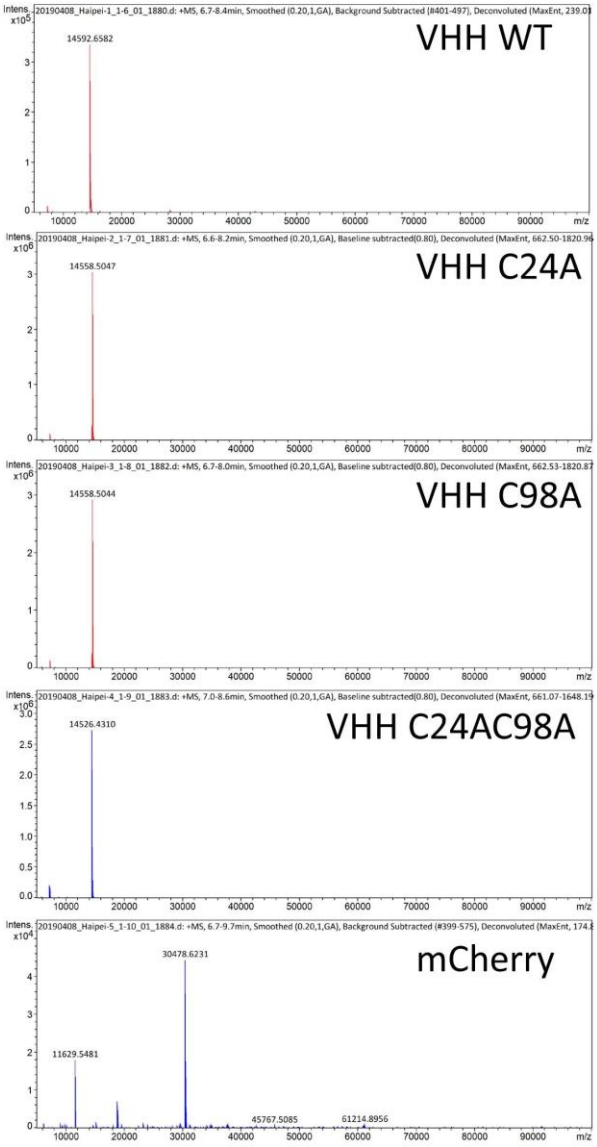
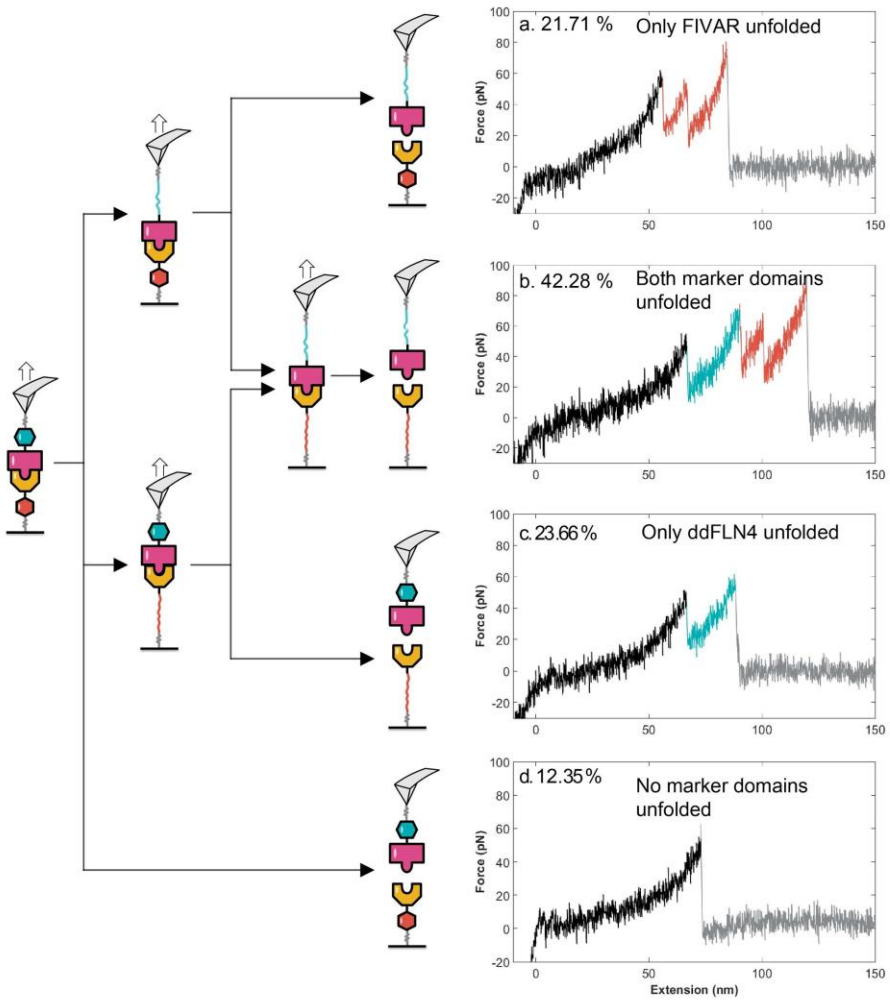


