A streptavidin variant with slower biotin dissociation and increased mechanostability

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Streptavidin binds biotin conjugates with exceptional stability, but dissociation does occur, limiting its use in imaging, DNA amplification and nanotechnology. We identified a mutant streptavidin, traptavidin, with more than tenfold slower biotin dissociation, increased mechanical strength and improved thermostability; this resilience should enable diverse applications. FtsK, a motor protein important in chromosome segregation, rapidly displaced streptavidin from biotinylated DNA, whereas traptavidin resisted displacement, indicating the force generated by FtsK translocation.

The remarkably strong interaction between the small molecule biotin and the proteins streptavidin or (neutr)avidin is widely exploited in biological research. Biotin-binding proteins have been isolated from a wide range of species, but streptavidin binds the most stably to biotin conjugates. Streptavidin is used in imaging, protein purification and nano-assembly, and is showing success in cancer clinical trials. Streptavidin and biotin have low nonspecific binding and biotinylation generally does not disrupt biomolecule function. Alternative targeting methods, such as those using HaloTag or SNAP-tag, form irreversible covalent bonds to their ligand and are valuable for cellular labeling. However, these domains do not have the resistance of streptavidin to temperature, pH or denaturant, and so even though prebound ligand will remain attached under these harsh conditions, these covalent-binding proteins may unfold, aggregate and promote nonspecific binding. Also, unlike SNAP-tag and HaloTag ligands, biotin can be precisely targeted to proteins in vitro, on cells and in living animals using biotin ligase. An additional advantage is that many streptavidin and biotin conjugates are commercially available.

Despite its stable binding, the perception that the streptavidin-biotin interaction is essentially irreversible is far from correct. For example, in imaging, low endosomal pH led to dissociation of streptavidin being detected in 2 h, whereas the receptor of interest had a lifetime of ~4 days. Also, nanoparticle attachment can cause a surprising decrease in streptavidin-biotin stability; the dissociation constant ($K_d$) for a biotinylated peptide increased approximately a million-fold when streptavidin was attached to beads. In the presence of shear forces lower than those in a blood capillary, streptavidin-coated beads do not attach to a biotinylated surface but instead roll across, with arrests of 20 ms to tens of seconds. In addition, streptavidin cannot prevent the translocation of molecular motors such as helicases, RNA polymerase or DNA polymerase along DNA. Streptavidin can be used at high temperatures, such as for PCR, BEAMing and 454 DNA sequencing, but DNA has to be bis-biotinylated to reduce dissociation.

A streptavidin mutant containing a cysteine forms a disulfide with a thiol-linked biotin conjugate, giving controlled reversibility, but this mutant only enhances binding to certain biotin conjugates and in systems unaffected by changing redox state, precluding use in cells. We therefore aimed to engineer a streptavidin mutant that would bind more stably to any biotin conjugate.

In a highly optimized system, almost any change reduces performance. Over 200 streptavidin mutants have been described but none have improved biotin-binding stability. Streptavidin libraries have been screened for various properties by phage display and in vitro compartmentalization, yielding, for example, a streptavidin variant with improved desthiobiotin binding, but no pair with as strong binding as wild-type streptavidin-biotin has been identified. Based on this literature, we avoided mutations near the ureido or thiophene rings of biotin, which invariably impair binding, and explored many mutations adjacent to the biotin carboxyl and in the L3/4 loop. We randomized promising residues and evaluated purified proteins according to biotin–4-fluorescein dissociation (‘off’) rate and found the lowest off rate for the SS2G,R53D mutant of streptavidin, which we termed traptavidin. We hypothesize that the mutations in traptavidin reduce flexibility of the L3/4 loop (residues 45–50). Upon biotin binding, this loop becomes ordered and closes over the biotin-binding pocket. A more ordered loop may reduce the entropic cost of biotin binding and inhibit dissociation as well as decrease the association (‘on’) rate and enhance thermostability.

