Design of an allosterically modulated doxycycline and doxorubicin drug-binding protein

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Abstract

The allosteric interplay between distant functional sites present in a single protein provides for one of the most important regulatory mechanisms in biological systems. While the design of ligand-binding sites into proteins remains challenging, this holds even truer for the coupling of a newly engineered binding site to an allosteric mechanism that regulates the ligand affinity. Here it is shown how computational design algorithms enabled the introduction of doxycycline- and doxorubicin-binding sites into the serine proteinase inhibitor (serpin) family member α1-antichymotrypsin. Further engineering allowed exploitation of the proteinase-triggered serpin-typical S-to-R transition to modulate the ligand affinities. These design variants follow strategies observed in naturally occurring plasma globulins that allow for the targeted delivery of hormones in the blood. By analogy, we propose that the variants described in the present study could be further developed to allow for the delivery of the antibiotic doxycycline and the anticancer compound doxorubicin to tissues/locations that express specific proteases, such as bacterial infection sites or tumor cells secreting matrix metalloproteinases.

Significance

The design of binding sites for nonnatural ligands into natural proteins is a very topical subject in protein design. This also applies to the manipulation of allosteric coupling pathways with the goal of controlling protein function. Here it is shown that both topics can be addressed concurrently in a single protein. Thus, starting from human α1-antichymotrypsin, two drug-binding proteins were developed. Whereas ligand-binding design enables the binding of either the antibiotic doxycycline or the anticancer compound doxorubicin, the reengineering of an existing allosteric coupling mechanism enables the release of the bound ligands on occurrence of a proteinase trigger signal. It is proposed that such binding proteins can be further developed into drug-specific delivery shuttles.


Significance

The design of binding sites for nonnatural ligands into natural proteins is a very topical subject in protein design. This also applies to the manipulation of allosteric coupling pathways with the goal of controlling protein function. Here it is shown that both topics can be addressed concurrently in a single protein. Thus, starting from human α1-antichymotrypsin, two drug-binding proteins were developed. Whereas ligand-binding design enables the binding of either the antibiotic doxycycline or the anticancer compound doxorubicin, the reengineering of an existing allosteric coupling mechanism enables the release of the bound ligands on occurrence of a proteinase trigger signal. It is proposed that such binding proteins can be further developed into drug-specific delivery shuttles.


Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID codes SOM2 (DBS-I), SOM3 (DBS-II), SOM5 (DBS-I-allo), SOM6 (DBS-I-allo), SOM7 (DBS-II), 6FTP (DBS-II-apollo, crystal form 1), and SOM8 (DBS-II-apollo, crystal form 2)). The computer scripts reported in this paper have been deposited at zenodo.org (doi: 10.5281/zenodo.1239768).

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release of the anticancer drug doxorubicin in DBS-II-αllo are allosterically modulated by proteinases, such as matrix metalloproteinases (MMPs) secreted by tumor cells.

**Results**

**Engineering a Doxycycline and Doxorubicin-Binding Pocket into ACT.**

A doxycycline-binding pocket (DBS-I variants) and a doxorubicin-binding pocket (DBS-II variant) were designed into human ACT by reengineering the surface of β-sheet B (9) (SI Appendix, Fig. S1). The top of β-sheet B is framed by helices A and H (hA and hH) and is devoid of any ligand-binding properties in ACT and most serpins (SI Appendix, Fig. S24). In two atypical serpin members, namely CBG and TBG, the respective ligands are bound at this position (SI Appendix, Fig. S2B) (10, 11). The design effort reported here started out not from CBG or TBG, but rather from human ACT onto which in a first step the corticosteroid-binding site of CBG has been grafted following the substitution of 12 residues (SI Appendix, Table S1). This variant, termed DBS-0, binds cortisol with 7.6 μM affinity (Table 1 and SI Appendix, Fig. S34). Computational side chain-packing algorithms were then used to predict additional substitutions to transform the corticosteroid-binding pocket into either a doxycycline- or a doxorubicin-binding pocket (15) (SI Appendix, Fig. S1).

