Impact of subunit linkages in an engineered homodimeric binding protein to α-synuclein

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Aggregation of the protein α-synuclein (α-syn) has been implicated in Parkinson’s disease and other neurodegenerative disorders, collectively referred to as synucleinopathies. The β-wrapin AS69 is a small engineered binding protein to α-syn that stabilizes a β-hairpin conformation of monomeric α-syn and inhibits α-syn aggregation at substoichiometric concentrations. AS69 is a homodimer whose subunits are linked via a disulfide bridge between their single cysteine residues, Cys-28. Here we show that expression of a functional dimer as a single polypeptide chain is achievable by head-to-tail linkage of AS69 subunits. Choice of a suitable linker is essential for construction of head-to-tail dimers that exhibit undiminished α-syn affinity compared with the solely disulfide-linked dimer. We characterize AS69-GS3, a head-to-tail dimer with a glycine-serine-rich linker, under oxidized and reduced conditions in order to evaluate the impact of the Cys28-disulfide bond on structure, stability and α-syn binding. Formation of the disulfide bond causes compaction of AS69-GS3, increases its thermostability, and is a prerequisite for high-affinity binding to α-syn. Comparison of AS69-GS3 and AS69 demonstrates that head-to-tail linkage promotes α-syn binding by affording accelerated disulfide bond formation.

Keywords: alternative scaffold/amyloid/disulfide bond/α-synuclein/β-wrapin

Introduction

Protein aggregates are a feature of several diseases, including many neurodegenerative diseases. For example, senile plaques consisting of the amyloid-β peptide (Aβ) are a neuropathological feature of Alzheimer’s disease (Querfurth and LaFerla, 2010), while Lewy bodies containing α-synuclein (α-syn) as the main protein component are a characteristic of Parkinson’s disease, dementia with Lewy bodies and other synucleinopathies (Lashuel et al., 2013). The development of protein aggregation inhibitors constitutes a promising therapeutic approach (Härder and Lendel, 2012). Several classes of molecules have been explored to counteract the deleterious effects of fibrils and of the particularly toxic oligomeric assemblies of α-syn. Small molecules interfering with α-syn aggregation, e.g. various polyphenols, generally act by complex mechanisms, including direct binding to the intrinsically disordered protein, oxidation, covalent modification and stabilization of non-toxic α-syn oligomers (Li et al., 2004; Masuda et al., 2006; Ehmcke et al., 2008; Meng et al., 2009; Zhou et al., 2009). The propensity to self-associate into chemical aggregates is an essential property of aggregation-inhibiting compounds (Feng et al., 2008; Lendel et al., 2009; Lamberto et al., 2011). Different hot spots for small molecule interactions were identified in the α-syn sequence, in the N-terminal region as well as in the central non-Aβ component (NAC) region and in the C-terminal region (Norris et al., 2005; Herrera et al., 2008; Lamberto et al., 2009; Lendel et al., 2009). Aggregation-inhibiting peptides with the potential to add to α-syn aggregates and to block any further aggregate growth were designed by modification of short amino acid stretches from the aggregation-prone NAC region, either by fusion of solubilizing amino acid residues or by N-methylation (El-Agnaf et al., 2004; Madine et al., 2008). Antibody-based approaches are particularly promising for the therapy of neurodegenerative diseases (Valera and Masliah, 2013). The predominantly intracellular localization of α-syn suggests the application of intrabodies, and anti-α-syn scFv antibodies indeed inhibit aggregation and toxicity upon transfection in cell culture models (Zhou et al., 2004; Lynch et al., 2008). However, recent evidence demonstrates that accumulation of α-syn oligomers in the plasma membrane and their propagation from cell-to-cell play crucial roles in the synucleinopathies (Lee et al., 2014). Therefore, extracellular targeting of α-syn is a viable approach for immunotherapy of synucleinopathies. Both active and passive immunization against α-syn proved successful in mouse models (Masliah et al., 2005, 2011). Passive immunization with the antibody 9E4 recognizing a C-terminal epitope resulted in reduced α-syn accumulation, reduced neurodegeneration, and reduced motor deficits (Masliah et al., 2011). α-Syn immunotherapy appears to be effective through a combination of mechanisms, including the binding of membrane-bound α-syn oligomers, followed by receptor-mediated endocytosis and degradation by autophagy, as well as the blocking of the propagation of misfolded α-syn (Valera and Masliah, 2013; Tran et al., 2014). One active and one passive immunization study are currently tested clinically, i.e. the vaccine AFFITOPE PD01 (Schneeberger et al., 2012) and PRX002, a humanized version of 9E4.

