Published in final edited form as:

J Mol Biol. 2012 January 13; 415(2): 393–405. doi:10.1016/j.jmb.2011.12.019.

Teaching an old scaffold new tricks: Monobodies constructed using alternative surfaces of the FN3 scaffold

Akiko Koide, John Wojcik, Ryan N. Gilbreth, Robert J. Hoey, and Shohei Koide*

Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637, USA

Abstract

The fibronectin type III domain (FN3) has become one of the most widely used non-antibody scaffolds for generating new binding proteins. Because of its structural homology to the immunoglobulin domain, combinatorial libraries of FN3 designed to date have primarily focused on introducing amino acid diversity into three loops that are equivalent to antibody complementarity-determining regions. Here, we report an FN3 library that utilizes alternative positions for presenting amino acid diversity. We diversified positions on a β -sheet and surface loops that together form a concave surface. The new library produced binding proteins (termed “monobodies”) to multiple target proteins, generally with similar efficacy as the original, loop-focused library. The crystal structure of a monobody generated from the new library in complex with its target, the Abl SH2 domain, revealed that a concave surface of the monobody, as intended in our design, bound to a convex surface of the target with the interface area being among the largest of published structures of monobody-target complexes. This mode of interaction differs from a common binding mode for single-domain antibodies and antibody mimics in which recognition loops recognize clefts in targets. Together, this work illustrates the utilization of different surfaces of a single immunoglobulin-like scaffold to generate binding proteins with distinct characteristics.

Keywords

protein-protein interaction; protein design; antibody mimic; combinatorial library; phage display

INTRODUCTION

Highly specific molecular recognition is a hallmark of protein-ligand interactions. Generating new binding interfaces to diverse target molecules is a major goal of protein engineering and design in both academic and pharmaceutical settings. Among many approaches, those utilizing a molecular scaffold in combination with high-throughput directed evolution techniques have proven highly successful.^{1; 2; 3; 4} A molecular scaffold is a molecule that is capable of presenting diverse amino acid sequences on a contiguous surface that can be used for molecular recognition. Although the immunoglobulins are the most prominent examples of such molecular scaffolds, a number of “alternative” scaffolds

© 2011 Elsevier Ltd. All rights reserved.

*Corresponding author: Shohei Koide, Department of Biochemistry and Molecular Biology, The University of Chicago, 929 East 57th Street, Chicago, IL 60637, U.S.A.; Telephone: 1-773-702-5073; Fax: 1-773-702-0439; skoide@uchicago.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

have been developed using proteins that are not involved in adaptive immunity.^{3; 5} Large combinatorial libraries are constructed in which portions of a scaffold are diversified, and functional molecules are identified from such libraries using molecular display techniques such as phage display and yeast display⁶. Because only a very small portion of the theoretically possible amino acid combinations can be experimentally sampled for a binding interface of typical size (15–20 positions), effective library design requires careful choices of the positions diversified and the amino acid compositions used so as to maximize the likelihood of generating functional molecules.^{4; 7}

Since its development as a molecular scaffold in 1998,⁸ the fibronectin type III domain (FN3) has become the most widely used non-antibody scaffold today.^{9; 10; 11} FN3 is similar in global fold to the immunoglobulin domains (Figure 1A). However, unlike the immunoglobulin domains, the folding of FN3 does not rely on the formation of an intradomain disulfide bond, making both production and intracellular applications straightforward. The structural homology between the FN3 and immunoglobulin domains has inspired the design of a number of FN3 combinatorial libraries in which the FN3 loops that are equivalent to the complementarity determining regions (CDRs) of antibodies are diversified. Numerous target-binding proteins have been generated from libraries of this type.¹¹ The crystal structure of a monobody (a term referring to a FN3-based binding protein) in complex with maltose-binding protein shows that the diversified loop regions indeed form a contiguous surface used for molecular recognition (Fig. 1B).^{12; 13} This mode of binding is analogous to that commonly observed in the camelid single domain antibodies (V_HHs).¹⁴

Although antibody-inspired, loop-based FN3 libraries are effective in producing highly functional monobodies, recent crystal structures have suggested the possibility of alternative design of monobody libraries based on positions distinct from those diversified in libraries reported to date. A surface comprised by a single loop and the face of a β -sheet of the FN3 molecule has been observed to form a binding surface in some cases (Fig. 1C).¹⁵ Interestingly, the monobodies using this “side and loop” mode of interaction to date have all been isolated from libraries in which diversity was introduced only into positions in the three loops at the end of the molecule (Fig. 1D). The use of the side-and-loop interaction mode, instead of the loop-dominated one intended by the library design (Fig. 1B), suggests an inherent suitability of the side and loop surface in mediating protein-protein interactions. Indeed, the FN3 domain family includes many members, and some of them use the surfaces of the β -sheet regions for interacting with other molecules.¹⁶ The observation of these two distinct modes of FN3-target interaction further suggests that alternative library designs focused on the side and loop surface could be more effective than conventional loop-based strategies in generating monobodies to some targets. There are several, well-established systems of non-antibody scaffolds that use non-loop positions for presenting amino acid diversity, including anticalins, affibodies and DARPINs.^{3; 4; 5; 17; 18; 19} In the case of anticalins, different residues were diversified in libraries for protein targets and for small molecules, illustrating the utility of tailoring the location of amino acid diversity.^{20; 21} The successes of these systems demonstrate that one need not use exclusively loop positions for generating a new recognition surface. In this work, we sought to explore the capacity of the side-and-loop surface of the FN3 scaffold for generating new monobodies.

RESULTS

Library Design

The FN3 scaffold has two β -sheets (Figure 1A), one constituted by β -strands A, B and E, and the other by β -strands C, D, F and G. The crystal structure of a monobody in complex with its target, the Abl SH2 domain, revealed extensive interactions made by residues in the

CDFG β -sheet region of the FN3 scaffold that were not diversified in our library (Fig. 1C).¹⁵ Alanine scanning mutagenesis experiments demonstrated the energetic importance of these residues in binding.¹⁵ Similar use of the surface of this β -sheet was observed in a monobody that bound to yeast small ubiquitin-like modifier (ySUMO).²² These observations suggest that it may be possible to construct a target-binding surface that is distinct from the conventional design relied upon in antibody-mimic engineering, i.e. interactions dominated by loops equivalent to the antibody CDRs. Also, the surface of the CDFG β -sheet is slightly concave, suggesting it is suitable for producing recognition surface complementary to convex surfaces found in most globular macromolecules.