Two variants were obtained that bind ligands of medical interest: DBS-I, engineered to bind doxycycline, and variant DBS-II, engineered to bind doxorubicin. These ligands are not known to bind to ligand-specific binding globulins such as TBG and CBG, and their chemical structures differ quite considerably from those of the natural TBG and CBG ligands (Fig. 1 B–D). Successful design led to the generation of specific DBS-I proteins that carry 22–24 amino acid replacements compared with wild-type ACT and 13–15 amino acid replacements compared with DBS-0 (SI Appendix, Table S1). These DBS-I proteins bind doxycycline with affinities corresponding to $K_d$ values of approximately 80 μM (Fig. 2A and Table 1). DBS-II carries 24 substitutions and binds doxorubicin with $K_d$ values of 1.8 μM affinity (Fig. 2B and Table 1). The designed binding sites display distinct ligand-binding specificities. DBS-I and DBS-II do not bind cortisol, the ligand of DBS-0 (Table 1 and SI Appendix, Fig. S3). Conversely, DBS-0 does not bind either doxycycline or doxorubicin. DBS-II and DBS-I, which differ by two amino acid substitutions (DBS-I vs. DBS-II; SI Appendix, Table S1) share similar doxorubicin-binding affinities, however, in DBS-II, the ligand-binding affinity for doxycycline is slightly reduced compared with the DBS-I proteins (104.8 vs. 80 μM), indicating a slight specificity gain in DBS-II (Table 1). Inspecting the thermodynamic binding parameters reveals that doxycycline binding is purely enthalpy-driven (average $\Delta H = -61.6$ kJ/mol; $-\Delta T\Delta S = 38.2$ kJ/mol), whereas doxorubicin binding is favored by both enthalpy and entropy ($\Delta H = -16.3$ kJ/mol; $-\Delta T\Delta S = -16.8$ kJ/mol) (SI Appendix, Table S2).

Crystal structure analyses reveal details of the atomic interactions in the engineered ligand-binding sites. The RCLs of the DBS-I and DBS-II variants were proteolytically cleaved before crystallization to exploit the fact that crystallization of serpins is facilitated in the R-conformation. In these variants (and in contrast to the “allo” variants discussed below), the ligand-binding affinities do not significantly differ between the S- and R-conformations (Table 1), and therefore the ligand-binding interactions are expected to be highly similar in the two states. In the 1.5-Å resolution crystal structure of DBS-I in complex with doxycycline (SI Appendix, Table S3), the ligand binds on top of β-sheet B in an orientation as intended by the computational design (ligand rmsd, 1.6 Å, binding pocket side-chain rmsd, 1.8 Å, comparing the design model and crystal structure) (SI Appendix, Fig. S1).

The single most characteristic feature of the binding site is a ligand-binding clamp consisting of Trp386 from the loop that interconnects strands s4B and s5B and residue Phe277 from helix H (Fig. 2C). Introduction of Trp386 was motivated by the occurrence of a tryptophan at this position in the corticosteroid-binding site of CBG; in contrast, Phe277 emerged from side chain-packing calculations using the MUMBO program. Doxycycline is sandwiched between Trp386 and Phe277, but the three ring systems are not entirely coplanar. The side chains of Trp386 and Phe277 are orientated at an angle of approximately 20° with respect to the plane of the doxycycline ring system (SI Appendix, Fig. S2C). In total, eight residues participate directly in doxycycline binding by forming a hydrophobic binding pocket and providing numerous polar interaction partners (SI Appendix, Fig. S2D). Hydrogen bonds are formed either directly between doxycycline and His383 and Asp278 or indirectly via water molecules as is the case for Glu270, Asp278, and Trp386.