The Affibody molecule ZAβ3 is a potent inhibitor of Aβ aggregation, obtained by phage display of a scaffold derived from staphylococcal protein A (Grönwall et al., 2007). The small size of ZAβ3 (two subunits of ~60 amino acids each) has facilitated the detailed analysis of its interaction with Aβ (Hoyer et al., 2008; Hoyer and Härder, 2008). ZAβ3 wraps the aggregation-prone sequence stretches of Aβ by forming a hydrophobic tunnel upon coupled folding and binding. Bound Aβ adopts a β-hairpin conformation, with those sequence regions forming an intramolecular β-sheet that otherwise build the core of the intermolecular β-sheets in amyloid fibrils (Hoyer et al., 2008). The amyloid-like conformation of ZAβ3-bound Aβ prompted us to exploit ZAβ3 as a scaffold for the generation...
of binders to other amyloidogenic targets. We generated ZAβ3-based phage display libraries from which ligands are selected that we term β-wrapins (β-wrapin), referring to the observation that ZAβ3 wraps around its target which adopts a β-structure in the complex. AS69 is a β-wrapin selected to bind to α-syn, exhibiting an affinity of 240 nM (Mirecka et al., 2014). In complex with AS69, α-syn forms a β-hairpin in the sequence region of amino acids 37–54, which contains most of the reported disease-related mutations (Fig. 1A) (Mirecka et al., 2014). Concomitantly, α-syn aggregation is inhibited, with substantial increases in the lag time of aggregation even at low substoichiometric concentrations of AS69 (Mirecka et al., 2014).

Like ZAβ3, AS69 is a homodimer covalently linked by a disulfide bridge between the subunits’ single cysteine residues, Cys-28 (Fig. 1A). The disulfide bond connects the helices α1 of both subunits and is located close to the interface with the β-hairpin of α-syn. In addition to the disulfide linkage, two β-wrapin subunits can be fused on the nucleic acid level to yield head-to-tail linked dimers. Head-to-tail linkage of ZAβ3 subunits proved to be advantageous for treatment of a Drosophila melanogaster model of Alzheimer’s disease (Luheshi et al., 2010). Expression of a head-to-tail variant of ZAβ3 inhibited the toxicity of wild-type Aβ42 and of the arctic mutant Aβ42(E22G) almost completely, while expression of individual ZAβ3 subunits was only partially effective. In order to evaluate the effect of AS69 on α-syn pathology in cell culture and animal models of synucleinopathies, it would thus be desirable to likewise employ a single-chain AS69 dimer. Such a construct would also be a preferable starting point for combinatorial protein engineering for affinity maturation of AS69, since it would allow independent optimization of the subunits (Lindberg et al., 2013).

Here we investigate the linker requirements for head-to-tail fusion of AS69 subunits. We generated AS69-GS3, a single-chain construct containing a glycine-serine-rich linker that exhibits the same affinity for α-syn as solely disulfide-linked AS69, and employed it to evaluate the impact of the Cys-28 disulfide bond on AS69 and its interaction with α-syn. Finally, we examined the effect of head-to-tail linkage on the kinetics of disulfide formation.