The off rate for free biotin at 37 °C and pH 7.4 was more than tenfold lower for traptavidin than streptavidin ($4.2 \pm 0.5 \times 10^{-6} \text{s}^{-1}$ for traptavidin and $6.8 \pm 0.3 \times 10^{-5} \text{s}^{-1}$ for streptavidin; Fig. 1b). As a substantial part of (strept)avidin’s binding energy comes from interaction with the carboxyl group of biotin, it is important to establish how derivatization at the carboxyl group changes binding strength. Traptavidin also had a dramatically reduced off rate to biotin conjugates ($P = 0.0008$; Fig. 1c). After the ~2% dissociation at the initial time point, there was little dissociation from traptavidin over the subsequent 12 h. In contrast, streptavidin dissociated steadily, and avidin dissociated even faster than streptavidin. At pH 5, traptavidin dissociation was faster than at pH 7.4 but was still significantly slower than streptavidin dissociation ($P = 0.001$; Fig. 1d). The on rate of

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traptavidin for biotin–4-fluorescein was reduced twofold, from 2.0±0.1×10^7 M^{-1}s^{-1} for streptavidin to 1.0±0.03×10^7 M^{-1}s^{-1} for traptavidin (P=0.004), whereas the on rate of traptavidin for [3H]biotin was also reduced (Supplementary Fig. 1). The slower on rate on traptavidin means that longer incubations are required to reach equilibrium.

Streptavidin is often used at high temperature. Traptavidin had increased thermostability compared to streptavidin before splitting into monomers; the midpoint of transition was ~10 °C higher than for streptavidin (Fig. 2a). We also assessed biotin-conjugate binding stability at high temperatures (Fig. 2b). At 70 °C, there was complete dissociation from streptavidin, but most ligand was still bound to traptavidin.

Imaging of cell-surface proteins using biotin ligase and streptavidin is rapid and sensitive, and the target protein needs only to be modified with a 15-amino-acid tag. The altered charge of traptavidin may affect nonspecific cellular binding, as seen for avidin compared to lower-pI mutants. We investigated whether traptavidin had similar specificity to streptavidin in mammalian cells. We fused the type 1 insulin-like growth factor receptor IGFR1 to the acceptor peptide (AP-IGFR1), biotinylated the acceptor peptide with co-expressed biotin ligase (BirA-ER) and detected biotinylated AP-IGFR1 with fluorescently labeled traptavidin or streptavidin (Supplementary Fig. 2).

Traptavidin showed high specificity for imaging, with a strong signal on cells expressing AP-IGFR1 and BirA-ER, and minimal binding when we pre-blocked traptavidin with biotin. Staining with traptavidin and streptavidin was comparable (Supplementary Fig. 2). However, with shorter staining durations, the cell staining was more intense with streptavidin (data not shown), consistent with the slower on rate of traptavidin.

The relationship between binding stability over time versus resistance to force is complex: force changes the height and landscape of the activation energy for dissociation. We probed the mechanical strength of traptavidin at the single-molecule level by atomic force microscopy (AFM). Traptavidin had greater mechanical binding stability than streptavidin over a range of loading rates (P<0.0001) (Fig. 3a). We observed a distribution of binding strengths (Supplementary Fig. 3a) because of the importance of thermal fluctuations to traversing the activation barrier. From the relationship between loading rate and rupture force, we could estimate the difference in the dissociation rate between streptavidin and traptavidin at a given force (Supplementary Fig. 3b).

We applied traptavidin to study FtsK, one of the fastest molecular motor proteins, translocating along DNA at 5 kb s^{-1} (refs. 13,14). Before bacteria divide, FtsK translocates along DNA until encountering XerC and XerD; then FtsK activates site-specific recombination by XerC/D, separating chromosome dimers and ensuring faithful partition of one chromosome to each daughter cell. In vivo DNA is bound to many proteins, including repressors, transcription factors, RNA polymerases and DNA-bending architectural proteins. To study how Pseudomonas aeruginosa FtsK copes with obstacles in its path, we used a short DNA substrate containing KOPS, an 8-bp sequence that loads FtsK directionally, with a biotinylated nucleotide near the end, so that FtsK would load on to the DNA in a defined orientation and then translocate until encountering streptavidin or traptavidin bound to the biotin (Fig. 3b).