Supplementary Figure 1. Mass spectrometry results on VHH(WT), VHH(C24A), VHH(C98A), VHH(C24AC96A) and mCherry.



Supplementary Figure 2. Representative force *vs.* extension curves showing various unfolding pathways occurring prior to VHH:mCherry complex rupture. Orange, ddFLN4; Blue, FIVAR.

Sample	Condition	Abs @ 412 nm	Free Cys %	Disulfide %
VHH	PBS	0.60	17.5	82.5
	6M GuHCl, PBS	0.37	24.2	75.8
C24A	PBS	1.52	87.7	ND
	6M GuHCl, PBS	0.79	96.3	ND
C98A	PBS	1.65	107.7	ND
	6M GuHCl, PBS	0.81	102.8	ND
C24AC98A	PBS	0.03	ND	ND
	6M GuHCl, PBS	0.01	ND	ND

Supplementary Table 1. Quantification of disulfide bond content in purified VHH(WT) and VHH mutants expressed in *E. coli* SHuffle cells. Phosphate buffered saline (PBS); Guanidinium hydrochloride (GuHCl); Not detected (ND).

Materials and Methods

Materials

Sodium dodecyl sulfate(SDS), N-hydroxysuccinimid(NHS), sodium hydroxide and ethanolamine hydrochloride were purchased from Sigma-Aldrich cooperation (St. Louis, Missouri, USA). The SPR chips (carboxymethyldextran, 200nm) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were ordered from XanTec bioanalytics GmbH (Düsseldorf, Germany).

Cloning of mCherry-FIVAR-His-ybbR and VHH-ddFLN4-His-ybbR

The plasmid pET28a_ybbr-HIS-ddFLN4-8GS-FIVAR-Doc was a gift from Hermann Gaub and Lukas Milles[1] at Ludwig-Maximilians-Universität Munich. A pBAD vector encoding mCherry was obtained from Addgene (plasmid #54630; depositing labs: Michael Davidson, Roger Tsien[2]). The pET28a vector containing the FIVAR marker domains, and a pET28a receiver plasmid containing a previously cloned C-terminal His-ybbR were subjected to Gibson Assembly[3] to build the mCherry-FIVAR-His-ybbr construct. The gene encoding the anti-mCherry VHH domain (LaM-4, Addgene #70696; depositing labs: Kazuhisa Nakayama)[4,5] was cloned with ddFLN4, followed by a C-terminal His-ybbR tag into pET28a vectors again by Gibson Assembly. Gene sequences lacking the ddFLN4 and FIVAR marker domains were made from the AFM genes by full plasmid PCR and PCR product circularization ligation. All sequences were verified by Sanger sequencing.

Site-directed mutagenesis

A Q5 site-directed mutagenesis kit (New England BioLabs) was used to mutate the two cysteines (C24 and C98) in VHH(WT) to alanine. Three constructs were made: VHH(C24A)-ddFLN4, VHH(C98A)-ddFLN4, and VHH(C24A, C98A)-ddFLN4. All mutagenesis products were confirmed by Sanger sequencing.