To explore the efficacy of such alternative FN3 library designs and how their performance compares with conventional loop-focused FN3 engineering strategies, we constructed two distinct monobody libraries. One library, which we call the side and loop library, utilizes residues in β -strands C (residues 31 and 33) and D (residues 47 and 49) as well as residues in the FG and CD loops (Figs. 1E and 2A) to present a binding surface centered around the β -sheet as described above. The other library, which we call the loop-only library, constitutes the conventional FN3 library design utilizing positions in the BC, DE and FG loops with no residues diversified in the β -sheet regions (Figs 1D and 2B).

In both libraries, we used highly biased amino acid diversity and various loop lengths for the FG loop, as described previously.¹⁵ In the loop-only library, this diversity was also used for positions in the BC loop, which was also varied in length. The DE loop in the loop-only library was fixed in length and diversified only to Tyr or Ser with Gly also included at position 52 (Fig 2B). In the side-and-loop library, we used codons that exclude Pro and Gly, amino acids that are probably detrimental to the structural integrity of the scaffold, for positions in β -strand C, an internal β -strand. For β -strand D, an edge strand, a small subset of amino acids, Ala, Glu, Lys and Thr, was used. Ala and Thr were used so as to avoid large side chains that might prevent target binding, and Glu and Lys were included as negative design elements to prevent aggregation mediated by the formation of an intermolecular β -sheet.²³ We did not diversify Tyr73 with a hope that this Tyr would always contribute to target interaction, as Tyr is highly suitable for making a protein-interaction interface.²⁴ Both libraries were constructed in the phage display format with estimated numbers of independent sequences of 2.0×10^{10} and 1.5×10^{10} , for the loop-only and side-and-loop libraries, respectively.

High-affinity FN3 monobodies from the new libraries

We examined the performance of the new “side and loop” library along with the conventional “loop only” library¹⁵ using three targets, Abl SH2, human small ubiquitin-like modifier 1 (hSUMO1), and green fluorescent protein (GFP). The molecular platform for these libraries was identical, except for the locations of the diversified residues and the two libraries contained similar numbers of independent sequences. For each combination of target and library, we performed the following steps to generate monobodies. We first enriched monobodies from the phage display libraries. We then “shuffled” the N-terminal segment and C-terminal segment among monobody clones in the enriched population for a given target, with a junction in the E strand to create a second-generation library in the yeast surface display format.¹³ The gene shuffling step was incorporated to increase the sequence space beyond that sampled in the starting, phage-display library. Finally, the yeast surface display library was sorted using flow cytometry.

We were able to generate monobodies to all the targets from both the side-and-loop and loop-only libraries. Many monobodies exhibited high affinity with K_d values in the low nM range as measured in the yeast display format (Figs. 2A–2C). As demonstrated previously,^{25; 26} these K_d values from yeast display experiments were in good agreement

with those determined using purified monobody samples and surface plasmon resonance (Fig. 2D). As in previously generated monobodies, residues in the FG loop were mutated in all the monobodies selected from both libraries, suggesting the central importance of the FG loop residues in target recognition. Some of the monobodies originating from the side-and-loop library contained the wild-type CD loop and the D strand, suggesting either that these residues are not involved in target recognition in these monobodies or that substitutions of the wild-type residues did not confer affinity improvement. In contrast, the diversified positions in the C strand were mutated in all of the selected monobodies, and monobodies to different targets exhibited different amino acid sequences (Fig. 2A). When we changed position 33 in the C strand of two Abl SH2 domain-binding monobodies, AS15 and AS27, back to the wild type residue (H33R and Y33R, respectively), their affinity was reduced by 4 and 8 times, respectively (Fig. 2C), although these mutants were slightly more stable than their respective parent proteins (Fig. 3B). Together, these results support the importance of the C strand positions in target binding of monobodies derived from the side-and-loop library.

In order to further assess the functionality of the selected monobodies, we characterized the oligomerization state and stability of several of them (Figure 3). The monobodies from the side-and-loop library were predominantly monomeric as tested using size-exclusion chromatography, and they exhibited cooperative denaturation with the midpoints of transition ($C_{0.5}$) similar to that for the “shaved” FN3 scaffold. The shaved FN3 scaffold contains Ser mutations at most of positions in the BC and FG loops, and accordingly we consider it to be representative of monobodies from the loop-only library. The GS2 monobody was eluted from the size-exclusion chromatography at the volume corresponding to a molecular weight much smaller than expected, suggesting it interacted with the column matrix. Nevertheless, it exhibited a single peak, consistent with a view that it is predominantly monomeric. It was substantially less stable than the rest of monobodies tested here in terms of the $C_{0.5}$ value. These results indicate that most of the monobodies from the side-and-loop library retained structural integrity despite the presence of mutations in the β -sheet segment of the FN3 scaffold.

Crystal structures of monobody-target complexes confirm library designs

In order to characterize how a monobody from the new side-and-loop library recognizes its target, we determined the crystal structure of a monobody isolated from the side and loop library termed SH13 in complex with its target, the SH2 domain of Abl kinase at a resolution of 1.83Å (Figs. 2A and 4A; Table 1). SH13 was among the initial monobody clones generated directly from the phage-display libraries without loop shuffling and yeast display screening. Accordingly, it has low affinity with a K_d value of $\sim 4 \mu\text{M}$. The SH13 monobody maintained the FN3 scaffold structure as evidenced by its minimal deviation from a previously determined monobody structure (C^α RMSD $< 0.7 \text{ \AA}$, excluding mutated residues).^{15; 27} The overall structure of the Abl SH2 domain is likewise in good agreement with a previously published crystal structure of the Abl SH2 domain in complex with another monobody (C^α RMSD $< 0.5 \text{ \AA}$).¹⁵ The phospho-Tyr binding pocket of the SH2 domain contained electron density consistent with a sulfate ion, which was present in the crystallization solution.

