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Crystal structure of streptavidin mutant with low immunogenicity

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We previously created a low-immunogenic core streptavidin mutant No. 314 (LISA-314) by replacing six amino-acid residues for use as a delivery tool for an antibody multistep pre-targeting process (Yumura et al., Protein Sci., 22, 213–221, 2013). Here, we performed high-resolution X-ray structural analyses of LISA-314 and wild-type streptavidin to investigate the effect of substitutions on the protein function and the three-dimensional structure. LISA-314 forms a tetramer in the same manner as wild-type streptavidin. The binding mode of D-biotin in LISA-314 is also completely identical to that in wild-type streptavidin, and conformational changes were observed mostly at the side chains of substituted sites. Any large conformational changes corresponding to the reduction of *B* factors around the substituted sites were not observed. These results demonstrated the LISA-314 acquired low immunogenicity without losing structural properties of original wild-type streptavidin.

[**Key words:** Streptavidin; Low immunogenicity; Pre-targeting; Protein engineering; Crystal structure; X-ray structural analysis; *B* factor]

Cancer is the leading cause of death in economically developed countries and the second in developing countries. Worldwide, cancer accounts for 7.6 million deaths, and this is predicted to increase, with an estimated 13.1 million cancer-related deaths in 2030 (1). Development of innovative cancer therapies is therefore of utmost importance.

Targeted therapies such as antibody-drug conjugates, which treat cancer by targeting cancer-specific molecules, have been attracting much attention recently as they result in fewer side effects compared with standard chemotherapy (2,3). Pre-targeting drug delivery systems are expected to be especially beneficial by increasing both bio-imaging sensitivity and therapeutic effect over conventional systems, in which fluorescent markers for imaging or radioisotopes for treatment are directly attached to antibodies (4–6). In the pre-targeting method, treatment is first performed with antibody vehicle, followed by administration of fluorescent marker or radioisotope, displaying more favorable tumor-targeting properties. Several preclinical studies have validated the advantages of this approach using the streptavidin-biotin system proposed by Goodwin (4–12).

The interaction between streptavidin (SA) and D-biotin (BTN) is known to have the highest non-covalent affinity (K_d = 10^{-14}–10^{-15} M) in nature (13,14), and this interaction has been used in many biotechnological applications (15–18). Anti-CD20 antibodies with streptavidin have been shown to augment the efficacy of radioimmunotherapy and decrease toxicity compared with a directly-labeled antibody (19). In the process of pre-targeting, cancer cells can be captured by the SA-fused antibody as the first drug. After the excretion of excess of the first drug, a biotinylated imaging marker or radioisotope as the second drug can be specifically delivered to the SA-fused antibody on the cancer cells.

However, SA from *Streptomyces avidinii* exhibits high immunogenicity in humans, and the direct use of wild-type SA (SA-WT) is not suitable for clinical use (4,20–22). A reduction of immunogenicity by site-directed mutagenesis is necessary for medical applications of SA, without impairing its function and stability. Subramanian and Adiga (23) studied immunologic properties of SA and performed a detailed comparison of the epitopes of SA with rabbit antisera. Six linear epitopes regions were proposed using epitope mapping, (residues 17–23, 33–43, 84–87, 96–103, 114–121, and 128–131). Meyer et al. (21) also reported a mutant SA with lowered reactivity for an anti-SA antibody by substituting charged or aromatic amino-acid residues that make-up putative conformational epitopes on the protein surface required for interaction with B cells. The substitutions, however, also increased the dissociation rate from biotin, suggesting that the BTN-binding function has been reduced. The mechanism of acquiring low immunogenicity is obscure, because no structural studies have been performed for the mutants with decreased immunogenicity.

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Recently, we have succeeded in creating a low immunogenic SA mutant No. 314 (LISA-314), which has greatly decreased immunoreactivity against monkey antiserum without impairing biophysical properties including biotin binding and thermal stability (24,25). LISA-314 was designed for reducing reactivity against anti-SA-WT serum and the number of *in silico* T-cell epitopes, by introducing six amino-acid substitutions (Y22S/Y83S/R84K/E101D/R103K/E116N) at solvent-exposed charged and aromatic residues that were proposed to be involved in its immune recognition. Unlike previous strategies mainly using alanine or glycine, we considered the substitutions so that the electrostatic charge and functional moieties of the WT residues were preserved.