VHH(C24A)-ddFLN4 was constructed using the following primers:

5'-GCGGCAGCAAGCGGTCGTTTTGCAG-3'

5'-GCTCAGACGCAGACTACCACCAG-3'

VHH(C98A)-ddFLN4 and VHH(C24A, C98A)-ddFLN4 were from starting plasmids VHH(WT) and VHH(C24A), respectively, using the following primers:

5'-AGTGTATTATGCGGCAGCCAATCTGGGTAAC-3'

5'-GCGGTATCATCCGGTTTC-3'

Protein expression and purification

mCherry-FIVAR was expressed in E. coli BL21(DE3) in TB^{kan} medium using 0.5 mM IPTG induction at 37°C for 4 hrs. The VHH(WT) and VHH mutants C24A, C98A and C24A/C98A with and without SMFS marker domains were expressed in *E. coli* SHuffle cells in LB^{kan} medium using 0.4 mM IPTG at 16°C overnight. Cells were lysed by sonication and the His_6-tagged proteins were purified using a His-Trap FF column, followed by desalting using a His-Trap Desalting column on AKTA Pure system followed by size exclusion. Protein concentrations were determined by absorbance at 280 nm.

AFM Sample Preparation

Ybbr tagged proteins were immobilized on functionalized AFM cantilevers and cover glasses according to previously published protocols[6,7]. In brief, AFM cantilevers and coverglasses were first cleaned by UV-ozone treatment for 40 minutes followed by soaking in piranha etching solution and rinsing in water. Next, levers and coverglasses were treated with 3-Aminopropyl (diethoxy) methylsilane (APDMES, ABCR GmbH, Karlsruhe, Germany) to silanize the surface with amine groups. The amine groups were subsequently conjugated to a heterobifunctional NHS-PEG-Mal linker (5 kDa; Rapp Polymere, Tübingen, Germany) in 50 mM HEPES buffer pH 7.5 for 30 min, followed by incubating with Coenzyme A (CoA, 200 µM) in coupling buffer (50mM sodium phosphate, 50mM NaCl, 10mM EDTA, pH 7.2) for 2 hours at room temperature. Finally, the ybbr tagged proteins mCherry-FIVAR and VHH-ddFLN4 were covalently immobilized to the CoA surfaces or cantilevers by a Sfp-catalyzed coupling reaction in PBS measurement buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4, pH 7.4) at room temperature for 2 hours. This resulted in covalent and site-specific surface immobilization through the ybbR tag located at the C-terminus of the marker domains. Functionalized cantilevers and coverglasses were washed extensively and kept in PBS buffer prior to immediate use.

AFM-SMFS measurements and data analysis

Force spectroscopy was conducted using automated AFM-based SMFS (Force Robot 300, JPK Instruments). SMFS data were recorded in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4, pH 7.4), at room temperature and constant pulling speeds of 400 nm/s, 800 nm/s, 1600 nm/s and 3200 nm/s. Force *vs.* extension curves were filtered and analysed using a combination of software available on the AFM instrument and custom python scripts. Date traces

were filtered by searching for contour length increments that matched the characteristics of unfolding of the specific marker domains. FIVAR has a single contour length increment of around 28 nm and ddFLN4 has two contour length increments around 15 nm and 17 nm following a two-step unfolding pathway with an intermediate state. Dynamic force spectroscopy results from different pulling speeds were fitted with Dudko–Hummer–Szabo model with a cusp-like barrier[8,9] to determine values of energy barrier heights ($\Delta G^{\ddagger}$), effective distance to the transition state (Δx) and the intrinsic off-rate (k_{off}).

SPR experiments

SPR measurements were conducted on an SR7500DC instrument (Reichert technologies inc., Depew, New York, USA). Prior to use, the system was first flushed with 0.5% (m/m) SDS solution, followed by glycine (50 mM, pH 9.5) and deionized water. The system was conditioned with immobilization buffer (Acetate buffer, 10 mM, pH 3.9) and a new chip was installed. To activate the carboxylated dextran surface, a freshly prepared solution containing 0.23 M EDC and 0.095 M NHS was injected at 10 µL min^{-1} for 5 minutes. Subsequently, a 20 µM VHH(WT) or VHH mutant solution in immobilization buffer was injected for 20 minutes to immobilize the VHH domain on the chip. Thereafter, the remaining activated carboxylic groups blocked by injecting ethanolamine hydrochloride (1M, pH 8.5) for 5 minutes. The system was then conditioned with PBS prior to the kinetic analysis. To measure binding kinetics, buffer (1x PBS) was injected for 5 min at a flow rate of 30 µL min^{-1}, followed by the analyte mCherry at 30 µL min^{-1} for 2.5 minutes. For dissociation, the running buffer (1x PBS) was perfused at 30 µL min^{-1} for 5 minutes. To completely remove the analyte from the surface, a sodium hydroxide solution (10 mM) was injected at 50 µL min^{-1} to regenerate the surface for 2 minutes. This cycle was repeated six times,

once for each concentration of analyte, starting with low concentrations (see Supplementary Table 2).