In accordance with the design of the side-and-loop library, the SH13 monobody binds to the target chiefly using the side and loop surfaces (Fig. 4A). The mode of interaction observed in the crystal structure is consistent with the epitope mapped using NMR chemical shift perturbation (Fig. 4B). The concave surface presented by the monobody effectively complements a convex surface of the Abl SH2 domain. The total surface area buried at the interaction interface is $\sim 1770 \text{ \AA}^2$, with the SH13 monobody contributing $\sim 880 \text{ \AA}^2$. Notably, of the monobody surface area buried in the interface, $\sim 90\%$ is contributed by residues at

positions that were diversified in the generation of the library. Similarly, out of 21 monobody residues that are within 5 Å of an SH2 atom, 15 were located at positions that were diversified in our library. All but one of these 15 residues are directly involved in target recognition. The extensive contributions of diversified positions to the interface suggest that the library design is effective in concentrating amino acid diversity at positions that are capable of making direct contacts with a target. These characteristics also provide additional support for the utility of this face of the FN3 scaffold for constructing protein-interaction interfaces.

The epitope of the Abl SH2 domain recognized by SH13 is distinct from the phosphopeptide-binding interface that a previously reported monobody recognizes.¹⁵ However, the SH13 epitope is also a known functionally important surface of the SH2 domain. In the context of the full-length Abl kinase, this surface, centered on the α A helix, mediates interactions with the C-lobe of the kinase domain in the inactive conformation. Almost a half (~ 475 Å²) of the epitope for the SH13 monobody is contributed by a linear segment including the entire α A helix and residues immediately adjacent to this helix (Fig. 4B). The concave paratope of the SH13 monobody seems suitable for recognizing the convex surface presented by this helix. It is unlikely that a monobody with a convex paratope shape, typically observed in monobodies with exclusively loop-based binding surfaces, would be able to produce a highly complementary surface to this epitope.

In order to better compare and contrast the structural basis for target recognition in monobodies isolated from the two distinct types of libraries, we determined the crystal structure of the monobody ySMB-9 bound to hSUMO1 at 2.15 Å resolution (Fig. 4C; Table 1). The ySMB-9 monobody was recovered from the same “loop only” phage-display library using a slightly different selection scheme²⁸ and shows close homology to new hSUMO1 monobodies recovered in this study (Fig. 2B). Thus, the structure of the ySMB-9/hSUMO1 complex provides a good example of how “loop only” monobodies recognize their targets. The structure showed that ySMB-9 binds to hSUMO1 in a “head-on” fashion using all three loops to form a contiguous binding surface in precisely the manner envisioned in typical loop-based FN3 library designs (Fig. 4C). This interface buries a total of ~ 1190 Å² with monobody contributing ~ 630 Å². Thus this interface is substantially smaller than the interface between the SH13 monobody and Abl SH2 described above (1770 Å²). The BC, DE and FG loops contribute 54%, 6% and 40% of the total monobody buried surface area respectively with no buried surface contributed by the β -sheet regions of the FN3 scaffold.

The mode of interaction exhibited by the ySMB-9 monobody stands in stark contrast with the “side and loop” surface employed by SH13 in binding to Abl SH2 (Fig. 4A) and is also distinct from that previously observed for a yeast SUMO (ySUMO)-binding monobody, ySMB-1 (Figs. 4C and 4D).²² The monobody ySMB-1 used the FG loop and the wild-type FN3 scaffold to form a side-and-loop mode of interaction similar to that exhibited by SH13. Interestingly, both ySMB-1 and ySMB-9 bind to structurally equivalent, highly conserved epitopes in hSUMO1 and ySUMO, respectively (Figs. 4D and 4E). Thus, this pair of monobodies demonstrates that both the “loop only” and “side and loop” binding modes can be used to successfully recognize essentially the same target surface and further supports the validity of both library design strategies. Furthermore, the epitope recognized by ySMB-9 is flat in shape, demonstrating that, although loop-only binding surfaces tend to have convex shapes that would seem unsuitable for recognizing flat surfaces, it is possible to effectively produce binders to a flat epitope using a loop-only monobody library.

Discussion

In this work we have developed a new type of FN3 monobody library in which positions for amino acid diversification are distinct from those of conventional monobody libraries. The new “side and loop” library is effective in generating high-affinity monobodies. Although the small library sizes with respect to the total number of possible amino acid sequences encoded by design and the small number of targets used in this study preclude us from making definitive conclusions, it appears that the two classes of monobody libraries performed differently against different targets. For GFP, the side library clones had higher affinity than the counterparts from the loop library, but for hSUMO1 the trend was opposite. High-affinity monobodies were obtained from both libraries for Abl SH2. These results suggest that, whereas both libraries are capable of generating monobodies to these diverse targets, the use of two distinct libraries increases the likelihood of generating highly functional monobodies to a broader range of targets.

This work produced several loop-only monobodies with good affinity for hSUMO1 whereas our previous attempt was largely unsuccessful, with a monobody termed ySMB-9 being the only hSUMO1 binder that we recovered with a sub- μ M K_d .²² One notable difference between the two studies is the inclusion of a loop-shuffling step in the present study. The ySMB-9/hSUMO1 crystal structure strongly suggests that residues from all three loops are important for binding in ySMB-9 and the same is likely true for the highly homologous monobodies isolated from the present work (Fig. 2B). Thus, consistent with previous studies,²⁹ loop shuffling expands the sequence space that can be searched and thus increases the probability of generating high-affinity monobodies.

The apparent difference in the functionality of the two libraries can be rationalized on the basis of the crystal structure of a monobody from the new “side and loop” library. This monobody presents a concave paratope (Fig 3A), which is distinctly different from flat or convex paratopes often observed in monobodies from “loop only” libraries (Fig. 4C). The ability of the new library to produce concave paratopes is likely to be useful for generating monobodies toward surfaces involved in protein-protein interaction, as the majority of protein surfaces range from flat to convex in shape. The SH13 structure showed that residues in the β -sheet region of the FN3 scaffold underwent minimal backbone movements upon target binding. Thus, a small entropic penalty incurred by these residues upon binding may favorably contribute to achieving high affinity. In addition to differences in paratope shape, it is possible that the side-and-loop library is capable of creating a larger paratope than the loop only library, which may contribute to the different performance of the two libraries. Whereas it is difficult to calculate the size of paratopes that can be constructed by diverse members of a combinatorial library, the structure of the SH13-Abl SH2 complex has substantially larger interface area than the ySMB-9-hSUMO1 complex (1770 versus 1190 $\text{\AA}^2$). Together, these results clearly illustrate that the single FN3 scaffold can be used to produce diverse types of binding surfaces that collectively are capable of recognizing epitopes with distinct topography. This work therefore expands the utility of the FN3 scaffold for producing synthetic binding interfaces.