**Materials and methods**

**Protein preparation**

Genes encoding the head-to-tail constructs AS69-Oct1-TEV, AS69-GS2-TEV, as well as a direct head-to-tail fusion of two AS69 subunits, were obtained from Life Technologies. The gene encoding AS69-GS3 was generated from the AS69 gene by introduction of the linker sequence by PCR using specifically designed primers. Here, the first subunit was amplified employing the T7 promoter, 5’-TAA TAC GAC TCA CTA TAG GG, and the reverse primer 5’-GTA GAT AAC AAA TTC, and T7 terminator, 5’-A TAT GCC ACC CTG GCC ACT GCC ACC ACC TTC TTG CCG CCG CTG AGC, thus adding one copy of (G4S) to the first subunit. Amplification of the second subunit along with addition of two copies of (G4S) was achieved using the forward primer 5’-A TAT GCC CAG GTG GCC GGT GCC GAT GGT GCC GGT GCC AGT GTA GAT AAC AAA TTC, and T7 terminator, 5’-GCT AGT TAT TGC TCA TCG G. Genes were subjected to digestion using the appropriate restriction enzymes, i.e. EcoRI, AverII and BglII (New England Biolabs), followed by ligation into the pET-302/NT-His expression vector and subsequent transformation into electrocompetent Escherichia coli JM109 cells. Sequence congruence of all constructs was verified by DNA sequencing (MWG Biotech). AS69 and the head-to-tail constructs were expressed from the pET-302/NT-His vector and purified as described previously (Mirecka et al., 2014). In short, following expression and cell lysis, lysates were cleared by centrifugation and proceeded for purification on a HisTrap FF affinity column (GE Healthcare), followed by further purification on a HiLoad 16/60 Superdex 75 size exclusion chromatography column (GE Healthcare). Purity of the peak fractions was confirmed by application on 16.5% Tris-Tricine SDS–PAGE (Bio-Rad) and visualization by Coomassie Blue staining. Expression and purification of α-synuclein were performed as previously described (Mirecka et al., 2014).

**Isothermal titration calorimetry**

Isothermal titration calorimetry (ITC) was performed on a Microcal iTC200 calorimeter (GE Healthcare) at 30°C. The
buffer was 20 mM sodium phosphate, 50 mM NaCl, pH 7.4. The titrant in the syringe was at ~10-fold higher concentration than the titrant in the cell, which was applied at a concentration in the range of 35–180 μM. For the experiment investigating the α-syn affinity of AS69-GS3 under reducing conditions, 5 mM DTT was added to the protein samples. The heat of post-saturation injections was averaged and subtracted from each injection to correct for heats of dilution and mixing. Data were processed using MicroCal Origin software provided with the calorimeter. Apparent dissociation constants $K_{d}^{app}$ were obtained from a nonlinear least-squares fit to a 1 : 1 binding model. AS69 and its head-to-tail variants show partial thermal unfolding (see Fig. 6) at the temperature of the ITC experiment. For those constructs with experimentally determined unfolding (see Fig. 6) at the temperature of the ITC experiment. The thermal denaturation profiles, the $K_{d}^{app}$ values were therefore corrected for the contribution from the coupled folding equilibrium (Dincbas-Renqvist et al., 2004), yielding the $K_{d}$ values given in Table I.

**Aggregation assay**

Aggregation of α-syn was monitored by thioflavin T (ThT) fluorescence (LeVine, 1999). Aggregation reactions contained 50 μM of α-syn and 40 μM ThT in 20 mM sodium phosphate, 50 mM NaCl, pH 6.0, 0.04% Na-azide, in a final volume of 150 μL. Aggregation was performed at 37 °C with continuous orbital shaking (300 rpm) in a round-bottom 96-well black plate (Nunc) containing a 2 mm glass bead in each well. ThT fluorescence was excited at 440 nm and measured at 480 nm on an Infinite M1000 plate reader (Tecan). The signal of a buffer sample containing ThT was subtracted for background correction.

