Functional interplay between protein domains in a supramodular structure involving the postsynaptic density protein PSD-95

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Cell scaffolding and signaling are governed by protein–protein interactions. Although a particular interaction is often defined by two specific domains binding to each other, this interaction often occurs in the context of other domains in multidomain proteins. How such adjacent domains form supertertiary structures and modulate protein–protein interactions has only recently been addressed and is incompletely understood. The postsynaptic density protein PSD-95 contains a three-domain supramodule (denoted PSG), which consists of PDZ, SH3, and guanylate kinase–like domains. The PDZ domain binds to the C terminus of its proposed natural ligand CXXC repeat–containing interactor of PDZ3 domain (CRIPT), and results from previous experiments using only the isolated PDZ domain are consistent with the simplest scenario for a protein–protein interaction, namely a two-state mechanism. Here, we analyzed the binding kinetics of the PSG supramodule with CRIPT. We show that PSG binds CRIPT via a more complex mechanism involving two conformational states interconverting on the second timescale. Both conformational states bound a CRIPT peptide with similar affinities but with different rates, and the distribution of the two conformational states was slightly shifted upon CRIPT binding. Our results are consistent with recent structural findings of conformational changes in PSD-95 and demonstrate how conformational transitions in supertertiary structures can shape the ligand-binding energy landscape and modulate protein–protein interactions.

The detailed characterization of protein-mediated interactions represents a critical task in understanding cellular processes, as well as in the identification of novel drug targets. From an experimental perspective, a quantitative description of protein-mediated networks demands the detection of specific interactions, followed by the attempt to understand larger systems. It should be noticed, however, describing protein interaction networks is a daunting task complicated by several factors that could be hard to estimate within the cellular environment, such as concentrations of individual proteins and compartmentalization. Additionally, proteins may experience conformational changes that may be difficult to capture but nevertheless modulate their function. Thus, a combination of structural, functional and computational approaches is generally necessary to understand the role of conformational changes.

Postsynaptic density protein-95 (PSD-95) is a multidomain protein, which is abundant in the postsynaptic density of neurons where it acts as a scaffolding protein (1). Starting from the N-terminus, this protein contains three "Postsynaptic density protein-95/Discs large/Zonula occludens-1" (PDZ) domains followed by an Src homology 3 (SH3) domain and a Guanylate kinase-like (GK) domain (2).
The PDZ domain is one of the most common protein interaction domains in our proteome (3). It presents a binding groove on its surface in which protein ligands can bind as a β-strand to form an extended β-sheet with the PDZ domain. PSD-95 contains supertertiary structure (4): the first two PDZ domains form one supramodule (5), whereas the third PDZ domain (PDZ3) forms another supramodule together with the SH3 and GK domains (denoted PSG) (6–9) (Fig. 1). This feature is shared by other membrane-associated GK family proteins (10), to which PSD-95 belongs. In general, PDZ domains display similar affinity for a range of protein ligands (11) such that specificity must rely on spatial and temporal control in the cell. Because of this it is often difficult to pinpoint protein ligands (11) such that specificity must rely on spatial and temporal control in the cell.