The “DCFG” β -sheet of the FN3 scaffold used for constructing a new binding site in this work corresponds structurally to the β -sheet of the immunoglobulin variable domain that mediates heterodimerization between the variable domains of the heavy and light chains.³⁰ Therefore, the immunoglobulin domains utilize this β -sheet surface for specific protein-protein interaction but not for recognizing foreign molecules. A very recent report of the structure of a disulfide-free VL domain in complex with a peptide demonstrates that this β -sheet surface can be utilized for target recognition when the heavy chain is absent.³¹ In the camelid single-domain antibodies (V_H Hs), the equivalent β -sheet contains several

mutations, with respect to the conventional variable domain, that prevent heterodimerization.^{32; 33} Although the paratopes of most camelid V_HHs reported to date are made with the three CDR loops and have convex topography,¹⁴ rare examples of V_HH that use a binding mode equivalent to the “side and loop” mode have been identified.³⁴ These examples suggest that the V_HH scaffold can also be used in the same manner as the FN3 scaffold to generate such “side binders”. The rarity of such V_HH molecules is likely to originate from the manner by which their amino acid diversity is generated in the natural immune system. The gene recombination mechanism underlying the generation of immunoglobulin sequence diversity focuses on the CDRs.³⁵ Consequently, the “side” positions on the β -sheet are not extensively diversified in the natural immune repertoire, limiting the chance of generating “side binder” V_HH molecules.

Whereas monobodies have been viewed as close mimics of antibodies due to their structural similarity, the design of the “side and loop” library represents a departure from this “antibody mimic” mind set. We emphasize that structural characterization of monobody-target complexes was instrumental in identifying the unanticipated mode of monobody-target interactions and the potential utility of the β -sheet surface for target recognition. Unlike immunoglobulin libraries derived from natural sources, monobody libraries are generated using in vitro mutagenesis. Consequently we have full control over the choice of locations for amino acid diversification in a library. This freedom is an obvious and important advantage of synthetic scaffold systems. A similar approach should be effective in identifying distinct surfaces useful for constructing binding interfaces in other scaffolds. Thus this work gives general insights into the design of molecular recognition interfaces.

Materials and Methods

Protein production and modification

Target proteins (Abl SH2, hSUMO1, and GFP) and monobody proteins were produced as His10-tag proteins using the pHFT2 vector,¹³ and purified as previously described.^{22; 36} The hSUMO1 sample used in this work contained the C52A mutation that prevents dimer formation.³⁷ Isotope-enriched samples were prepared as described previously.³⁸ For SPR experiments, the His-tag segment of the targets was cleaved using the TEV protease. For crystallization the His-tag segment was removed from both the targets and monobodies.

Target proteins used for yeast display were biotinylated using EZ-Link NHS-PEG4-Biotin (Thermo Fisher Scientific). We typically incubated 0.3–0.6 mg/ml of a target protein with 60 μ M reagent for 30 min, and quenched the reaction by adding Tris-Cl (pH8) at a final concentration of 0.1 M. Excess biotinylation reagent was removed by dialysis against 20 mM Tris Cl buffer, pH 8 containing 100 mM NaCl and 1 mM EDTA. The level of biotinylation was determined to be ~1 per molecule using MALDI-TOF mass spectroscopy.

Phage and yeast display, library construction and selection

The “loop only” library has been described.¹⁵ The “side and loop” library was constructed using the Kunkel mutagenesis method as described previously.^{9; 39} Phage display selection was performed according to the methods previously described.^{7; 36} The His-tagged target proteins were incubated with equimolar concentration of BTtrisNTA, a high affinity Ni-NTA compound containing a biotin moiety, for 30 min to form a BTtrisNTA/his-tagged protein complex, and the complex was incubated with monobody phage-display libraries. The target concentrations used for rounds 1, 2 and 3 were 100, 100 and 50 nM for Abl SH2, 100, 50 and 50 nM for GFP, respectively, and 100 nM throughout for hSUMO1. Monobody-displaying phages bound to the BTtrisNTA/target complexes were captured using Streptavidin (SAV)-coated magnetic beads. The captured phages were eluted with 10 mM

EDTA solution that disrupts the linkage between the targets and BTrisNTA. The recovered phages were amplified in the presence of 0.2 mM IPTG to induce the expression of monobody-p3 fusion genes.

After three rounds of the phage-display library selection, we transferred the genes of selected monobodies to a yeast-display vector to make yeast libraries, using homologous recombination in yeast.⁴⁰ We also incorporated gene shuffling during the construction of yeast-display libraries as follows. We prepared a linearized yeast display vector, pGalAgaCamR,²⁶ using NcoI and XhoI digestion. We amplified monobody gene segments respectively encoding residues 1–74 and those for residues 54–94 separately using PCR from the enriched pool after the phage selection. We then transformed yeast strain EBY100 using a mixture of the three DNA fragments. Correctly recombined clones contained the fusion gene for Aga2-monobody-V5 tag. The transformants were selected in tryptophan-deficient media and Aga2-monobody fusion protein was expressed as previously described.^{13; 25}

The yeast display libraries were sorted using 30 nM biotinylated Abl-SH2, 10 nM biotinylated hSUMO1, and 3 nM biotinylated GFP as described previously.¹³ The surface-displayed monobodies were detected with anti-V5 antibody (Sigma). We used neutravidin (NAV)-PE (Invitrogen) or SAV-PE (Invitrogen) and Alexa Fluor® 647-conjugated chicken anti-rabbit IgG (Invitrogen) as the secondary detection reagents for biotinylated protein and anti V5 antibody, respectively. A total of two rounds of library sorting were performed for Abl SH2 and hSUMO1, and one round for GFP.