Doxorubicin binding to DBS-II makes use of the same ligand-binding clamp (Fig. 2D). In the 1.7-Å crystal structure of DBS-II in complex with doxorubicin, the A−D rings of doxorubicin are bound in a slightly different orientation compared with the doxycycline bound to DBS-I. The orientation differs by a 40° rotation around an axis oriented perpendicular to the respective ring systems (SI Appendix, Figs. S2E and S4). The three ring systems of doxorubicin, Phe277, and Trp386 are oriented almost perfectly coplanar, and an extensive π-stacking interaction is achieved (SI Appendix, Fig. S2C). Mutation of either Phe277 or Trp386 to alanine leads to a complete loss of binding affinity in DBS-II (Table 1), further emphasizing the central role of the ligand-binding clamp. DBS-I and DBS-II differ in sequence at positions 278 and 383, and Glu278 and Asn383 directly contribute to doxorubicin binding to DBS-II (SI Appendix, Fig. S2F). The mutation of Asn383 to alanine leads to a 13-fold reduction in binding affinity; in the Glu278 to alanine mutation, no significant reduction is observed (Table 1).

A remarkable interaction occurs between the amino group of the daunosamine moiety (ring E, Fig. 1D) of doxorubicin and DBS-II. The amino group adopts the function of a C-terminal helix cap, as it is in hydrogen-bond distance to the carbonyl oxygen of residues Phe277, Glu278, and Leu280 of helix H (Fig. 2D). In doxycycline-bound DBS-I (DBS-I), a water molecule is bound at exactly the same position, suggesting that this is a favorable position for hydrogen-bond interactions with the protein scaffold, and that release of this water molecule during doxorubicin binding might be a key contributor to the doxorubicin-associated entropy gain (SI Appendix, Table S2).
Table 1. Binding affinities determined by ITC

<table>
<thead>
<tr>
<th>Drug-specific binding protein</th>
<th>Titrated ligand</th>
<th>S-conformation $K_d$, µM</th>
<th>R-conformation $K_d$, µM</th>
<th>x-fold change in affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBS-0</td>
<td>Cortisol</td>
<td>7.6</td>
<td>9.6</td>
<td>1.3</td>
</tr>
<tr>
<td>DBS-0</td>
<td>Doxycycline</td>
<td>No measurable binding heats</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>DBS-0</td>
<td>Doxorubicin</td>
<td>No measurable binding heats</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>DBS-I</td>
<td>Cortisol</td>
<td>No measurable binding heats</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>DBS-I</td>
<td>Doxycycline</td>
<td>86.7</td>
<td>124.2</td>
<td>1.4</td>
</tr>
<tr>
<td>DBS-I</td>
<td>Doxycycline</td>
<td>78.6</td>
<td>101.5</td>
<td>1.3</td>
</tr>
<tr>
<td>DBS-I</td>
<td>Doxycycline</td>
<td>76.3</td>
<td>114.3</td>
<td>1.5</td>
</tr>
<tr>
<td>DBS-I</td>
<td>Doxycycline</td>
<td>76.3</td>
<td>153.0</td>
<td>2.0</td>
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<tr>
<td>DBS-I</td>
<td>Doxycycline</td>
<td>79.2</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>DBS-I-allo</td>
<td>Doxycycline</td>
<td>80.5 ± 7.4*</td>
<td>No measurable binding heats</td>
<td>≥10</td>
</tr>
<tr>
<td>DBS-I-allo2</td>
<td>Doxycycline</td>
<td>81.5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>DBS-I</td>
<td>Doxycycline</td>
<td>104.8</td>
<td>194.8</td>
<td>1.8</td>
</tr>
<tr>
<td>DBS-I</td>
<td>Doxycycline</td>
<td>1.8</td>
<td>2.5</td>
<td>1.4</td>
</tr>
<tr>
<td>DBS-I</td>
<td>Doxycycline</td>
<td>24.6</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>DBS-I</td>
<td>Doxycycline</td>
<td>24.6</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>DBS-I</td>
<td>Doxycycline</td>
<td>No measurable binding heats</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>DBS-I-allo</td>
<td>Doxorubicin</td>
<td>1.5 ± 0.1*</td>
<td>14.1 ± 2.8*</td>
<td>9.4</td>
</tr>
</tbody>
</table>

n.d., not determined.
*Mean ± standard deviation of three independent measurements.

Targeting the RCL of the Binding Proteins by Different Proteinases.