**Circular dichroism spectroscopy**

Far-UV circular dichroism (CD) spectra were measured on a JASCO J-815 spectropolarimeter in a 0.5 mm Quartz cuvette (Hellma) using protein samples at a concentration of 17.5 μM. The buffer was 20 mM sodium phosphate, 50 mM NaCl, pH 7.4. For measurements under reducing conditions, Tris-(2-carboxyethyl)-phosphine (TCEP) (Thermo Scientific) was added at a concentration of 10 mM. Melting curves were recorded at 222 nm with a heating rate of 1 °C min$^{-1}$. Melting temperatures were derived from fits of the melting curves to a two-state unfolding model (Pace et al., 1998).

**Analytical size exclusion chromatography**

Analytical size exclusion chromatography (SEC) was performed on a Superdex 75 10/300 GL column (GE Healthcare) connected to an ÄKTÀ Purifier system (GE Healthcare) at a temperature of 20 °C and a flow rate of 0.5 ml min$^{-1}$. Proteins were detected by absorbance at 280 nm. Samples of 0.1 ml at a concentration of 180 μM (AS69) or 160 μM (AS69-GS3) were injected and eluted with 20 mM sodium phosphate, 50 mM NaCl, pH 7.4. For SEC experiments under reducing conditions, the protein samples were reduced by incubation with 10 mM TCEP for 1 h at 20 °C, and 5 mM dithiothreitol (DTT) was added to the elution buffer. Molecular weight calibration was achieved with conalbumin, ovalbumin, carbonic anhydrase, ribonuclease A and aprotinin as globular protein standards.

**Analytical RP-HPLC**

Separation and quantification of the oxidized and reduced fractions of AS69 and AS69-GS3 were achieved by injecting 20 μl of protein solutions at a concentration of 115 μM (subunit concentration 230 μM) onto an analytical Zorbax 300SB-C8 RP-HPLC column (5 μm, 4.8 × 250 mm, Agilent) connected to an Agilent 1200 Infinity system at a column temperature of 80 °C and a flow rate of 1 ml/min. The analysis was performed using a gradient of 30–36% (vol/vol) acetonitrile, 0.1% (vol/vol) TFA, in water within 20 min, followed by an isocratic step at 36% (vol/vol) acetonitrile, 0.1% (vol/vol) TFA, in water for 3 min. Ultra-violet absorption at 214 nm was used for protein detection and relative quantification of the oxidized and reduced fractions of AS69 and AS69-GS3. Reverse phase-high performance liquid chromatography (RP-HPLC) of AS69 and AS69-GS3 in the absence and presence of 10 mM TCEP was performed to determine the elution volumes of the oxidized and reduced proteins. To follow the kinetics of disulfide bond formation, AS69 and AS69-GS3 were reduced by incubation with 10 mM TCEP for 30 min at 25 °C. TCEP was removed by size exclusion chromatography on Sephadex G-25 (two HiTrap Desalting 5 ml columns (GE Healthcare) connected in series to an ÄKTÀ Purifier system) using 20 mM sodium phosphate, 50 mM NaCl, pH 7.4, as buffer system at a flow rate of 1.5 ml min$^{-1}$. Protein containing fractions were collected and immediately used for analysis of reoxidation kinetics ($t = 0$ min). The reoxidation reaction was performed by incubation at 25 °C in closed reaction tubes without agitation. Aliquots were withdrawn at different time points and analyzed for the amount of oxidized and reduced states by RP-HPLC as described above.

**Kinetics of disulfide bond formation**

The kinetic data of reoxidation of AS69-GS3 were fitted as a single exponential in compliance with an intramolecular reaction, yielding the first-order rate constant $k_{AS69-GS3}$. AS69 reoxidation involves dimerization and was fitted to the dimerization rate law, providing the second-order rate constant $k_{AS69}$:

\[
\frac{1}{[\text{AS69}_{\text{red}}]} = k_{AS69}t + \frac{1}{[\text{AS69}_{\text{red}}]_{t=0}}
\]

The effective concentration of subunits in the disulfide formation reaction of AS69-GS3 was calculated from the above rate constants as $2k_{AS69-GS3}/k_{AS69}$ according to Robinson and Sauer (1996).