Affinity measurements using yeast display

Individual clones from sorted libraries were isolated on agar plates and grown in liquid media as described previously.^{13; 25} Fifty thousand yeast cells for each clone were incubated with various concentrations of biotinylated target in the final volume of 20 μ l in the BSS buffer (50 mM Tris Cl, 150 mM NaCl, pH 8, 1 mg/ml BSA) in the wells of a polypropylene 96-well plate (Greiner 650201) on ice for 30 min with shaking. The wells of a 96-well filter plate (MultiScreenHTS HV, 0.45 μ m pore size; Millipore) were washed by adding 100 μ l BSS and then removing the liquid by applying a vacuum. The cell suspensions from the binding reactions were transferred to the washed wells of the 96-well filter plate. The binding solution was removed by vacuum filtration. The yeast cells in the wells were washed with 100 μ l of BSST (the BSS buffer containing 0.1% Tween 20) twice in the same manner. Next, 20 μ l of 10 μ g/ml NAV-PE (Invitrogen) in BSS was added to each of the wells. After incubation on ice with shaking for 30min, the cells were washed with BSST once. The cells were suspended in 300 μ l BSS and analyzed using a Guava EasyCyte 6/L flow cytometer (Millipore). The K_d values were determined from plots of the mean PE fluorescence intensity versus target concentration by fitting the 1:1 binding model using the KaleidaGraph program (Synergy Software).

Surface plasmon resonance

SPR measurements were carried out on Biacore 2000 and 3000 instruments. For kinetic experiments, Abl SH2 was immobilized on a CM5 chip using amine coupling following methods provided by the manufacturer. Monobodies at varying concentrations were flowed over the surface at a rate of 100 μ l/min and the binding signal was recorded. Quintuplicate data sets were processed and fit with a bimolecular model including mass transport using the Scrubber2 program (BioLogic Software, Campbell, Australia). The presence of mass transport was confirmed using varying flow rates. GS2 kinetic measurements were performed by immobilization of monobody on a NTA chip and flowing over varying concentrations of GFP at 30 μ l/min. Equilibrium experiments were performed as described

previously.²² Duplicate data sets were processed in Scrubber2 and saturation curves were fit with a 1:1 binding model using the Origin software (OriginLab, Northampton, MA).

Size-exclusion chromatography

Proteins were analyzed using a Superdex 75 5/150 GL column (GE Healthcare) in 20mM Tris HCl buffer pH 8.0 containing 150mM NaCl, and 1mM EDTA.

Stability measurements

Chemical denaturation reactions of monobodies were monitored using Trp fluorescence emission using a fluorometer equipped with an automated titrator as described previously.^{8; 41} Because the monobodies contained different numbers of aromatic amino acids, a different emission wavelength was chosen for each monobody that gave a good transition curve. The transition curves were fitted with the two-state unfolding model.^{7; 41}

Crystallization and structure determination

The SH13/Abl SH2 domain complex was purified with a Superdex 75 column (GE Lifesciences) and concentrated to ~10 mg/ml and crystallized in 0.2M Magnesium chloride, 0.1M Bis-Tris Cl pH 5.5 and 25% PEG 3350 at 19 °C by the hanging-drop vapor-diffusion method. The ySMB-9 and hSUMO1 proteins were mixed in a 1:1 molar ratio, concentrated to a total protein concentration of ~10 mg/mL and dissolved in 10 mM Tris HCl, 50 mM NaCl, pH 8.0. The complex was crystallized in 24% PEG-8000, 0.1 M Imidazole, pH = 8.0 at 19 °C using the hanging drop vapor diffusion method. Crystals were frozen in a mixture of 80% mother liquor and 20% glycerol as a cryoprotectant.

X-ray diffraction data were collected at the Advanced Photon Source beamlines (Argonne National Laboratory). Crystal and data collection information are reported in Table 1. X-ray diffraction data were processed and scaled with HKL2000.⁴² The SH13/Abl SH2 structure was determined by molecular replacement using Phaser in the CCP4 program suite.^{43; 44} A multicopy search was performed with the Abl SH2 domain and the FN3 scaffold, without the loop regions, as the search models (PDB IDs 2ABL and 1FNA, respectively). Simulated annealing, energy-minimization, B-factor refinement and map building were carried out using CNS.^{45; 46} The ySMB-9/hSUMO1 structure was determined by molecular replacement using sequential search with two different models with the program MOLREP in CCP4.⁴³ The hSUMO1 structure (residues 20–92 of chain B PDB ID code 1Z5S) was used as a search model, along with the FN3 structure with the variable loop regions deleted (PDB ID code 1FNA).^{47; 48} For both structures, model building and the search for water molecules was carried out using the Coot program.⁴⁹ TLS (Translation/Libration/Screw) and bulk solvent parameters, restrained temperature factor and final positional refinement were completed with REFMAC5.⁵⁰ Molecular graphics were generated using PyMOL (www.pymol.org). Surface area calculations were performed using the PROTORG protein protein interaction server.⁵¹

NMR spectroscopy

The following suite of spectra were taken on a uniformly ¹³C/¹⁵N enriched Abl SH2 domain (~200 μM) in 10 mM sodium phosphate buffer, pH 7.4 containing 150 mM NaCl, 50 μM EDTA and 0.005% sodium azide prepared in 90% H₂O and 10% D₂O, using a Varian (Palo Alto, CA) INOVA 600 NMR spectrometer equipped with a cryogenic probe using pulse sequences provided by the manufacturer: ¹H, ¹⁵N-HSQC, HNCO, CBCACONH, HNCACB, CCONH, HN(CA)CO. NMR data were processed and analyzed using NMRPipe and NMRView software.^{52; 53} Resonance assignments were obtained using the PINE server⁵⁴ and verified by visual inspection in NMR view. For epitope mapping, the ¹H, ¹⁵N-HSQC

spectra of the ^{15}N enriched Abl SH2 domain ($\sim 60\ \mu\text{M}$) in the absence and presence of 1.25 fold molar excess of unlabeled SH13 monobody were recorded. The ^1H , ^{15}N -HSQC cross peaks were classified according to the degree of migration upon SH13 binding as described previously.¹³

Accession numbers

Coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers, 3UYO and 3RZW.

Acknowledgments

This work was supported by the National Institutes of Health (NIH) grants R01-GM072688 and R01-GM090324 to SK and by the University of Chicago Comprehensive Cancer Center. JW was supported in part by the NIH grant 5T32GM07281-33. Structural characterization of the SH13/Abl SH2 complex is based upon research conducted at the Advanced Photon Source on the Northeastern Collaborative Access Team beamlines, which are supported by award RR-15301 from the National Center for Research Resources at the National Institutes of Health. The ySMB-9/hSUMO1 structure was determined using data collected at the Advanced Photon Source LS-CAT Sector 21 which is supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor for the support of this research program (Grant 085P1000817). Use of the Advanced Photon Source is supported by the U.S. Department of Energy, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357.