Serpins are cleaved by the RCL segment by the proteinases that they target. With the goal of developing a trigger-responsive binding protein, we explored the extent to which specific proteinase cleavage sites can be introduced into the RCL while at the same time retaining the ability of the serpin to undergo an S-to-R transition. Whereas the latter transition is expected to provide for a mechanism to actively expulse the bound ligand, proteinase specificity should ensure that ligand release is confined to locations where the proteinase occurs (Fig. 1A). To probe whether these premises can be met in DBS-I and DBS-II, the RCL segment was mutated to enable cleavage by one sequence-specific bona fide proteinase, the human rhinovirus 3C proteinase (HRV3CP), and two endogenous human proteinases, the MMPs MMP9 and MMP14, which were chosen because their levels are elevated in the stroma surrounding cancer cells where they promote extracellular matrix degradation (Table 2) (16). Thus, these specific proteinases represent interesting target proteinases that could be exploited for the triggered release of cytotoxic compounds at carcinomas.

The susceptibility of RCL cleavage between positions P1 and P1′ [using the P-nomenclature according to Schechter and Berger (17)] by specific proteinases can be readily altered by mutating up to nine residues. These residues cover six positions (P6–P1) preceding and three positions (P1–P3) following the putative cleavage site (Table 2). Initial evidence that DBS-I and DBS-II proteins can be efficiently and selectively cleaved by HRV3CP, MMP9, and MMP14 was obtained by SDS/PAGE analyses. Cleavage of the RCL between P1 and P1′ reduces the molecular weight of all studied protein samples by approximately 4.7 kDa as a result of the release of a short C-terminal peptide (residues 361–400) under denaturing conditions (Fig. 3A and SI Appendix, Fig. S5). Additional experiments provide insight into whether a serpin adopts the S-conformation or the R-conformation and thereby allow monitoring of the S-to-R transition. The S- and R-conformations differ by (i) an increased thermal stability of the R-conformation, (ii) differing secondary structure compositions, and (iii) an enhanced tendency of the S-conformation to form aggregates on heating (9). While the former two factors can be monitored by circular dichroism (CD) spectroscopy, aggregation can be visualized in a native PAGE (18). HRV3CP, MMP9, and MMP14-cleavable DBS-I and DBS-II constructs show similar thermal unfolding behavior in the S-conformation, with denaturing/aggregation between 45 °C and 50 °C (Fig. 3 B and C and SI Appendix, Fig. S6 A–H). Their thermal stability is only slightly impaired compared with the wild-type ACT protein, which is stable up to 55 °C under the same experimental conditions (SI Appendix, Fig. S6). After RCL cleavage, all DBS-I and DBS-II constructs display increased thermal stability, as expected for serpins in the R-conformation. Furthermore, the far-UV CD spectra of all cleaved samples suggest an increase in β-sheet content compared with the uncleaved samples, which is apparent from a shifted zero-crossing from 203 nm to 205 nm (Fig. 3D and SI Appendix, Fig. S6). Bioinformatics analyses of these spectra show a 5% increase in β-sheet content and a 4% decrease in disordered regions comparing cleaved with intact DBS proteins (here DBS-I) (19). This finding is in agreement with insertion of the RCL as an additional β-strand s4A upon the S-to-R transition. These combined analyses show that all DBS-I and DBS-II proteins adopt a defined S-conformation before and a serpin-typical R-conformation after proteinase-specific cleavage.

Insight into how the variants accommodate the altered RCL sequences is obtained from crystal structures. After the S-to-R transition, the newly formed β-strand s4A buries the residues preceding the P1-P1′ cleavage site and corresponding to positions P14–P3 of the RCL sequence into the interior of the serpin fold (9). The crystal structures show that the RCL sequences of the HRV3CP and MMP-cleavable DBS-I and DBS-II proteins can be readily accommodated into β-sheet A (Fig. 3 E–G), and that all proteins display a highly similar R-conformation. This also applies to MMP9 and MMP14-cleavable DBS-I variants, which display a proline at position P3. Prolines cannot be accommodated at the center of β-sheets; however, the crystal structures show that the cyclic side chain of proline has only a minor effect on the hydrogen-bond pattern at the C-terminal tip of β-strand s4A.