Despite the strength of the streptavidin-biotin interaction, 0.5 μM FtsK displaced the majority of streptavidin from the DNA within 3 min, whereas traptavidin resisted displacement (Fig. 3c).
Streptavidin displacement was detectable after only 2 s, but we observed little displacement of traptavidin even after 300 s (Fig. 3d). Increasing the FtsK concentration to 2 µM allowed substantial displacement of traptavidin (Fig. 3e), indicating that multiple FtsK motors could cooperate in exerting a stronger force. Traptavidin was equally as strong a roadblock to Escherichia coli FtsK and with DNA biotinylated at the 5’ terminus rather than at an internal thymidine (Supplementary Fig. 4). As FtsK rapidly broke the stable biotin-streptavidin interaction, in vivo FtsK should be sufficient to displace even strongly attached DNA-binding proteins.

The stall force for FtsK measured by pulling on the DNA with optical tweezers is > 65 pN, at which point the DNA double helix itself is deformed.14 Traptavidin displacement should enable testing of higher forces without distorting the DNA. Streptavidin has previously been used as an obstacle to motors, providing a simpler method to probe force generation than single-molecule assays.13,14 However, only wild-type streptavidin and the weak nitroavidin have been used, so traptavidin and the range of weaker streptavidin mutants could be used to generate a calibration curve to dissect force generation by proteins.15

Traptavidin binding was more stable for a range of biotin conjugates (Supplementary Fig. 5), not just one particular ligand. Also, traptavidin can be recombinantly expressed in comparable yields to streptavidin, and recombinant expression of streptavidin gives yields higher than purification from Streptomyces avidinii.16 Therefore, traptavidin has the potential to replace streptavidin in many applications in which dissociation is a limitation, for example, when used as a molecular anchor for arrays, surface-plasmon resonance or point-of-care diagnostics. Traptavidin-biotin recognition may also aid our understanding of the subtle intermolecular forces that govern interactions of extreme stability.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Accession codes. GenBank: GU952124.

Figure 3 | Traptavidin has increased mechanical stability compared to streptavidin. (a) Traptavidin and streptavidin displacement analysis by AFM: single-molecule rupture forces between a biotinylated bead and an AFM tip coated with streptavidin or traptavidin were measured at the indicated loading rates. Means are shown ± 1 s.e.m. (streptavidin, n = 400 and traptavidin, n = 562), with best fits and 95% confidence limits indicated by solid and dashed lines, respectively. (b) Cartoon of motor assay showing DNA containing a loading site for FtsK and a biotinylated thymidine near the terminus that was capped with traptavidin or streptavidin (green). FtsK α and β domains are shown in blue and γ in gray. In the presence of ATP, FtsK translocates along the DNA and collides with streptavidin or traptavidin. (c) Displacement of streptavidin (SA) or traptavidin (Tr) by FtsK after 180 s was determined by gel electrophoresis, with DNA fluorescence visualized. FtsK does not remain bound to DNA upon electrophoresis, but bound streptavidin or traptavidin causes the gel shift indicated with arrows. Controls are shown without streptavidin or traptavidin or without ATP, preventing FtsK activity. The percentage of free DNA and the percentage of DNA displaced by FtsK for duplicate assays are indicated under each lane. (d) Streptavidin or traptavidin displacement by FtsK analyzed by incubating each protein with FtsK for the indicated times (analyzed as in c). (e) Traptavidin is displaced by a high concentration of FtsK. Streptavidin and traptavidin displacement by varying amounts of FtsK analyzed by incubating each protein for 180 s with the indicated concentration of FtsK (analyzed as in c).

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

C.E.C., M.H., E.C., V.T.M. and C.C. performed the research; M.H., V.T.M. and D.J.S. designed research; all analyzed the data; M.H., D.J.S. and C.E.C. wrote the manuscript.

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ONLINE METHODS

General. Biotin (Sigma) was dissolved in DMSO at 100 mM. Avidin (Sigma) was dissolved in PBS at 23 μM. SDS-PAGE was performed at 200 V with the gel box (X Cell SureLock; Invitrogen) surrounded by ice to prevent dissociation of the streptavidin subunits during electrophoresis. Structures of streptavidin without biotin (1swa) and with biotin (1mk5) were displayed and aligned using PyMOL (DeLano Scientific). The cartoon in Figure 3b was constructed with PyMOL based on crystal structures 1swa, 2iuu and 2ve9. Note that FtsK-γ is shown in the conformation bound to KOPS and is likely to change when translocating14.