**Results**

**Linker sequence is critical to retain α-syn affinity in head-to-tail AS69 dimers**

In the case of ZAβ3, direct fusion of two subunits without introduction of an extra linker sequence was feasible for retaining a functional dimer (Hoyer and Härd, 2008; Luaheshi et al., 2010; Lindberg et al., 2013). The N-terminal ~13 residues of ZAβ3 remain disordered in the complex with Aβ and can therefore serve as a linker. Likewise, the N-terminus of α-syn-bound AS69 is disordered (Mirecka et al., 2014). Direct head-to-tail linkage of AS69 subunits, however, resulted in a complete loss of α-syn binding according to ITC (Fig. 2A, Table I). Therefore, we introduced different linker sequences (Fig. 1B) and tested their ability...
to restore the affinity for α-syn. Introduction of a 15-amino acid variant of the disordered, flexible linker of the Oct-1 POU domain (van Leeuwen et al., 1997) with an additional TEV protease cleavage site resulted in the head-to-tail construct AS69-Oct1-TEV. Titration of AS69-Oct1-TEV with α-syn gave very weak, slowly decaying binding heat indicative of very slow association kinetics, demonstrating that the head-to-tail linkage interferes with binding (data not shown). Glycine-serine-rich sequences such as (Gly4-Ser)3 are frequently used as flexible linkers, particularly to link antibody domains in a single-chain Fv format (Huston et al., 1988). We tested two glycine-serine-rich linkers, with and without an additional TEV protease cleavage site. In both constructs, AS69-GS2-TEV and AS69-GS3, the α-syn affinity of the solely disulfide-linked AS69 was recovered (Fig. 2B, Table I).

To test whether the head-to-tail linkage of AS69 interferes with its aggregation-inhibitory action, α-syn aggregation was followed in the presence and absence of AS69-GS3 (Fig. 3). The increased fluorescence of the dye ThT upon binding to the amyloid cross-β structure was used as an indicator of α-syn aggregation (LeVine, 1999). Stoichiometric amounts of AS69-GS3 completely inhibited α-syn aggregation, while substoichiometric amounts led to significant increases in the aggregation lag-time (Fig. 3), matching the data previously obtained for AS69 (Mirecka et al., 2014).

The Cys-28 disulfide causes compaction and increased thermostability of AS69-GS3 and is essential for α-syn binding

We studied the properties of AS69 and AS69-GS3 before and after reduction of the disulfide bond, in order to evaluate the impact of the head-to-tail and disulfide linkages on structure, stability and α-syn binding of AS69. The investigated AS69 configurations are schematically depicted in Fig. 1C. High-affinity binding of α-syn required the presence of the Cys-28 disulfide linkage, which is evidenced by an ~1000-fold lower affinity of AS69-GS3 as a consequence of disulfide bond cleavage upon reduction (Table I).

The secondary structure contents of AS69 and AS69-GS3 in their oxidized and reduced states were analyzed by CD spectroscopy (Fig. 4). The CD spectra of AS69 and AS69-GS3 show minima at 208 and 222 nm, in agreement with largely α-helical conformation. Upon disulfide bond reduction, a slight decrease in the 222:208 nm ellipticity ratio is observed for both, AS69 and AS69-GS3, in line with partial unfolding. SEC was performed for comparison of the hydrodynamic