Allosteric Modulation of Doxycycline- and Doxorubicin-Binding Affinities.

A hallmark of the proposed binding proteins is the intended coupling of the ligand release to the proteinase-triggered S-to-R transition. Such a coupling is absent in DBS-0, DBS-I, and DBS-II, since...
in these variants only 1.3- to 1.4-fold changes in affinity are observed on proteasme cleavage (Table 1). To achieve a pronounced change in affinity, different substitutions were introduced at RCL position P12, which becomes part of the newly formed strand s4α after the S-to-R transition. Isothermal titration calorimetry (ITC) measurements with doxorubicine titrated to intact and cleaved DBS-I P12 mutants show that a tyrosine at position P12 (DBS-I) has only a slight effect on the affinity change, whereas a lysine at P12 (DBS-I) induced a twofold reduction in binding affinity upon the S-to-R transition (Table 1). However, when substituting the P12 residue with arginine, no measurable binding heats were observed in the HRV3CP-cleaved protein, while the affinity of the intact protein remained unaltered (Fig. 4A and Table 1). We term this allosterically regulated variant DBS-I-allo and estimate an at least 10-fold reduction in affinity between intact and cleaved DBS-I-allo, when taking the ITC experimental setup and expected heat detection limits into account (20). When introducing the same P12 arginine substitution into the doxorubicin-binding protein, the resulting variant (DBS-II-alleo) shows again a very pronounced reduction in affinity on cleavage, namely a 9.4-fold reduction (Fig. 4B and Table 1).

The structural impact of the P12 A349R substitution onto the ligand-binding site is apparent from the 1.6-Å crystal structure of HRV3CP-cleaved DBS-I-alleo (SI Appendix, Table S3). As a consequence of the spatial requirements for the accommodation of the arginine side chain, β-sheet B and helix A become considerably displaced and helix D becomes slightly displaced compared with DBS-I, while the additionally present helix H remains unaffected (Fig. 4C). Since residues from all these secondary structure elements (except for helix D) participate in ligand binding, the simultaneous occurrence of shifted and nonshifted secondary structure elements has a considerable impact on the geometry of the ligand-binding site. A direct consequence is that in HRV3CP-cleaved DBS-I-alleo, the side chain of Trp386 swings inward and obstructs the binding site. This leads to a drastic reduction of the solvent-accessible pocket volume, from 77 Å³ to 41 Å³ (Fig. 4D) (21). Interestingly, the structural impact of the A349R substitution is restricted to the binding site only; HRV3CP-cleaved DBS-I, and DBS-I-alleo, can be superimposed with an rmsd as low as 0.6 Å for 316 Cα atoms.

A similar picture is obtained for the doxorubicin variant DBS-II-alleo. When crystallizing cleaved DBS-II-alleo in the presence of doxorubicin, two different types of diffraction datasets were recorded (SI Appendix, Table S5). In crystal form 1, residual density in and local disorder around the binding site can be observed. The ligand-binding site is partially occupied, and the pocket elements hA and the s4βs5β display two alternative conformations that together lead to narrowing of the binding pocket (SI Appendix, Fig. S7). The partial ligand occupancy is in agreement with the observed residual binding affinity of cleaved DBS-II-alleo (Table 1). In crystal form 2, the binding site is empty, and the doxorubicin-free structure shows how the relief of Van-der-Waals repulsions surrounding the newly introduced Arg349 leads to distortions in the ligand-free binding sites of cleaved DBS-II-alleo that are similar to those in DBS-I-alleo (SI Appendix, Fig. S7). Together, the crystal structures of cleaved DBS-I-alleo and DBS-II-alleo provide an explanation for how the A349R substitution introduces an effective mechanism that allosterically couples the already present S-to-R transition in ACT to a reduction in ligand-binding affinity.