References

1. Sidhu SS, Fellouse FA. Synthetic therapeutic antibodies. *Nat Chem Biol.* 2006; 2:682–8. [PubMed: 17108986]
2. Binz HK, Pluckthun A. Engineered proteins as specific binding reagents. *Curr Opin Biotechnol.* 2005; 16:459–69. [PubMed: 16005204]
3. Skerra A. Alternative non-antibody scaffolds for molecular recognition. *Curr Opin Biotechnol.* 2007; 18:295–304. [PubMed: 17643280]
4. Koide, S. Design and engineering of synthetic binding proteins using nonantibody scaffolds. In: Park, SJ.; Cochran, JR., editors. *Protein Engineering and Design*. CRC Press; Boca Raton, FL: 2010. p. 109-130.
5. Binz HK, Amstutz P, Pluckthun A. Engineering novel binding proteins from nonimmunoglobulin domains. *Nat Biotechnol.* 2005; 23:1257–68. [PubMed: 16211069]
6. Pluckthun A, Mayo SL. The design of evolution and the evolution of design. *Curr Opin Struct Biol.* 2007; 17:451–3.
7. Fellouse FA, Esaki K, Birtalan S, Raptis D, Cancasci VJ, Koide A, Jhurani P, Vasser M, Wiesmann C, Kossiakoff AA, Koide S, Sidhu SS. High-throughput Generation of Synthetic Antibodies from Highly Functional Minimalist Phage-displayed Libraries. *J Mol Biol.* 2007; 373:924–940. [PubMed: 17825836]
8. Koide A, Bailey CW, Huang X, Koide S. The fibronectin type III domain as a scaffold for novel binding proteins. *J Mol Biol.* 1998; 284:1141–1151. [PubMed: 9837732]
9. Koide A, Koide S. Monobodies: antibody mimics based on the scaffold of the fibronectin type III domain. *Methods Mol Biol.* 2007; 352:95–109. [PubMed: 17041261]
10. Bloom L, Calabro V. FN3: a new protein scaffold reaches the clinic. *Drug Discov Today.* 2009; 14:949–55. [PubMed: 19576999]
11. Lipovsek D. Adnectins: engineered target-binding protein therapeutics. *Protein Eng Des Sel.* 2011; 24:3–9. [PubMed: 21068165]
12. Gilbreth RN, Esaki K, Koide A, Sidhu SS, Koide S. A dominant conformational role for amino acid diversity in minimalist protein-protein interfaces. *J Mol Biol.* 2008; 381:407–418. [PubMed: 18602117]
13. Koide A, Gilbreth RN, Esaki K, Tereshko V, Koide S. High-affinity single-domain binding proteins with a binary-code interface. *Proc Natl Acad Sci U S A.* 2007; 104:6632–7. [PubMed: 17420456]

14. De Genst E, Silence K, Decanniere K, Conrath K, Loris R, Kinne J, Muyldermans S, Wyns L. Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. *Proc Natl Acad Sci U S A*. 2006; 103:4586–91. [PubMed: 16537393]
15. Wojcik J, Hantschel O, Grebien F, Kaupe I, Bennett KL, Barkinge J, Jones RB, Koide A, Superti-Furga G, Koide S. A potent and highly specific FN3 monobody inhibitor of the Abl SH2 domain. *Nat Struct Mol Biol*. 2010; 17:519–27. [PubMed: 20357770]
16. Kavran JM, Ward MD, Oladosu OO, Mulepati S, Leahy DJ. All mammalian Hedgehog proteins interact with cell adhesion molecule, down-regulated by oncogenes (CDO) and brother of CDO (BOC) in a conserved manner. *J Biol Chem*. 2010; 285:24584–90. [PubMed: 20519495]
17. Nord K, Gunneriusson E, Ringdahl J, Stahl S, Uhlen M, Nygren PA. Binding proteins selected from combinatorial libraries of an alpha-helical bacterial receptor domain. *Nat Biotechnol*. 1997; 15:772–777. [PubMed: 9255793]
18. Beste G, Schmidt FS, Stibora T, Skerra A. Small antibody-like proteins with prescribed ligand specificities derived from the lipocalin fold. *Proc Natl Acad Sci U S A*. 1999; 96:1898–903. [PubMed: 10051566]
19. Binz HK, Amstutz P, Kohl A, Stumpp MT, Briand C, Forrer P, Grutter MG, Pluckthun A. High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat Biotechnol*. 2004; 22:575–82. [PubMed: 15097997]
20. Korndorfer IP, Schlehuber S, Skerra A. Structural mechanism of specific ligand recognition by a lipocalin tailored for the complexation of digoxigenin. *J Mol Biol*. 2003; 330:385–96. [PubMed: 12823976]
21. Vogt M, Skerra A. Construction of an artificial receptor protein (“anticalin”) based on the human apolipoprotein D. *Chembiochem*. 2004; 5:191–9. [PubMed: 14760740]
22. Gilbreth RN, Truong K, Madu I, Koide A, Wojcik JB, Li NS, Piccirilli JA, Chen Y, Koide S. Isoform-specific monobody inhibitors of small ubiquitin-related modifiers engineered using structure-guided library design. *Proc Natl Acad Sci U S A*. 2011; 108:7751–7756. [PubMed: 21518904]
23. Richardson JS, Richardson DC. Natural beta-sheet proteins use negative design to avoid edge-to-edge aggregation. *Proc Natl Acad Sci U S A*. 2002; 99:2754–9. [PubMed: 11880627]
24. Koide S, Sidhu SS. The importance of being tyrosine: lessons in molecular recognition from minimalist synthetic binding proteins. *ACS Chem Biol*. 2009; 4:325–34. [PubMed: 19298050]
25. Boder ET, Wittrup KD. Yeast surface display for directed evolution of protein expression, affinity, and stability. *Methods Enzymol*. 2000; 328:430–44. [PubMed: 11075358]
26. Koide A, Tereshko V, Uysal S, Margalef K, Kossiakoff AA, Koide S. Exploring the capacity of minimalist protein interfaces: interface energetics and affinity maturation to picomolar KD of a single-domain antibody with a flat paratope. *J Mol Biol*. 2007; 373:941–53. [PubMed: 17888451]
27. Gilbreth RN, Esaki K, Koide A, Sidhu SS, Koide S. A dominant conformational role for amino acid diversity in minimalist protein-protein interfaces. *J Mol Biol*. 2008; 381:407–18. [PubMed: 18602117]
28. Hogrefe HH, Mullinax RL, Lovejoy AE, Hay BH, Sorge JA. A bacteriophage lambda vector for the cloning and expression of immunoglobulin Fab fragments on the surface of filamentous phage. *Gene*. 1993; 128:119–126. [PubMed: 8508952]
29. Hackel BJ, Kapila A, Wittrup KD. Picomolar affinity fibronectin domains engineered utilizing loop length diversity, recursive mutagenesis, and loop shuffling. *J Mol Biol*. 2008; 381:1238–52. [PubMed: 18602401]
30. Amzel LM, Poljak RJ. Three-dimensional structure of immunoglobulins. *Annu Rev Biochem*. 1979; 48:961–97. [PubMed: 89832]
31. Schiefner A, Chatwell L, Korner J, Neumaier I, Colby DW, Volkmer R, Wittrup KD, Skerra A. A Disulfide-Free Single-Domain V(L) Intrabody with Blocking Activity towards Huntingtin Reveals a Novel Mode of Epitope Recognition. *J Mol Biol*. 2011; 10.1016/j.jmb.2011.09.034
32. Desmyter A, Transue TR, Ghahroudi MA, Thi MH, Poortmans F, Hamers R, Muyldermans S, Wyns L. Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme. *Nat Struct Biol*. 1996; 3:803–11. [PubMed: 8784355]

33. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R. Naturally occurring antibodies devoid of light chains. *Nature*. 1993; 363:446–8. [PubMed: 8502296]
34. Kirchhofer A, Helma J, Schmidthals K, Frauer C, Cui S, Karcher A, Pellis M, Muyldermans S, Casas-Delucchi CS, Cardoso MC, Leonhardt H, Hopfner KP, Rothbauer U. Modulation of protein properties in living cells using nanobodies. *Nat Struct Mol Biol*. 2010; 17:133–8. [PubMed: 20010839]
35. Wu TT, Johnson G, Kabat EA. Length distribution of CDRH3 in antibodies. *Proteins: Struct Funct Genet*. 1993; 16:1–7. [PubMed: 8497480]
36. Koide A, Wojcik J, Gilbreth RN, Reichel A, Piehler J, Koide S. Accelerating phage-display library selection by reversible and site-specific biotinylation. *Protein Eng Des Sel*. 2009; 22:685–90. [PubMed: 19737805]
37. Tatham MH, Jaffray E, Vaughan OA, Desterro JM, Botting CH, Naismith JH, Hay RT. Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem*. 2001; 276:35368–74. [PubMed: 11451954]
38. Pham TN, Koide S. NMR studies of *Borrelia burgdorferi* OspA, a 28 kDa protein containing a single-layer β -sheet. *J Biomol NMR*. 1998; 11:407–414. [PubMed: 9691284]
39. Sidhu SS, Lowman HB, Cunningham BC, Wells JA. Phage display for selection of novel binding peptides. *Methods Enzymol*. 2000; 328:333–63. [PubMed: 11075354]
40. Swers JS, Kellogg BA, Wittrup KD. Shuffled antibody libraries created by in vivo homologous recombination and yeast surface display. *Nucleic Acids Res*. 2004; 32:e36. [PubMed: 14978223]
41. Koide A, Jordan MR, Horner SR, Batori V, Koide S. Stabilization of a fibronectin type III domain by the removal of unfavorable electrostatic interactions on the protein surface. *Biochemistry*. 2001; 40:10326–33. [PubMed: 11513611]
42. Otwinowski Z, Minor W. Processing of X-ray Diffraction Data Collected in Oscillation Mode. *Methods in Enz*. 1997; 276:307–326.
43. Collaborative_Computational_Project. The CCP4 Suite: Programs for Protein Crystallography. *Acta Cryst*. 1994; D50:760–763.
44. Potterton E, Briggs P, Turkenburg M, Dodson E. A graphical user interface to the CCP4 program suite. *Acta Crystallogr D Biol Crystallogr*. 2003; 59:1131–7. [PubMed: 12832755]
45. Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr*. 1998; 54:905–921. [PubMed: 9757107]
46. Brunger AT. Version 1.2 of the Crystallography and NMR system. *Nature protocols*. 2007; 2:2728–33.
47. Dickinson CD, Veerapandian B, Dai X-P, Hamlin RC, Xuong N-H, Ruoslahti E, Ely KR. Crystal structure of the tenth type III cell adhesion module of human fibronectin. *J Mol Biol*. 1994; 236:1079–1092. [PubMed: 8120888]
48. Reverter D, Lima CD. Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. *Nature*. 2005; 435:687–92. [PubMed: 15931224]
49. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr*. 2004; 60:2126–32. [PubMed: 15572765]
50. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr*. 1997; 53:240–55. [PubMed: 15299926]
51. Reynolds C, Damerell D, Jones S. ProtorP: a protein-protein interaction analysis server. *Bioinformatics*. 2009; 25:413–4. [PubMed: 19001476]
52. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6:277–293. [PubMed: 8520220]
53. Johnson BA, Blevins RA. NMRView: A computer program for the visualization and analysis of NMR data. *J Biomol NMR*. 1994; 4:603–614.

54. Bahrami A, Assadi AH, Markley JL, Eghbalnia HR. Probabilistic interaction network of evidence algorithm and its application to complete labeling of peak lists from protein NMR spectroscopy. *PLoS Comput Biol.* 2009; 5:e1000307. [PubMed: 19282963]

Highlights

- FN3 is a widely used scaffold for constructing synthetic binding proteins.
- Combinatorial libraries of FN3 reported to date only diversified residues in loops.
- We report a new library design that diversifies residues in β -sheet regions.
- The new library is effective in generating high-affinity binding proteins.

Figure 1.

Monobody library design. (A) A comparison of the V_HH scaffold (left) and the FN3 scaffold (right). The two β-sheet regions are colored in cyan and blue, respectively. The CDR regions of the V_HH and the corresponding loops in FN3 are colored and labeled. The β-strands of FN3 are labeled with A–G. (B) The structure of a monobody bound to its target, maltose-binding protein.¹² The monobody is depicted in the same manner as in A. Only a portion of maltose-binding protein is shown as a surface model. (C) The structure of a monobody bound to the Abl SH2 domain depicted as in B.¹⁵ (D) The locations of diversified residues in the “loop only” library shown as spheres on the FN3 structure. (E) The locations of diversified residues in the “side and loop” library.