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In the present study, we investigated the binding kinetics of PSG supramodule and a 6 amino acid CRIPT peptide displayed a biphasic behavior. We monitored the binding of CRIPT to PSG using an N-terminal dansyl group attached to the six C-terminal residues of CRIPT (D-RIPT). The dansyl quenches the fluorescence of an engineered Trp in the PSG. The Trp was situated in the same position in PDZ3 (residue 337) as in previous studies on the isolated PDZ3 domain where it did not affect affinity or kinetics of binding (16). The quenching of the Trp upon binding results in kinetic traces with high signal-to-noise (Fig. 2A). We have previously performed extensive experiments with isolated PDZ3 and D-RIPT6, which yielded single exponential traces as pseudo-first order conditions are approached, consistent with an apparent two state binding mechanism (16, 24). However, fitting of the kinetic binding data of the PSG supramodule and D-RIPT6 to a single exponential function consistently yielded residuals displaying a clear non-random distribution around zero, even at D-RIPT6 concentrations 10-fold higher than PSG (Fig. 2), suggesting a more complex mechanism than two state. A double exponential equation fitted well to the experimental traces recorded over 1 s, and the observed rate constants $k_{\text{obs}1}$ and $k_{\text{obs}2}$ obtained from the fit were determined over a range of D-RIPT6 concentrations (Fig. 2B). The apparently linear dependence of both rate constants $k_{\text{obs}1}$ and $k_{\text{obs}2}$ on D-RIPT6 concentration suggests that the PSG sample contained two distinct species, both of which could bind D-RIPT6 one with an association rate constant $k_{\text{on}1}$ of $5.8 \times 10^6$ M$^{-1}$s$^{-1}$ (from $k_{\text{obs}1}$) and the other with a $k_{\text{on}2}$ of approximately $0.56 \times 10^6$ M$^{-1}$s$^{-1}$ (from $k_{\text{obs}2}$). In terms of amplitudes of the kinetic traces, $k_{\text{obs}1}$ had a relatively large and $k_{\text{obs}2}$ a relatively small amplitude (Fig. 2C). The $k_{\text{off}}$ values could not be determined directly from these binding data since they are too low to allow an accurate extrapolation of $k_{\text{obs}}$ to zero D-RIPT6 concentration.

Displacement experiments corroborated the presence of two distinct molecular species. In order to investigate the dissociation kinetics of the PSG:RIPT complex, we performed displacement experiments in which a preformed complex of PSG: D-RIPT6 (1.5 μM final concentration)
concentrations) was rapidly mixed with an excess (50-100 \( \mu M \)) of unlabeled CRIPT peptide. In contrast to what we have previously observed for the isolated PDZ3 domain (16, 24), the dissociation kinetics of PSG:D-CRIPT \(_6\) were also biphasic (Fig. 3A), which strongly supports the presence of two distinct binding competent PSG conformers. In fact, in a displacement experiment, any sequential mechanism such as induced fit will yield kinetic dissociation transients that are well described by single exponential kinetics. The observed rate constants, which approximates the overall apparent dissociation rate constants \( k_{\text{off}^{\text{app}}} \) were approximately \( k_{\text{off}^{\text{app}}} = 1.35 \text{ s}^{-1}\) and \( k_{\text{off}^{\text{app}}} = 0.17 \text{ s}^{-1}\), respectively (Fig. 3B). The errors in the amplitudes were large because of co-variation of the parameters, a common phenomenon in curve fitting of complex models (25). Nevertheless, based on six independent experiments, we found that the fast phase (described by \( k_{\text{off}^{\text{app}}} \)) was associated with a relatively smaller amplitude and the slow phase (described by \( k_{\text{off}^{\text{app}}} \)) with a relatively higher amplitude (Fig. 3C).

**Affinity between PSG and CRIPT determined by isothermal titration calorimetry confirmed an affinity in the low \( \mu M \) range.** From the kinetic data it is not clear which \( k_{\text{off}^{\text{app}}} \) belongs to which \( k_{\text{on}} \). We therefore performed ITC experiments to obtain an independent estimate of \( K_d \). ITC experiments at the temperature of the stopped flow (10°C) resulted in poor thermograms but experiments at 20, 25, 30 and 35°C yielded good data (Fig. 4). The \( K_d \) values at 20-30°C were all around 0.2 \( \mu M \) and we deem it likely that the affinity is similar at 10°C. The two \( K_d \) values obtained from the ratios of rate constants, \( k_{\text{off}^{\text{app}}}/k_{\text{on}1} \) (0.23 \( \mu M \)) and \( k_{\text{off}^{\text{app}}}/k_{\text{on}2} \) (0.30 \( \mu M \)), respectively, are in very good agreement with the ITC data. This \( K_d \) is also close to that obtained for the isolated PDZ3 domain (15, 16). On the other hand \( k_{\text{off}^{\text{app}}}/k_{\text{on}2} \) and \( k_{\text{off}^{\text{app}}}/k_{\text{on}1} \) would give \( K_d \) values of 29 nM and 2.4 \( \mu M \), respectively. Thus, the equilibrium binding data were most consistent with a scenario where \( k_{\text{off}^{\text{app}}} \) and \( k_{\text{on}1} \) are associated with the binding to one conformer (denoted PSG\(_A\)) and \( k_{\text{off}^{\text{app}}} \) and \( k_{\text{on}2} \) to the other conformer (PSG\(_B\)).

