1 **Crystal Structure of a Streptavidin Mutant with Low Immunogenicity**

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25 **Abstract**

26 We previously created a low-immunogenic core streptavidin mutant No. 314
27 (LISA-314) by replacing six amino acid residues for use as a delivery tool for an
28 antibody multistep pre-targeting process (Yumura et al., *Protein science*, **22**, 213-21
29 (2013)). Here, we performed high-resolution X-ray structural analyses of LISA-314 and
30 wild-type streptavidin to investigate the effect of substitutions on the protein function
31 and the three-dimensional structure. LISA-314 forms a tetramer in the same manner as
32 wild-type streptavidin. The binding mode of D-biotin in LISA-314 is also completely
33 identical to that in wild-type streptavidin, and conformational changes were observed
34 mostly at the side chains of substituted sites. Any large conformational changes
35 corresponding to the reduction of *B* factors around the substituted sites were not
36 observed. These results demonstrated the LISA-314 acquired low immunogenicity
37 without losing structural properties of original wild-type streptavidin.

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43 **Introduction**

44 Cancer is the leading cause of death in economically developed countries and the
45 second in developing countries. Worldwide, cancer accounts for 7.6 million deaths, and
46 this is predicted to increase, with an estimated 13.1 million cancer-related deaths in
47 2030 (1). Development of innovative cancer therapies is therefore of utmost importance.

48 Targeted therapies such as antibody-drug conjugates, which treat cancer by targeting
49 cancer-specific molecules, have been attracting much attention recently as they result in
50 fewer side effects compared with standard chemotherapy (2, 3). Pre-targeting drug
51 delivery systems are expected to be especially beneficial by increasing both bio-imaging
52 sensitivity and therapeutic effect over conventional systems, in which fluorescent
53 markers for imaging or radioisotopes for treatment are directly attached to antibodies
54 (4-6). In the pre-targeting method, treatment is first performed with antibody vehicle,
55 followed by administration of fluorescent marker or radioisotope, displaying more
56 favorable tumor-targeting properties. Several preclinical studies have validated the
57 advantages of this approach using the streptavidin-biotin system proposed by Goodwin
58 (4-11).

59 The interaction between streptavidin (SA) and D-biotin (BTN) is known to have the
60 highest non-covalent affinity ($K_d = 10^{-14}$ – 10^{-15} M) in nature (12, 13), and this

61 interaction has been used in many biotechnological applications (14-17). Anti-CD20
62 antibodies with streptavidin have been shown to augment the efficacy of
63 radioimmunotherapy and decrease toxicity compared with a directly-labeled antibody
64 (18). In the process of pre-targeting, cancer cells can be captured by the SA-fused
65 antibody as the first drug. After the excretion of excess of the first drug, a biotinylated
66 imaging marker or radioisotope as the second drug can be specifically delivered to the
67 SA-fused antibody on the cancer cells.

68 However, SA from *Streptomyces avidinii* exhibits high immunogenicity in humans,
69 and the direct use of wild-type SA (SA-WT) is not suitable for clinical use (4, 19-21). A
70 reduction of immunogenicity by site-directed mutagenesis is necessary for medical
71 applications of SA, without impairing its function and stability. Subramanian et al.
72 studied immunologic properties of SA and performed a detailed comparison of the
73 epitopes of SA with rabbit antisera (22). Six linear epitopes regions were proposed
74 using epitope mapping, (residues 17–23, 33–43, 84–87, 96–103, 114–121, and 128–
75 131). Meyer et al. also reported a mutant SA with lowered reactivity for an anti-SA
76 antibody by substituting charged or aromatic amino acid residues that make-up putative
77 conformational epitopes on the protein surface required for interaction with B cells (20).
78 The substitutions, however, also increased the dissociation rate from biotin, suggesting

79 that the BTN binding function has been reduced. The mechanism of acquiring low
80 immunogenicity is obscure, because no structural studies have been performed for the
81 mutants with decreased immunogenicity.

82 Recently, we have succeeded in creating a Low Immunogenic SA mutant No. 314
83 (LISA-314), which has greatly decreased immunoreactivity against monkey antiserum
84 without impairing biophysical properties including biotin binding and thermal stability
85 (23, 24). LISA-314 was designed for reducing reactivity against anti-SA-WT serum and
86 the number of *in silico* T-cell epitopes, by introducing six amino-acid substitutions
87 (Y22S/Y83S/R84K/E101D/R103K/E116N) at solvent-exposed charged and aromatic
88 residues that were proposed to be involved in its immune recognition. Unlike previous
89 strategies mainly using alanine or glycine, we considered the substitutions so that the
90 electrostatic charge and functional moieties of the WT residues were preserved.