### Table 1. Affinity for α-syn of AS69 variants with different dimer linkages determined by ITC at 30°C

<table>
<thead>
<tr>
<th>Head-to-tail linkage</th>
<th>Cys-28 disulfide</th>
<th>$K_{d}^{app}$ (μM)</th>
<th>$K_{d}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Oxidized</td>
<td>0.24</td>
<td>0.18</td>
</tr>
<tr>
<td>Direct head-to-tail linkage</td>
<td>Oxidized</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Oct1-TEV</td>
<td>Oxidized</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>GS2-TEV</td>
<td>Oxidized</td>
<td>0.14</td>
<td>0.25</td>
</tr>
<tr>
<td>GS3</td>
<td>Oxidized</td>
<td>0.25</td>
<td>0.21</td>
</tr>
<tr>
<td>GS3</td>
<td>Reduced</td>
<td>280</td>
<td>200</td>
</tr>
</tbody>
</table>

*Corrected for coupled AS69 folding equilibrium.

n.d., not detected.

n.d., not determined, weak heat signal with a time profile indicative of very slow association kinetics.

![Fig. 2. Impact of the linker on the α-syn affinity of head-to-tail AS69 constructs determined by ITC. (A) No heat of binding were detected when 465 μM α-syn was titrated into a 54 μM solution of a head-to-tail construct in which two AS69 subunits are directly fused without an extra linker. (B) Titration of 716 μM AS69-GS3 into 75 μM α-syn, yielding an apparent affinity of $K_{d}^{app} = 250$ nM.](image)

![Fig. 3. Inhibition of α-syn aggregation by AS69-GS3. Kinetics of α-syn aggregation in the absence and presence of the indicated concentrations of AS69-GS3 monitored by ThT fluorescence.](image)
volumes of the different β-wrapin configurations (Fig. 5). According to calibration with globular standard proteins, AS69 and AS69-GS3 eluted as proteins with apparent masses of 24 or 27 kDa, respectively, although their real molecular weight (MW) is ~15 kDa. The high apparent MW reflects the presence of disorder in the N-termini, as previously observed for ZAβ3 (Hoyer and Hard, 2008). Upon disulfide bond reduction, elution of AS69 is strongly retarded, demonstrating the separation of the homodimer into its subunits. In contrast, AS69-GS3 elutes earlier from SEC after disulfide bond reduction, at an apparent MW of 32 kDa. The increased hydrodynamic volume of reduced AS69-GS3 suggests that the interface between the subunits’ α1-helices is not fully established if the disulfide bond is not formed.

Thermal melting profiles were obtained by CD at 222 nm for AS69 and AS69-GS3 in their free and α-syn-bound states and were analyzed by a two-state unfolding model (Fig. 6). Disulfide bond reduction led to a decrease in the melting temperatures of AS69 and AS69-GS3 by 11 and 8K, respectively, revealing a strong impact of the Cys-28 disulfide linkage on thermostability. Comparison of melting profiles of AS69-GS3 and AS69 showed that the head-to-tail linkage also enhanced thermostability, with an increase in melting temperatures of ~5K (Fig. 6). An additional increase in the melting temperature of 13K is observed upon complex formation with α-syn (Fig. 6), which was dependent on formation of the Cys-28 disulfide bond, in agreement with the ITC data (Fig. 2, Table I).

**Head-to-tail linkage affords accelerated disulfide bond formation**

The kinetics of formation of the Cys-28 disulfide bond was monitored starting from reduced AS69 or reduced AS69-GS3, both at a subunit concentration of 230 μM (Fig. 7). The fractions of oxidized and reduced molecules were separated by HPLC and quantified by their absorbance (Fig. 7A). Disulfide bond formation in AS69-GS3 was significantly accelerated compared with AS69, even at the high protein concentrations used in this experiment, which foster the intermolecular dimerization reaction of AS69 (Fig. 7B). The first-order rate constant obtained for intramolecular disulfide bond formation in AS69-GS3 was $k_{\text{AS69-GS3}} = 1.56 \pm 0.07 \times 10^{-4}$ s$^{-1}$. The second-order rate constant determined for intermolecular disulfide bond formation in AS69 was $k_{\text{AS69}} = 7.7 \pm 0.5 \times 10^{-2}$ M$^{-1}$ s$^{-1}$. The effective concentration of subunits in the disulfide formation reaction of AS69-GS3 can be calculated from the above rate constants (Robinson and Sauer, 1996) and is $4.1 \pm 0.5$ mM.