However, a model with two non-interconverting conformers predicts that the larger association amplitude should be connected to the larger dissociation amplitude. This is inconsistent with the relative amplitudes of the observed kinetic phases. Although the large variation in dissociation amplitudes precludes a detailed quantitative analysis, it is qualitatively clear that the kinetic amplitude of \( k_{\text{off}^{\text{app}}} \) (Fig. 3C) is more prominent than that associated with \( k_{\text{on}2} \) in the binding experiment (Fig. 2C). The most likely explanation for this discrepancy is that there is an equilibrium between PSG\(_A\) and PSG\(_B\) and that this equilibrium is shifted such that relatively more PSG\(_B\) is present upon binding of D-CRIPT\(_6\) (Fig. 5).

**Direct detection of the conformational transition in a double jump interrupted binding experiment.** In an attempt to directly monitor a transition between PSG\(_A\) and PSG\(_B\) in their bound conformation according to the scheme in Fig. 5, we performed interrupted binding experiments in a double jump setup in the stopped flow instrument (Fig. 6A). Here 2 \( \mu M \) PSG was mixed with 4 \( \mu M \) D-CRIPT\(_6\) in the first jump (concentrations after 1:1 mixing). After a defined delay (or ageing) time ranging between 10 ms and 200 s, this complex was dissociated by mixing with a large excess of unlabeled CRIPT in the second jump. Following this second jump the apparently irreversible dissociation kinetics of PSG:D-CRIPT\(_6\) were monitored as described for a regular dissociation experiment, which in practice corresponds to an interrupted binding experiment with a very long delay time. We collected fourteen kinetic traces from these double jump experiments with delay times between 10 ms and 200 s. The trace at 10 ms delay time was excluded because the mismatch between expected and actual delay time was deemed to large. Next, all remaining dissociation traces (delay times between 24 ms and 200 s) were fitted simultaneously to a double exponential function with shared values for \( k_{\text{off}^{\text{app}}} \) and \( k_{\text{on}2} \), either locked to the average values reported in Fig. 3D or free fitted (Fig. 6B). This is possible since the final conditions at which kinetics are measured were identical for each double jump experiment. However, it is also necessary to perform a simultaneous fit since the kinetic amplitudes at short delay times are too small for accurate free fitting of \( k_{\text{off}^{\text{app}}} \) and \( k_{\text{off}^{\text{app}}} \). Next, the kinetic amplitudes associated with \( k_{\text{off}^{\text{app}}} \) and \( k_{\text{off}^{\text{app}}} \) would give \( K_d \) values of 29 nM and 2.4 \( \mu M \), respectively. Thus, the equilibrium binding data were most consistent with a scenario where \( k_{\text{off}^{\text{app}}} \) and \( k_{\text{on}1} \) are associated with the binding to one conformer (denoted PSG\(_A\)) and \( k_{\text{off}^{\text{app}}} \) and \( k_{\text{on}2} \) to the other conformer (PSG\(_B\)).