91 In this study, we performed structural analysis by X-ray crystallography, to evaluate
92 the effect of substitutions inside LISA-314 on the protein function and the
93 three-dimensional structure. The results demonstrate that LISA-314 acquired lowered
94 immunogenicity while maintaining an intact tetrameric assembly, main chain
95 frameworks, and BTN-binding mode at the same level as SA-WT. We demonstrated

96 that LISA-314 has identical structural property to SA-WT and potential for clinical
97 applications in pre-targeting drug-delivery systems.

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99 **Material and methods**

100 **Expression and Purification**

101 The core SA-WT and LISA-314 were cloned into cleavable eXact tag fusion pPAL7
102 vector (Bio-Rad Laboratories). For the expression, SA-WT and LISA-314 plasmids
103 were transformed into the *Escherichia coli* BL21Star(DE3) strain, which was cultivated
104 in Luria-Browth medium supplemented with 100 µg/mL ampicillin at 37 °C. When the
105 optical density ($\lambda = 600$ nm) reached a value of 0.5, 0.4 mM
106 isopropyl-β-D-thiogalactopyranoside (IPTG) was added for induction. Cultivation was
107 continued for approximately 24 h at 16 °C. Cells were harvested by centrifugation at
108 4 °C at 8,000 × g for 20 min and resuspended in extraction buffer (10 mM phosphate,
109 100 mM NaCl, Complete EDTA-free protease inhibitor cocktail tablets (Roche) and
110 Benzonase (Novagen) at pH 7.2). The sample was lysed by passing through a French
111 press twice at 12,000 lb/in². The lysate was centrifuged at 10,000 × g for 30 min to
112 remove the insoluble fraction. The supernatant was loaded onto a HiTrap SP HP (GE
113 Healthcare) and eluted with a linear gradient of 0.1–1.0 M NaCl in 10 mM phosphate

114 buffer at pH 7.2. The eluted protein was dialyzed against binding buffer for the eXact
115 column (Bio-Rad Laboratories) containing 10 mM phosphate and 300 mM sodium
116 acetate at pH 6.8. The sample was then loaded onto an eXact column and the column
117 was washed with the binding buffer. The column was further washed with the binding
118 buffer supplemented with 100 mM NaF and incubated to allow cleavage of the
119 eXact-tag at 25 °C for 1 h. The cleaved protein was collected in the flow-through and
120 dialyzed against 10 mM phosphate buffer at pH 6.0 for SA-WT and pH 6.8 for
121 LISA-314. The sample was loaded onto a hydroxyapatite column and eluted with a
122 phosphate gradient (Bio-rad Laboratories). The eluted protein was buffer-exchanged
123 into gel-filtration buffer (150 mM Tris-HCl and 150 mM NaCl at pH 7.5) using a
124 Vivaspin 30-kDa cutoff (GE Healthcare). BTN was added to the sample at excess molar
125 ratio for the SA-WT or LISA-314. Further purification was carried out by gel-filtration
126 chromatography using a HiLoad 26/600 Superdex 75 column (GE Healthcare). The
127 purified sample was then concentrated to approximately 9.0 mg/mL, estimated by the
128 absorbance at 280 nm.

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132 **Crystallization**

133 Crystallization was performed by the sitting-drop vapor-diffusion methods at 20 °C
134 in Viologam 96-well plates (As One, Osaka, Japan). Sixty microliters of the reservoir
135 solution was added to each well of the 96-well plates. Crystals of SA-WT were obtained
136 by mixing 0.5 µL of protein solution (9.0 mg/mL SA-WT, 150 mM Tris-HCl and 150
137 mM NaCl at pH 7.5) and 0.5 µL of reservoir solution (0.2 M ammonium sulfate, 0.1 M
138 sodium acetate trihydrate and 24%(w/v) PEG4000 at pH 5.2). On the other hand,
139 crystals of LISA-314 were prepared by mixing 0.5 µL of protein solution (9.0 mg/mL
140 LISA-314, 150 mM Tris-HCl and 150 mM NaCl at pH 7.5) and 0.5 µL of reservoir
141 solution (0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate and 24%(w/v)
142 PEG4000 at pH 5.2). Crystals were cryoprotected by the reservoir solution containing
143 30% glycerol.

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145 **X-ray data collection and processing**

146 All datasets were collected on the beamline BL44XU at SPring-8 (Harima, Japan)
147 under -173 °C. Data were indexed and scaled with the programs *DENZO* and
148 *SCALEPACK* from the *HKL2000* program suite (HKL Research). The structures were
149 solved by the molecular replacement with the program *Phaser* (25) from the *CCP4i*

150 package (26) using a core streptavidin structure (PDB ID: 2F01) as the search model.
151 The resultant structures were manually modified to fit into the experimental electron
152 density maps, using the program *Coot* (27), then refined with the program *Refmac* (28)
153 from the *CCP4i* package. The results of the structural analysis are summarized in Table
154 1. Figures were prepared with *Pymol* (<http://www.pymol.org/>). The final structure
155 coordinates and structure factor amplitudes were deposited into the Protein Data Bank
156 with IDs 3WYP for SA-WT and 3WYQ for LISA-314.