Plasmid construction. Streptavidin in this paper refers to core streptavidin with 6His at the C terminus, with the gene encoding the protein in pET21a(+). Traptavidin was generated by introducing the S52G,R53D mutation into streptavidin by QuikChange (Stratagene) mutagenesis using the primer S52G-R53D F (Supplementary Table 1) and its reverse complement. The mutations were confirmed by DNA sequencing. AP-IGFIR was constructed from pcDNA3 containing human IGF1R (a kind gift from V. Macaulay, University of Oxford) by PCR-amplifying two fragments: the first fragment with primers IGFA and IGFB and the second fragment with primers IGFIC and IGFID (Supplementary Table 1). The fragments were joined by overlap extension PCR, digested with Nhel and NotI and ligated into pcDNA3.1. The acceptor peptide (GLNDIFEAQKIEWHE) was then inserted after the IGFIR signal sequence along with a 6-amino-acid spacer sequence before the start of sequence encoding the N terminus of mature IGF1R. BirA-ER (E. coli biotin ligase targeted to the endoplasmic reticulum) and pECFP-H2B (human histone H2B fused to enhanced CFP) have been previously described18.

Streptavidin and traptavidin expression and purification. An overnight culture of streptavidin or traptavidin, picked from a freshly grown colony of E. coli BL21 (DE3) Ripl (Stratagene), was diluted 100-fold into LB ampicillin, grown to OD600 0.9 at 37 °C, induced with 0.5 mM IPTG and incubated for an additional 4 h at 37 °C. Inclusion bodies were isolated from the cell pellet of a 750 ml culture by rocking with 10 ml 300 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.8 mg ml−1 lysozyme, 1% Triton X-100 pH 7.8 for 30 min at 25 °C followed by 9 min of pulsed sonication on ice at 40% amplitude on a Sonics Vibra-Cell sonicator. After centrifugation at 27,000 g for 15 min, the inclusion body pellet was washed three times in 10 ml 100 mM NaCl, 50 mM Tris, 0.5% Triton X-100 (pH 7.8) and then dissolved in 6 M guanidinium hydrochloride (pH 1.5) (GuHCl). Protein in GuHCl was refolded by rapid dilution into PBS at 4 °C and stirring overnight19. Ni-NTA resin (Qiagen), equilibrated in 300 mM NaCl, 50 mM Tris, 10 mM imidazole (pH 7.8), was added and rotated overnight at 4 °C. The next day, the resin was isolated by centrifugation, washed once with 5 ml of 300 mM NaCl, 50 mM Tris, 30 mM imidazole (pH 7.8) and then added to a poly-prep column (Bio-Rad) for elution with 5 ml 300 mM NaCl, 50 mM Tris, 200 mM imidazole (pH 7.8). The eluate was dialyzed three times against PBS. Protein concentration was determined after dialysis from OD280 using ε280 of 34,000 M−1 cm−1 (ref. 20). Typical yields were 8 mg per liter of culture for streptavidin and 5 mg per liter of culture for traptavidin. Streptavidin and traptavidin were labeled with Alexa Fluor 555 by adding 1/10 volume of 1 M NaHCO3 (pH 8.3) and then a tenfold molar excess of Alexa Fluor 555 succinimidyl ester (Invitrogen) (stock dissolved at 1 mg ml−1 in dry dimethylformamide) and incubating for 4 h at 25 °C. Free dye was separated using 1 ml packed volume Sephadex G-25 (Sigma) in a poly-prep column. Fractions containing labeled protein were pooled and free dye was removed by three rounds of dialysis in PBS.