The active release of doxorubicin from DBS-II-alleo upon proteinase-triggered S-to-R transition was monitored in real time in a fluorescence experiment. Whereas the solution of a preformed complex of DBS-II-alleo and doxorubicin exhibited a constant fluorescence intensity for more than 2 h, addition of the HRV3CP proteinase to the complex resulted in a slow 2.5-fold increase of fluorescence with a time constant of 70 min (Fig. 4E). Analysis of the fluorescence properties shows that the initial fluorescence of the DBS-II-alleo–doxorubicin complex is caused by the presence of a small proportion of unbound doxorubicin (SI Appendix, Fig. S8). When bound to DBS-II-alleo in the S-conformation, doxorubicin showed no detectable fluorescence. The doxorubicin fluorescence was completely quenched in the binding site, which can be explained by the direct stacking contacts with Trp386. RCL cleavage and transition to the R-conformation did not result in a doxorubicin fluorescence that could be attributed to the existence of any remaining protein-bound doxorubicin population (i.e., with a longer diffusion time, a longer fluorescence lifetime, or a significantly increased fluorescence anisotropy decay time). Instead, the observed fluorescence increase is related solely to the cleavage-induced release kinetics of freely diffusing doxorubicin (SI Appendix, Fig. S8).

Table 2. Altered RCL sequences for selective cleavage by targeted proteinases

<table>
<thead>
<tr>
<th>Targeted proteinase</th>
<th>RCL sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Chymotrypsin</td>
<td>V K I T L L S A L</td>
</tr>
<tr>
<td>MMP9</td>
<td>L E V L F Q G P L</td>
</tr>
<tr>
<td>MMP14</td>
<td>L F I P L S L R M</td>
</tr>
<tr>
<td>HRV3CP</td>
<td>L E V L F Q G P L</td>
</tr>
</tbody>
</table>

*a-P-nomenclature according to Schechter and Berger (17).
†Proteinase-specific cleavage sites as described previously (32–34).
Discussion

The current study shows that binding sites for the nonnatural ligands doxycycline and doxorubicin can be introduced into the ACT scaffold. At the same time, it is possible to allosterically couple the ligand affinities to the serpin-specific S-to-R transition. Since we also demonstrate that it is possible to trigger the S-to-R transition for the modulation of the ligand-binding affinity. To achieve this, an arginine was introduced at RCL position P12. Interestingly, the same P12 mutation has been structurally characterized previously in ACT in an entirely different context (22). The authors proposed that β-sheet B serves as a “shock absorber” as a result of the additional space required to accommodate the arginine side chain. This is in accordance with our observations in cleaved DBS-I-allo and DBS-II-allo variants with the additional bonus that the doxycycline- and doxorubicin-binding pockets narrow and ligand binding is impeded. A possible route for providing additional energy for disrupting the binding site could be the inclusion of an additional mutation, such as A351R (P10), since the P10 residue also points toward β-sheet B on completion of the S-to-R transition (22).

Fig. 3. Altered RCL sequences are compatible with the serpin-typical S-to-R transition. (A) RCL cleavage of DBS-I by HRV3CP as visualized on SDS/PAGE. Lane S depicts uncleaved protein (black arrow) and lane R cleaved protein (gray arrow) with the released C-terminal peptide (white arrow) and the proteinase HRV3CP (red arrow) highlighted. (B) Thermal denaturation of uncleaved DBS-I-ell (solid line) and of cleaved DBS-I-ell (dotted line). (C) Native PAGE illustrating temperature-induced oligomerization of uncleaved DBS-I-ell, while cleaved DBS-I-ell is highly temperature-stable. (D) Far-UV CD spectra of HRV3CP-cleavable DBS-I-ell in its uncleaved (solid line) and cleaved (dotted line) state. (∼G) Close-up views of the central β-sheet A with the RCL conformation following the S-to-R transition and yielding new β-strand s4A for HRV3CP-cleaved DBS-I-ell (E), MMP9-cleaved DBS-I-ell-α (F), and MMP14-cleaved DBS-I-ell (G). The mF-DF map is contoured at 1.2 σ (gray mesh) and displayed in a 2-Å radius of the depicted atoms. The mF-DF, density maps are displayed at +3 σ in green and −3 σ in red.