157

158 **Results**

159 **Overview of the structure of LISA-314**

160 Many structural analyses of SA have been reported previously (29-34). To perform a
161 detailed structural comparison between LISA-314 and SA-WT, we determined their
162 high-resolution crystal structures in complex with biotin (Table 1) by using the same
163 protein expression, purification, and crystallization conditions. In the asymmetric unit,
164 two protomers are contained in LISA-314 and four in SA-WT.

165 LISA-314 forms a tetramer in a similar manner to SA-WT (Fig. 1A). Each subunit
166 consists of an eight-stranded β -barrel with a ligand binding site at one end of the barrel.
167 The root mean squared deviation (RMSD) value for the C α carbon atoms of the whole

168 structure between LISA-314 and SA-WT is calculated to be 0.70 Å, but the RMSD
169 value for each protomer is actually calculated to be lower (0.50–0.70 Å). Except for part
170 of the C-terminal region, the main chains of the protomer in LISA-314 superimpose
171 well with those in SA-WT, even at substituted sites (Fig. 1B, Table 2).

172

173 **The BTN-binding mode of LISA-314**

174 In 2/4 protomers of SA-WT, the sulfur atom of BTN is oxidized, so we compared
175 the BTN-binding mode of LISA-314 with the other 2/4 chains of SA-WT containing
176 non-sulfoxide BTN. The sulfoxide form of BTN is sometimes seen in the crystal
177 structure of the SA-BTN complex, even though it was not added in purification or
178 crystallization step (35, 36). The BTN-binding mode of LISA-314 is the same as that of
179 SA-WT, showing that the RMSD value for the BTN molecule between SA-WT and
180 LISA-314 is only 0.05 Å. The BTN molecule was stabilized by an extensive network of
181 hydrogen bonds and van der Waals interactions as is seen in SA-WT (Fig. 2).

182

183 **Conformational changes at substituted sites and *B* factors**

184 The structural change at every substitution site is summarized in Fig. 3, and the
185 change of temperature (*B*) factors for all C α carbon atoms is shown in Fig. 4. The *B*

186 factor reflects the local mobility of protein backbones. We observed significant
187 increases for *B* factors, which was not caused by packing effect, around the substitution
188 sites of Y83S and R84K. The electron density maps for the side chain of Y83 of SA-WT
189 and S83 of LISA-314 are well ordered, and main chains near the residues are well
190 superimposed (Fig. 3B). However, the hydrogen bond between R84 and N49, which
191 was found in SA-WT, was broken owing to the substitutions of R84K and Y83S in
192 LISA-314 (Fig. 3C), and the *B* factors of the residues around these substituted residues
193 were increased (Fig. 4). In all the substitutions, a slight decrease in *B* factor was only
194 observed by the substitution of E101D. The electron density map for D101 of LISA-314
195 was improved after the introduction of shorter aspartate residue and the reduction of
196 flexibility compared to E101 of SA-WT (Fig. 3D). Interestingly, the space group of the
197 crystal of LISA-314 changed to *C2* from the *P2*₁ of the SA-WT, even though both
198 crystals were obtained using the same crystallization conditions. We try to rationalize
199 this difference in the crystal packing below.

200 The conformational changes were mainly caused by the substitutions of Y22S and
201 R103K (Fig. 3A, E), but the *B* factors were not affected (Fig. 4). One change is the flip
202 of the C-terminal loop (residues 133–134) owing to the substitution of Y22S. The
203 disappearance of the CH/ π interaction between Y22 and K134 near the C-terminus is

204 thought to be the reason for the flip as it is sometimes observed in the SA structures
205 deposited in the Protein Data Bank. The second is the change in the hydrogen bond
206 network resulting from the substitution of R103K. The hydrogen bond between R103
207 and T129, which was formed in 2/4 chains of SA-WT, was changed to that between
208 K103 and T131 (a new hydrogen bond partner) as a result of the substitution. The last
209 substitution site at E116N did not change the *B* factor, although the electron density for
210 the side chain of N116 was clearly obtained (Fig. 3F).

211 Other than these substitution sites, we observed decreased *B* factors at residue
212 numbers 32–35. The crystal packing was changed from *C*2 to *P*2₁ owing to the
213 substitution of Y22S, and this region interacted with a neighboring molecule in the new
214 crystal form, which may be the reason for the decrease in *B* factors.