Biotin–4-fluorescein off-rate assay. The off rate of biotin–4-fluorescein from avidin, streptavidin or traptavidin was measured using a PHERArst plate-reader with 480 nm excitation and 520 nm emission (BMG LabTech). In this assay, the binding of biotin–4-fluorescein to an excess of binding protein results in quenching of fluorescein emission21. As the biotin–4-fluorescein dissociates, the fluorescence recovers. The assay was performed using excess biotin, so that sites left open by biotin–4-fluorescein dissociation are re-filled by biotin immediately. We added 1 μM protein in 10 μl of PBS to 12 nM biotin–4-fluorescein (Invitrogen) with 0.12 mg ml−1 BSA in 170 μl of PBS and incubated the solutions for 1 h at 37 °C. Then, 20 μl PBS or 20 μl 1 M biotin in PBS was added, and fluorescence measurements were immediately started at 37 °C. Percentage dissociation was calculated as (signal with biotin − signal without biotin)/ (signal without quenching − signal without biotin) × 100. For the signal without quenching, no biotin-binding protein was added to the biotin–4-fluorescein (B4F).

For the low pH off-rate assay, 100 nM streptavidin or traptavidin was incubated with 12 nM B4F in 100 mM NaCl with 30 mM sodium citrate (pH 5.0) for 3 h at 25 °C before incubating at 37 °C with 100 μM biotin for 0.5, 1, 3, 6 and 18 h. As B4F fluorescence is decreased at low pH, samples were then placed on ice to block further dissociation, adjusted to pH 7.2 with 1 M HEPES at pH 8.3, and fluorescence intensity was immediately measured as above. P values were calculated using two-tailed Student’s t-tests from the triplicate data at the 6 h time point.

[3H]biotin off-rate assay. The dissociation kinetics of biotin from streptavidin and traptavidin were determined using a method modified from that described previously22. We incubated 10 nM 8,9-[3H]biotin (PerkinElmer LAS) for 1 h at 25 °C with 250 nM streptavidin or traptavidin in PBS. To initiate dissociation, non-radioactive biotin was added to a final concentration of 50 μM and incubated at 37 °C. At each time point, the protein-biotin complex was pulled down by incubation with a 50% slurry of Ni-NTA resin, equilibrated in 300 mM NaCl, 50 mM Tris, 10 mM imidazole (pH 7.8), for 1 h at 25 °C, followed by centrifugation. Then, 25 μl supernatant, containing unbound radioactive biotin, was added to the scintillation cocktail and counted in a liquid scintillation counter (LS-500TD; BeckmanCoulter). The average radioactivity of the supernatant at each time point (x) and the radioactivity of the protein-biotin complex before addition of cold biotin (a) enabled the first-order dissociation rate constant to be determined from the plot of ln(fraction bound) (ln(a−x/a)) against time. Excel was used for linear regression and to calculate error bars, using the “LINEST” linear least-squares curve-fitting routine.

Biotin–4-fluorescein on-rate assay. The on rate of B4F for streptavidin or traptavidin was measured in PBS on a PHERArst plate-reader. We added 20 μl of 10 nM streptavidin or traptavidin...
COS7 cells were grown 27–29°C. AFM is described in 23,26−1.

run at 6.0 V cm−1 in a Thermal Cycler and cooling to 10 °C. A 1.5% agarose gel was at 25 °C, before heating at the indicated temperature for 3 min.

20 mM potassium glutamate (pH 7.5) and incubated for 5 min at 25 °C, before heating at the indicated temperature for 3 min in a Bio-Rad DNA Engine Peltier Thermal Cycler and overnight for optimum biotinylation by BirA-ER. The next day, cells were washed 3 times in PBS with 5 mM MgCl2 (PBS-Mg) and kept thereafter at 4 °C. Cells were incubated for 15 min in PBS-Mg with 1% dialyzed BSA and 0.4 µM Alexa Fluor 555−conjugated traptavidin or streptavidin. For pre-blocking, 50 µM biotin was added to the fluorescent traptavidin 5 min before adding to cells. Cells were washed with PBS/Mg 3 times before imaging live. Cells were imaged using a wide-field DeltaVision Core fluorescent microscope (AppliedPrecision) with a 40× oil-immersion lens.

ECPF (436DF20 excitation, 480DF40 emission, Chroma 8602v1 dichroic) and Alexa Fluor 555 (540DF50 excitation, 600DF50 emission, Chroma 84100bs polychroic) images were collected and analyzed using softWoRx 3.6.2 software. Typical exposure times were 0.1 – 0.5 s and fluorescence images were background-corrected. Different samples in the same experiment were prepared, imaged and analyzed under identical conditions.