**Discussion**

In the present study, we investigated the requirements for subunit linkages of the β-wrapin AS69, an engineered binding protein to α-syn. While the head-to-tail linkage of AS69 subunits can generate functional single-chain binders to α-syn, the
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binding by entailing accelerated disulfide formation, providing an effective subunit concentration of 4.1 ± 0.5 mM for the disulfide formation reaction. This value is in good agreement with the effective subunit concentration in a single-chain variant of the Arc repressor dimer of phage P22, containing a 15-residue, glycine-rich linker (4.5 ± 1.8 mM, calculated from the bimolecular and unimolecular refolding reactions) (Robinson and Sauer, 1996).

AS69 exhibits a unique mode of interaction with α-syn (Mirecka et al., 2014). It sequesters a sequence region that is critical for α-syn dysfunction, judging from the clustering of disease-related mutations. Monomeric α-syn is stabilized at low substoichiometric concentrations of AS69, indicating that AS69 interferes with the nucleation of aggregation. The β-wrapin thus offers a distinct therapeutic approach to the synucleinopathies. The small size of the β-wrapin might support its uptake into the brain and limits the costs of production. AS69-GS3 is an advantageous construct to evaluate the therapeutic potential of β-wrapin interference with α-syn assembly in cell culture and animal models of synucleinopathies. AS69-GS3 can moreover be employed for affinity maturation of β-wrapins to α-syn as it is compatible with the independent adaption of its subunits to the target.

Acknowledgements

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References


Fig. 7. Head-to-tail linkage leads to accelerated formation of the Cys-28 disulfide bond. The kinetics of disulfide bond formation was monitored starting from reduced AS69 and AS69-GS3, respectively. (A) The oxidised and reduced fractions were separated and quantified by HPLC, exemplified for the time point of 158 min, indicated by arrows in (B). (B) Time traces of disulfide bond formation. The lines represent fits to first-order reaction kinetics in the case of AS69-GS3 (intramolecular disulfide bond formation) and to second-order dimerisation kinetics in the case of AS69 (intermolecular disulfide bond formation).

affinity is sensitive to the identity of the linker sequence. In contrast to ZAβ3 (Hoyer and Här;d, 2008; Luheshi et al., 2010; Lindberg et al., 2013), direct fusion of two AS69 results in the loss of affinity for the target. On the other hand, head-to-tail constructs with glycine-serine linkers, commonly employed in the construction of single-chain antibody fragments (Huston et al., 1988), recover the α-syn affinity of solely disulfide-linked AS69.

The presence of the Cys-28 disulfide bond is a prerequisite for high-affinity binding to α-syn. This can be explained by its potential to fix the contact between the subunits’ α1-helices, which in turn establishes the interaction surface for α-syn binding (Fig. 1A). In line with this, Cys-28 disulfide bond formation causes compaction and increased stability of AS69 as demonstrated by SEC and thermal melting experiments. The critical importance of the Cys-28 disulfide bond also for the ZAβ3 : Aβ interaction is highlighted by the conserved occurrence of Cys-28 in combinatorial engineering of Affibody molecules to Aβ (Grönwall et al., 2007; Lindberg et al., 2013). Head-to-tail linkage via a glycine-serine linker in the construct AS69-GS3 leads to increased protein stability, similar to the observations reported before for a single-chain variant of the Arc repressor dimer of phage P22 (Robinson and Sauer, 1996, 1998). In addition, head-to-tail linkage promotes α-syn
Subunit linkages in a β-wrap to α-synuclein