215

216 **Discussion**

217 The X-ray crystallography analyses of LISA-314 and SA-WT at high resolution
218 revealed that LISA-314 forms a tetramer in the same manner as SA-WT, and the main
219 chains are essentially identical to those of SA-WT except for a part of the C-terminus.
220 The current study demonstrates that the binding mode of BTN in LISA-314 is
221 completely identical to that of SA-WT, as all hydrogen bonds and van der Waals

222 interactions are well conserved. This result strongly indicates that LISA-314 has the
223 same BTN-binding function as that of SA-WT, although the binding constants measured
224 by isothermal titration calorimetry (ITC) in previous work (24) exceeded the
225 measurement limit of ITC.

226 Generally, the higher the *B* factor value, the more flexible the corresponding region
227 in the protein, and vice versa. This attribute has been used to predict linear B-cell
228 epitopes (37). However, we did not observe any large conformational changes
229 corresponding to the large reduction of *B* factors around the substituted sites. Even
230 higher *B* factors were measured because of the substitutions. The effect of substitutions
231 is restricted mainly to the changes of side chains at the substituted sites. From these
232 results, if the lowered immunogenicity of LISA-314 is caused by the conformational
233 changes, we presume that it is not caused by the conformational change of the whole
234 structure, but by the local conformational changes at the substituted sites. In this study,
235 we have proven that LISA-314 has both low immunogenicity and high biotin-binding
236 affinity without a striking change in the whole structure, despite introducing six
237 amino-acid substitutions. Practically, we expect LISA-314 to be most useful in
238 pre-targeting cancer therapy.

239 Some groups have discussed the problem of competing endogenous BTN in blood

240 and tissues (38, 39). Specifically, the endogenous BTN can effectively block the
241 BTN-binding pocket of administrated SA, impairing the efficiency of the pre-targeting
242 method. To solve this problem, the binding site of LISA-314 has to be modified to
243 selectively bind to the non-natural BTN analog without interference of endogenous
244 BTN. Detailed structural information of the present study will be useful for future
245 improvement of LISA-314.

246

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385 **Figure Legends**

386 FIG. 1. Crystal structures of SA-WT and LISA-314. (A) The overall structure of
387 SA-WT depicted in ribbon diagram. In the tetramer, one of the protomers is shown in
388 green, and the other three protomers are shown in gray. In the four protomers, 2 BTN
389 (shown in red) and 2 biotin-D-sulfoxide (shown in blue) molecules are bound in the
390 active sites. (B) Main chain tracing for SA-WT (pale cyan) and LISA-314 (blue) after
391 superposition on protomer. Substituted residues are shown by a red circle.

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393 FIG. 2. Schematic representation of the interactions between BTN and LISA-314 or
394 SA-WT. Dashed lines represent hydrogen bonds. The distances ($\text{\AA}$) between residues of
395 LISA-314 or SA-WT (parentheses) and BTN are shown.

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397 FIG. 3. Structural change of SA induced by six amino-acid substitutions. The electron
398 density maps of SA-WT (cyan) and LISA-314 (blue) are shown in the first and the
399 second lanes, respectively. Superimposed structures between SA-WT and LISA-314 are
400 shown in the third lane. The panels are displayed around (A) Y22S, (B) Y83S, (C)
401 R84K, (D) E101D, (E) R103K, and (F) E116N, respectively.

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403 FIG. 4. Comparison of relative temperature (B) factors between SA-WT (thin line) and
 404 LISA-314 (thick line). The B factor for $C\alpha$ atom of each amino acid is standardized by
 405 the equation: $B \text{ factor}/B_{\text{ave}}$ (B_{ave} : the average B factor of the whole $C\alpha$ atoms).

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408 **Figure 1**

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410 **Figure 2**

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412 **Figure 3**

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414 **Figure 4**

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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	104,885	101,796
R_{work} (%) ^b / R_{free} (%) ^c	13.1/17.9	13.7/16.3
No. of atoms		
Protein	3,697	1,805
Ligand/ion	136	109
Water	480	254
<i>B</i> 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		
Favoured (%)	95.30	96.04
Allowed (%)	3.42	3.52
Outliers (%)	1.28	0.44

2 Values in parentheses are for the highest-resolution shell.

3 ^a R_{sym} is calculated as $\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of

4 an individual measurement of the reflection with Miller indices hkl and $\langle I(hkl) \rangle$ is the

5 average intensity from multiple observations.

6 $^bR_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated

7 structure-factor amplitudes, respectively.

8 $^cR_{\text{free}}$ is computed in the same manner as R_{work} but using only a small set (5%) of

9 randomly chosen intensities that were not used in the refinement of the model.

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TABLE 2. RMSD values^a (Å) for superposed streptavidin subunits.

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		SA-WT			
		A	B	C	D
LISA-314	A	0.54	0.59	0.62	0.50
	B	0.64	0.69	0.73	0.68

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^aCα atoms were used for the structural superpositions.

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