Injection number	1	2	3	4	5	6
Concentration [µM]	0.5	1.0	1.5	2.0	2.5	3.0

Supplementary Table 2. Injection concentrations for the SPR experiment.

The raw data of the SPR was extracted, fitted and evaluated in the software package Origin. The dissociation constant (k_{off}) was fitted globally over the curves using Equation 1, which takes the baseline offset R_0 into account. The association constant (k_{on}) was extracted by fitting each curve with Equation 2, where the dissociation constant (k_{off}) was given as a fixed parameter obtained from the fit with equation (1). The 95%-confidence intervals reported for each sample in the main text Table 1 represent the standard error from 6 curves analyzed.

$$f(t) = (R_{max} - R_0) \cdot e^{-\boldsymbol{k_{off}} \cdot t} + R_0 \quad \text{Eq. 1}$$

$$f(t) = \frac{(R_{max} - R_0)[A]}{\left(\frac{k_{off}}{k_{on}}\right) + [A]} \left(1 - e^{-(\boldsymbol{k_{on}}[A] + \boldsymbol{k_{off}}) \cdot \boldsymbol{t}}\right) + R_0 \qquad \text{Eq. 2}$$

Ellman's assay protocol

Protein samples were prepared at 60-120 µM in phosphate buffered saline (PBS) or in PBS containing additionally 6M guanidinium hydrochloride (6M GuHCl) to denature VHH tertiary structure. Samples were then incubated with Ellman's reagent for 15 minutes at room temperature

and the absorbance was measured at 412 nm (Abs @ 412nm). The concentration of free cysteine was determined based on a calibration curve using free cysteine.

Measurement protocol for ITC

The binding affinity between mCherry and VHH mutants were measured using an isothermal titration calorimeter (VP200-ITC system, MicroCal LLC). A 15 μM solution of VHH was transferred into the sample cell (cell volume 1.42 mL). All ITC measurements were conducted at 25 °C. A 130 μM solution of mCherry protein was added to the protein solution by syringe in 1 time 1 μL injection followed by 2 μL injections every 2 minutes. Data were plotted as the power needed to maintain the reference cell and sample cell at the same temperature against time and as kcal/mol of injectant against the molar ratio of ligand and protein. The data from titrations were then analyzed using Orgin7 (OriginLab) and the calculated dissociation constants are summarized in Table 1 in the main text. Each of the 95%-confidence intervals reported in the main text Table 1 represents the fitting error from a single titration curve consisting of 27 injections.

Measurement protocol for nanoDSF

The melting temperatures (T_m) of the samples were measured by nano differential scanning fluorimetry (nanoDSF) using a Prometheus NT.48 system (Nanotemper Technologies) based on a tryptophan fluorescence ratio 350/330 nm. Protein samples were prepared at 0.1 mg/ml in phosphate buffered saline (PBS) before the nano DSF. The protein denaturation curves were determined in a range between 20 °C and 95 °C with a slope of 1 °C/min. T_m were calculated as the inflection point of the denaturation curve by first derivative analysis. Each sample was measured in triplicate and the 95%-confidence intervals are provided for Tm reported in Table 1.

Protein sequences for AFM-SMFS including marker domains:

VHH(WT)-ddFLN4-His-ybbR:

MAQVQLVESGGSLVQPGGSLRLSCAASGRFAESSSMGWFRQAPGKEREFVAAISWSGG

ATNYADSAKGRFTLSRDNTKNTVYLQMNSLKPDDTAVYYCAANLGNYISSNQRLYGY

WGQGTQVTVSSPFTGSGSGSGSAGTGSGADPEKSYAEGPGLDGGESFQPSKFKIHAVDP

DGVHRTDGGDGFVVTIEGPAPVDPVMVDNGDGTYDVEFEPKEAGDYVINLTLDGDNV

NGFPKTVTVKPAPSGHHHHHHGSDSLEFIASKLA

VHH(C24A)-ddFLN4-His-ybbR:

MAQVQLVESGGSLVQPGGSLRLSAAASGRFAESSSMGWFRQAPGKEREFVAAISWSGG

ATNYADSAKGRFTLSRDNTKNTVYLQMNSLKPDDTAVYYCAANLGNYISSNQRLYGY

WGQGTQVTVSSPFTGSGSGSGSAGTGSGADPEKSYAEGPGLDGGESFQPSKFKIHAVDP

DGVHRTDGGDGFVVTIEGPAPVDPVMVDNGDGTYDVEFEPKEAGDYVINLTLDGDNV

NGFPKTVTVKPAPSGHHHHHHGSDSLEFIASKLA

VHH(C98A)-ddFLN4-His-ybbR:

MAQVQLVESGGSLVQPGGSLRLSCAASGRFAESSSMGWFRQAPGKEREFVAAISWSGG

ATNYADSAKGRFTLSRDNTKNTVYLQMNSLKPDDTAVYYAAANLGNYISSNQRLYGY

WGQGTQVTVSSPFTGSGSGSGSAGTGSGADPEKSYAEGPGLDGGESFQPSKFKIHAVDP

DGVHRTDGGDGFVVTIEGPAPVDPVMVDNGDGTYDVEFEPKEAGDYVINLTLDGDNV

NGFPKTVTVKPAPSGHHHHHHGSDSLEFIASKLA

VHH(C24A, C98A)-ddFLN4-His-ybbR:

MAQVQLVESGGSLVQPGGSLRLSAAASGRFAESSSMGWFRQAPGKEREFVAAISWSGG

ATNYADSAKGRFTLSRDNTKNTVYLQMNSLKPDDTAVYYAAANLGNYISSNQRLYGY

WGQGTQVTVSSPFTGSGSGSGSAGTGSGADPEKSYAEGPGLDGGESFQPSKFKIHAVDP

DGVHRTDGGDGFVVTIEGPAPVDPVMVDNGDGTYDVEFEPKEAGDYVINLTLDGDNV

NGFPKTVTVKPAPSGHHHHHHGSDSLEFIASKLA

mCherry-FIVAR-His-ybbR:

MVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGP

LPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDS

SLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKD

GGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMD

ELYKGSGSGSGSDKTNLGELINQGKSLLDESVEGFNVGEYHKGAKDGLTVEINKAEEVF

NKEDATEEEINLAKESLEGAIARFNSLLIEESTGSVVPGWLHHHHHHGSDSLEFIASKLA

Protein sequences for ITC, SPR, and DSF:

VHH(WT)-His:

MAQVQLVESGGSLVQPGGSLRLSCAASGRFAESSSMGWFRQAPGKEREFVAAISWSGG

ATNYADSAKGRFTLSRDNTKNTVYLQMNSLKPDDTAVYYCAANLGNYISSNQRLYGY

WGQGTQVTVSSPFTHHHHHH

VHH(C24A)-His:

MAQVQLVESGGSLVQPGGSLRLSAAASGRFAESSSMGWFRQAPGKEREFVAAISWSGG

ATNYADSAKGRFTLSRDNTKNTVYLQMNSLKPDDTAVYYCAANLGNYISSNQRLYGY

WGQGTQVTVSSPFTHHHHHH

VHH(C98A)-His:

MAQVQLVESGGSLVQPGGSLRLSCAASGRFAESSSMGWFRQAPGKEREFVAAISWSGG

ATNYADSAKGRFTLSRDNTKNTVYLQMNSLKPDDTAVYYAAANLGNYISSNQRLYGY

WGQGTQVTVSSPFTHHHHHH

VHH(C24A, C98A)-His:

MAQVQLVESGGSLVQPGGSLRLSAAASGRFAESSSMGWFRQAPGKEREFVAAISWSGG

ATNYADSAKGRFTLSRDNTKNTVYLQMNSLKPDDTAVYYAAANLGNYISSNQRLYGY

WGQGTQVTVSSPFTHHHHHH

His-mCherry:

HHHHHHGMASMTGGQQMGRDLYDDDDKDPSSSMVSKGEEDNMAIIKEFMRFKVHME

GSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPA

DIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVM

QKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGA

YNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYK

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