Fig. 4. Allosteric modulation of the binding affinity. (A and B) ITC experiments showing the titration of doxycycline into uncleaved (black) and cleaved (red) DBS-I-α (A) and doxorubicin into uncleaved (black) and cleaved (red) DBS-II-α (B). Open symbols indicate ligand-into-buffer reference titrations in the lower parts of A and B. (C) A structural comparison of selected secondary structure elements of variants DBS-I-ell (green) and DBS-I-α (red), providing insight into the allosteric coupling mechanism. Doxycycline bound to cleaved DBS-I (green) and the A349R substitution at position P12 in DBS-I-α (red) are shown in a stick presentation. (D) Surface presentation of the open pocket of cleaved DBS-I, with bound doxycycline (top) and the closed pocket of cleaved DBS-I-α (Bottom). (E) Fluorescence trace of unbound doxorubicin. Black, fluorescence signal of the DBS-II-α-doxorubicin complex. Red, fluorescence signal showing the release of doxorubicin from the DBS-II-α-doxorubicin complex on addition of the protease after 5 min. Blue, monoeponential fit of doxorubicin release kinetics.
Approximately 10-fold reductions in ligand-binding affinities were observed in DBS-I-αllo and DBS-II-αllo on RCL cleavage (Table 1). In the two naturally occurring serpin-based binding globulins TBG and CBG, which bind their respective ligands with 2.2 and 45 nM affinity, respectively, the reduction in ligand-binding affinity upon the S-to-R transition is also only 5.5-fold (TBG) and 10-fold (CBG), yet these changes are of functional importance (22–25). It should also be noted that in CBG and TBG, an entirely different allosteric coupling mechanism has been identified that relies on the extension of helix D as responsible for the coupling between the RCL insertion site and the ligand-binding site (10, 11, 25).

Taken together, the modulation of the ligand-binding affinity that we achieved in the present study equals or even exceeds those observed in natural binding globulins. That ligand release is substantial and directly increases the local concentration of the free ligand, could be monitored in fluorescence experiments. When considering the experimental setup, the concentration of the released doxorubicin is estimated at approximately 1 μM, which compares well with the IC50 Values reported for the cytotoxicity of doxorubicin for different cell lines (0.1–1.0 μM) (26). This shows that in the reported setup, DBS-II-αllo is able to release doxorubicin in concentrations that could kill surrounding cells.

The proof-of-concept study presented here holds many promises for the development of artificial binding globulins for therapeutic applications. Since we started out with ACT, a blood-circulating human protein, and introduced only a limited number of mutations, any antigenic response can be expected to be low. In addition, the general topology of the binding site, which is located on top of a β-sheet and is flankned by two helices, has previously been recognized as being ideal for ligand binding (27). Thus, this should facilitate the incorporation of binding sites for highly diverse ligands. The targetted delivery of therapeutic compounds is of increasing interest (8). The serpin-based drug-binding protein described in the present study opens up interesting new venues for achieving this goal.

Materials and Methods

The design effort followed a step-by-step strategy alternating between computational design and validation cycles (SI Appendix, Fig. 51). MUMBO (https://www.biotechnik.nat.fau.de/research/downloads/) was used for designing the ligand-binding sites (15). Protein mutagenesis, production, and purification started out from a purchased pQE-T7 expression vector harboring the gene encoding for ACT (UNIPROT P01011; Qiagen). CD measurements were recorded on a Jasco J-815 spectropolarimeter, ITC experiments were performed in a Nano ITC standard cell (TA Instruments) and fluorescence measurements were obtained with a custom-designed confocal microscope modified to record doxorubicin fluorescence for λ > 605 nm (28). Diffraction data were recorded at synchrotron beamlines BL14.1 and BL14.2 of BESSY II (Berlin, Germany) and beamline P14 of PETRA III (Hamburg, Germany). Crystal structures were determined using XDS, CCP4, and PHENIX software suites (29–31). Detailed descriptions of the computational design, mutagenesis, protein expression, purification and analysis, CD, ITC, fluorescence measurements, and structure determinations are provided in SI Appendix, Materials and Methods.

ACKNOWLEDGMENTS

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