In this study, we performed structural analysis by X-ray crystallography, to evaluate the effect of substitutions inside LISA-314 on the protein function and the three-dimensional structure. The results demonstrate that LISA-314 acquired lowered immunogenicity while maintaining an intact tetrameric assembly, main chain frameworks, and BTN-binding mode at the same level as SA-WT. We demonstrated that LISA-314 has identical structural property to SA-WT and potential for clinical applications in pre-targeting drug-delivery systems.

MATERIALS AND METHODS

Expression and purification The core SA-WT and LISA-314 were cloned into cleavable eXact tag fusion pPAL7 vector (Bio-Rad Laboratories). For the expression, SA-WT and LISA-314 plasmids were transformed into the *Escherichia coli* BL21Star(DE3) strain, which was cultivated in Luria-Broth medium supplemented with 100 μg/mL ampicillin at 37°C. When the optical density ($\lambda = 600$ nm) reached a value of 0.5, 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added for induction. Cultivation was continued for approximately 24 h at 16°C. Cells were harvested by centrifugation at 4°C at 8000 ×*g* for 20 min and resuspended in extraction buffer (10 mM phosphate, 100 mM NaCl, Complete EDTA-free protease inhibitor cocktail tablets (Roche) and Benzonase (Novagen) at pH 7.2). The sample was lysed by passing through a French press twice at 12,000 lb/in^2. The lysate was centrifuged at 10,000 ×*g* for 30 min to remove the insoluble fraction. The supernatant was loaded onto a HiTrap SP HP (GE Healthcare) and eluted with a linear gradient of 0.1–1.0 M NaCl in 10 mM phosphate buffer at pH 7.2. The eluted protein was dialyzed against binding buffer for the eXact column (Bio-Rad Laboratories) containing 10 mM phosphate and 300 mM sodium acetate at pH 6.8. The sample was then loaded onto an eXact column and the column was washed with the binding buffer. The column was further washed with the binding buffer supplemented with 100 mM NaF and incubated to allow cleavage of the eXact-tag at 25°C for 1 h. The cleaved protein was collected in the flow-through and dialyzed against 10 mM phosphate buffer at pH 6.0 for SA-WT and pH 6.8 for LISA-314. The sample was loaded onto a hydroxyapatite column and eluted with a phosphate gradient (Bio-Rad Laboratories). The eluted protein was buffer-exchanged into gel-filtration buffer (150 mM Tris–HCl and 150 mM NaCl at pH 7.5) using a Vivaspin 30-kDa cutoff (GE Healthcare). BTN was added to the sample at excess molar ratio for the SA-WT or LISA-314. Further purification was carried out by gel-filtration chromatography using a HiLoad 26/600 Superdex 75 column (GE Healthcare). The purified sample was then concentrated to approximately 9.0 mg/mL, estimated by the absorbance at 280 nm.

Crystallization Crystallization was performed by the sitting-drop vapor-diffusion methods at 20°C in Violamo 96-well plates (As One, Osaka, Japan). Sixty microliters of the reservoir solution was added to each well of the 96-well plates. Crystals of SA-WT were obtained by mixing 0.5 μL of protein solution (9.0 mg/mL SA-WT, 150 mM Tris–HCl and 150 mM NaCl at pH 7.5) and 0.5 μL of reservoir solution (0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate and 24% (w/v) PEG4000 at pH 5.2). On the other hand, crystals of LISA-314 were prepared by mixing 0.5 μL of protein solution (9.0 mg/mL LISA-314, 150 mM Tris–HCl and 150 mM NaCl at pH 7.5) and 0.5 μL of reservoir solution [0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate and 24% (w/v) PEG4000 at pH 5.2]. Crystals were cryoprotected by the reservoir solution containing 30% glycerol.