AFM analysis. AFM is described in Supplementary Note 1.

FtsK displacement assay. For the experiments shown in Figure 3, a 439 bp monobiotinylated DNA fragment was generated by PCR with Taq DNA polymerase using primers Fts1 and the internally biotinylated primer bioFts2 (Eurofins) from plasmid pJEG41-P1. This fragment contained two KOPS loading sites (GGGCAGGGGGGAGGG) positioned such that upon addition of ATP, it would take FtsK loaded at KOPS−0.5 s to translocate to streptavidin or traptavidin13. Pseudomonas aeruginosa FtsK PAK4, a soluble fragment containing the C-terminal 578 residues including the α, β and γ domains and a C-terminal 6His tag, was a kind gift of J. Graham (Oxford University). The protein was overexpressed in E. coli and purified by ammonium sulfate precipitation, nickel affinity chromatography and heparin affinity chromatography. The FtsK displacement assay was performed at 25 °C in 20 mM Tris acetate, 2 mM magnesium acetate, 20 mM potassium glutamate and 1 mM dithiothreitol (DTT) (pH 7.5). 16 nM DNA fragment was incubated with 2 µM streptavidin or traptavidin for 10 min, followed by 100 µM biotin to block free biotin binding sites. PAK4 FtsK was added at 0.5 µM in the experiments shown in Figure 3c,d and at the indicated concentrations in the experiment shown in Figure 3e and allowed to bind to the DNA for 5 min. We added 2 mM ATP to start the reaction and incubated the samples for 3 min for the experiments shown in Figure 3c,e and for the indicated times for the experiment shown in Figure 3d. Reactions were stopped by adding 1 µl 0.1% SDS with 200 mM EDTA (pH 8.0). Samples were
incubated for a further 15 min to allow FtsK to dissociate from the DNA, mixed with 6× gel loading buffer (1.2 M sucrose, 0.75 mM bromophenol blue; 0.93 mM xylene cyanol FF) and loaded on a 1.5% agarose gel in TAE at 6.0 V cm\(^{-1}\) for 45 min at 25 °C. Ethidium bromide–stained DNA was visualized on a ChemiDoc XRS imager. Percentage free = 100 × intensity of band for free DNA / (intensity of band for free DNA + intensity of band for bound DNA). Percentage displaced = 100 × (% free – % free for negative control)/(100 − percentage free for negative control). Negative values of percentage displaced were rounded up to zero.

For the experiment shown in Supplementary Figure 4, a 598-bp monobiotinylated DNA fragment, containing two 8 bp KOPS loading sites 226 bp from the biotinylated end, was generated by PCR with Taq DNA polymerase using primer Fts1 and the terminally biotinylated primer bioFts3 (Sigma-Genosys) (Supplementary Table 1) from plasmid pJEG41. E. coli FtsK\(_{50C}\), a soluble fragment containing the α, β and γ domains of FtsK, was purified as described\(^{30}\). The FtsK displacement assay was performed at 25 °C in 25 mM Tris (pH 7.5), 10 mM MgCl\(_2\), 5.9 nM DNA fragment was incubated with 0.5 µM streptavidin or traptavidin for 15 min, followed by 100 µM biotin to block free biotin binding sites. 1 µM FtsK\(_{50C}\) was added and allowed to bind to the DNA for 5 min. 2.5 mM ATP was added to start the reaction, which was stopped after 2 min with a final concentration of 0.1% SDS and 20 mM EDTA. Samples were incubated for 20 min and then mixed with 10× gel loading buffer (250 mM Tris (pH 7.5), 20 mM EDTA, 50% glycerol, 2.5% bromophenol blue) before loading on a 1.5% agarose gel in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.3) at 10 V cm\(^{-1}\) for 2 h at 25 °C. Gels were stained with SYBR Green (Invitrogen) for 2 h, washed in ddH\(_2\)O for 30 min, imaged using a Fuji FLA3000 scanner and quantified using Image Gauge software (Fuji).