Figure 2.

Monobody library designs and generated clones. Amino acid sequences of monobodies generated from the new “side and loop” library (A) and the “loop only” library (B). “X” denotes a mixture of 30% Tyr, 15% Ser, 10% Gly, 5% Phe, 5% Trp and 2.5% each of all the other amino acids except for Cys; “B”, a mixture of Gly, Ser and Tyr; “J”, a mixture of Ser and Tyr; “O”, a mixture of Asn, Asp, His, Ile, Leu, Phe, Tyr and Val; “U”, a mixture of His, Leu, Phe and Tyr; “Z”, a mixture of Ala, Glu, Lys and Thr. (C) Binding measurements by yeast surface display of representative monobodies. The mean fluorescence intensities of yeast cells displaying a monobody are plotted as a function of the concentration of the target as indicated in panel A. The errors indicated are the standard deviations from curve fitting of the 1:1 binding model. A portion of data for point mutants of As15 and As27 is shown for clarity. (D) SPR sensorgrams for target binding of representative monobodies. The thin lines show the best global fit of the 1:1 binding model. The insets show dose-dependence analysis of the sensorgrams (black) and the best fit of the 1:1 binding model (gray). The errors indicated are the standard deviation of the global fits from at least triplicate data sets for the kinetic experiments, or the standard deviation from global curve fitting of duplicate data sets for the equilibrium experiments.

Figure 3.

Oligomerization state and stability of monobodies. **(A)** Size-exclusion chromatograms of monobodies. The chromatograms are shown with vertical offsets for clarity. The labels show the identities of analyzed samples. The void volume (V_0) and elution positions for bovine serum albumin (BSA; 67 kDa) and ribonuclease A (RNaseA; 13.7 kDa) are indicated with the arrows. The monobodies have an average molecular weight of ~14 kDa. The GS2 monobody is exhibited a monodispersed peak but appeared to interact with the chromatography media, resulting in delayed elution. **(B)** Fluorescence emission intensity of monobodies plotted as a function of guanidine thiocyanate concentration. The emission wavelengths that gave good transitions were different for different monobodies, and they are indicated on the vertical axis. The curves are the best fit of the two-state transition model.

The vertical scales of the data for AS15[H33R] and AS27[Y33R] have been adjusted so that their native and denatured baselines coincide with the counterparts of their respective parent proteins. The denaturant concentration at the mid point of the denaturation transition ($C_{0.5}$) is shown for each protein. The errors are the standard deviations from curve fitting of the two-state model.

Figure 4.

The crystal structures of monobodies originating from the two libraries. The structures are shown with the monobodies in similar orientations. (A) The structure of the SH13 monobody bound to the Abl SH2 domain depicted as in Fig. 1C. (B) NMR-based epitope mapping of the SH13/Abl SH2 complex. The red, yellow and gray spheres show residues of Abl SH2 whose amide resonances were strongly affected (shift of >1.5 peak width), weakly affected (shift of 0.5–1.5 peak width) and minimally affected (shift of <0.5 peak width) by monobody binding, respectively. (C) The crystal structures of the ySMB-1/ySUMO complex (left) and ySMB-9/hSUMO1 complex (right). (D) The two SUMO-binding monobodies bound to equivalent epitopes on the targets using distinct modes. The top panel shows a comparison of the two crystal structures shown in C with ySUMO and hSUMO1 superimposed. The bottom panel shows ySUMO and hSUMO1 in equivalent orientations with the epitopes for the indicated monobodies highlighted in green and blue, respectively. Epitope residues are defined as those containing at least one non-hydrogen atom within 5 Å of a non-hydrogen atom of the bound monobody in the crystal structures. (E) Alignment of portions of the ySUMO (top) and hSUMO1 (bottom) sequences indicating epitopes for ySMB-1 and ySMB-9, respectively. Residues in the ySMB-1 epitope are shaded in blue, and residues in the ySMB-9 epitope in green. Residue numbering is according to the ySUMO sequence.

Table 1

Data collection and refinement statistics (molecular replacement)

	Monobody SH13/Abl1 SH2 complex (3UYO)	Monobody ySMB9/hSUMO1 complex (3RZW)
Data collection*		
Space group	P2 ₁ 2 ₁ 2	C222 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	65.55, 49.18, 60.95	93.35, 97.83, 96.58
α, β, γ (°)	90, 90, 90	90, 90, 90
Beamline	APS 24 ID-E	APS 21 ID-F
Wavelength (Å)	0.97917	0.97872
Resolution (Å)	1.83 (1.90-1.83)	2.15 (2.19-2.15)
<i>R</i> _{sym} or <i>R</i> _{merge}	11.5 (52.9)	8.2 (52.2)
<i>I</i> / σ <i>I</i>	24.4 (2.6)	17.9 (2.1)
Completeness (%)	87.4 (83.0)	98.2 (96.6)
Redundancy	4.0 (3.1)	6.7 (6.0)
Refinement		
Resolution (Å)	1.83	2.15
No. reflections	15737	22,699
<i>R</i> _{work} [‡] / <i>R</i> _{free} [§]	0.188/0.237	0.186/0.237
No. atoms	1753	2,807
Protein	1614	2,657
Ligand/ion	5	12
Water	134	138
<i>B</i> -factors		
Protein	25.6	29.51
Ligand/ion	37.4	64.29
Water	33.3	32.24
R.m.s. deviations		
Bond lengths (Å)	0.012	0.019
Bond angles (°)	1.337	1.828
Ramachandran values	98.5% favored 1.5% allowed 0% outliers	97.2% favored 2.2% allowed 0.6% outliers

APS, Advanced Photon Source.

* Values for highest resolution shell shown in parentheses

[‡] $R_{\text{merge}} = \sum_{\text{hkl}} \sum_i |I(\text{hkl})_i - \langle I(\text{hkl}) \rangle| / \sum_{\text{hkl}} \sum_i \langle I(\text{hkl}) \rangle$ over *i* observations of a reflection *hkl*.[‡] $R = \sum | |F(\text{obs})| - |F(\text{calc})| | / \sum |F(\text{obs})|$.[§] *R*_{free} is *R* with 5% of reflections sequestered before refinement.