X-ray data collection and processing All datasets were collected on the beamline BL44XU at SPring-8 (Harima, Japan) under −173°C. Data were indexed and scaled with the programs DENZO and SCALEPACK from the HKL2000 program suite (HKL Research). The structures were solved by the molecular replacement with the program Phaser (26) from the CCP4i package (27) using a core streptavidin structure (PDB ID: 2F01) as the search model. The resultant structures were manually modified to fit into the experimental electron density maps, using the program *Coot* (28), then refined with the program Refmac (29) from the CCP4i package. The results of the structural analysis are summarized in Table 1. Figures were prepared with Pymol (http://www.pymol.org/). The final structure coordinates and structure-factor amplitudes were deposited into the Protein Data Bank with IDs 3WYP for SA-WT and 3WYQ for LISA-314.

TABLE 1. Data collection and refinement statistics.

	SA-WT	LISA-314
Data collection		
Space group	$P2_1$	$C2$
Unit-cell parameters (Å, °)	$a = 50.35$, $b = 97.70$, $c = 52.57$, $\beta = 112.29$	$a = 63.22$, $b = 67.07$, $c = 56.36$, $\beta = 116.68$
Wavelength	0.90000	0.82000
Resolution (Å)	50-1.30 (1.32-1.30)	50-1.00 (1.04-1.00)
R_{sym} (%)[a]	4.6 (34.5)	4.7 (29.6)
$I/\sigma(I)$	23.4 (2.9)	30.7 (3.6)
Completeness (%)	96.0 (93.3)	95.4 (91.6)
Redundancy	3.0 (2.7)	4.3 (3.2)
Refinement		
Resolution	1.30	1.00
No. of reflections	104885	101796
R_{work} (%)[b]/R_{free} (%)[c]	13.1/17.9	13.7/16.3
No. of atoms		
Protein	3697	1805
Ligand/ion	136	109
Water	480	254
B factors		
Protein	9.2	8.4
Ligand/ion	16.3	18.5
Water	29.3	28.1
R.m.s deviations		
Bond length (Å)	0.025	0.026
Bond angles (°)	2.23	2.43
Ramachandran plot		
Favored (%)	95.30	96.04
Allowed (%)	3.42	3.52
Outliers (%)	1.28	0.44

Values in parentheses are for the highest-resolution shell.

[a] R_{sym} is calculated as $\Sigma_{hkl}\Sigma_i|I_i(\mathrm{hkl}) - <I(hkl)>|/\Sigma_{hkl}\Sigma_i I(hkl)$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection with Miller indices *hkl* and $<I(hkl)>$ is the average intensity from multiple observations.

[b] $R_{work} = \Sigma_{hkl}||F_{obs}| - |F_{calc}||/\Sigma_{hkl}|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes, respectively.

[c] R_{free} is computed in the same manner as R_{work} but using only a small set (5%) of randomly chosen intensities that were not used in the refinement of the model.

RESULTS

Overview of the structure of LISA-314 Many structural analyses of SA have been reported previously (30–35). To perform a detailed structural comparison between LISA-314 and SA-WT, we determined their high-resolution crystal structures in complex with biotin (Table 1) by using the same protein expression, purification, and crystallization conditions. In the asymmetric unit, two protomers are contained in LISA-314 and four in SA-WT.

LISA-314 forms a tetramer in a similar manner to SA-WT (Fig. 1A). Each subunit consists of an eight-stranded β-barrel with a ligand binding site at one end of the barrel. The root mean squared deviation (RMSD) value for the Cα carbon atoms of the whole structure between LISA-314 and SA-WT is calculated to be 0.70 Å, but the RMSD value for each protomer is actually calculated to be lower (0.50–0.70 Å). Except for part of the C-terminal region, the main chains of the protomer in LISA-314 superimpose well with those in SA-WT, even at substituted sites (Fig. 1B, Table 2).

The BTN-binding mode of LISA-314 In 2/4 protomers of SA-WT, the sulfur atom of BTN is oxidized, so we compared the BTN-binding mode of LISA-314 with the other 2/4 chains of SA-WT containing non-sulfoxide BTN. The sulfoxide form of BTN is sometimes seen in the crystal structure of the SA-BTN complex, even though it was not added in purification or crystallization step (36,37). The BTN-binding mode of LISA-314 is the same as that of SA-WT, showing that the RMSD value for the BTN molecule between SA-WT and LISA-314 is only 0.05 Å. The BTN molecule