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were plotted as a function of delay time (Fig. 6C and 6D). The change in the kinetic amplitudes over time followed a biphasic behavior as clearly seen for the amplitude related to $k_{\text{off app}}$. This biphasic behavior reflects the initial build-up of the two complexes, PSG$_A$: D-CRIPT$_6$ and PSG$_B$: D-CRIPT$_6$, respectively, followed by their equilibration. The change in the amplitudes over time thus follows exponential kinetics. We could fit a double exponential function to the amplitude data and obtain two observed rate constants. However, the errors function to the amplitude data and obtain two kinetics. We could fit a double exponential amplitude over time thus follows exponential their equilibration. The change in the kinetic amplitudes over time follow ed a biphasic behavior as clearly seen for the amplitude related to $k_{\text{off app}}$. This biphasic behavior reflects the initial build-up of the two complexes, PSG$_A$: D-CRIPT$_6$ and PSG$_B$: D-CRIPT$_6$, respectively, followed by their equilibration. The change in the kinetic amplitudes over time thus follows exponential kinetics. We could fit a double exponential function to the amplitude data and obtain two observed rate constants. However, the errors function to the amplitude data and obtain two kinetics. We could fit a double exponential curve fitting because the binding step described by $k_{\text{on1}}$ and $k_{\text{off1 app}}$ gets too fast. It is clear that $k_{\text{on2}}$ is closer to 0.6 $\mu$M$^{-1}$s$^{-1}$ (Fig. 2C) than to 2.2 $\mu$M$^{-1}$s$^{-1}$ obtained from the global fit. The dissociation rate constants $k_{\text{off1}}$ and $k_{\text{off2}}$ are also more similar to each other in the global fit than $k_{\text{off1 app}}$ and $k_{\text{off2 app}}$ in the individual fits presented in Fig. 3. However, the values of $k_{\text{off1}}$ and $k_{\text{off2}}$ in the global fit are dependent on the relative values of $k_1$ and $k_3$, which are poorly defined. The rate constants $k_1$ and $k_3$ agree well with the fit of double jump data in Fig. 6 insofar that their sum is 0.53 s$^{-1}$. Finally, $k_4$ and $k_5$ are fitted as 0.007-0.018 s$^{-1}$, but they are obviously very poorly defined, except that this step must be relatively slow relative to the other ones.

In summary, despite low accuracy for several of the fitted rate constants, global fitting (Fig. 7) shows that experimental data is overall consistent with the proposed minimal model depicted in Fig. 5 for the interaction between PSG and D-CRIPT$_6$.

**Global fitting of a minimal model for the interaction between PSG and D-CRIPT$_6$.** To test the robustness of the kinetic scheme in Fig. 5 we attempted a global fit of kinetic transients from binding and dissociation experiments using the Kintek software (26, 27). Global fitting with numerical integration takes information from both rate constants and kinetic amplitudes into consideration. The information from kinetic amplitudes is often overlooked, but can in favorable cases be used to distinguish between kinetic models. However, in the case of fluorescence-monitored measurements, this requires knowledge about the fluorescence yields of all molecular species, and those of intermediates are usually unknown. Here, the information from the kinetic amplitudes was used to restrain the parameters in the model. Thus, a global fit of a data set including seven binding curves from Fig. 2C and all dissociation curves from the double jump experiment (Fig. 6) was performed and it resulted in reasonable kinetic parameters (Fig. 7).

A comparison of the globally fitted rate constants to those obtained from fitting binding and dissociation transients individually shows that while $k_{\text{on1}}$ is similar, $k_{\text{on2}}$ from the global fit is 5-fold larger than in Fig. 2C. The main reason is that we could not include the data points at high concentrations of D-CRIPT$_6$ in the global curve fitting because the binding step described by $k_{\text{on1}}$ and $k_{\text{off app}}$ gets too fast. It is clear that $k_{\text{on2}}$ is closer to 0.6 $\mu$M$^{-1}$s$^{-1}$ (Fig. 2C) than to 2.2 $\mu$M$^{-1}$s$^{-1}$ obtained from the global fit. The dissociation rate constants $k_{\text{off1}}$ and $k_{\text{off2}}$ are also more similar to each other in the global fit than $k_{\text{off1 app}}$ and $k_{\text{off2 app}}$ in the individual fits presented in Fig. 3. However, the values of $k_{\text{off1}}$ and $k_{\text{off2}}$ in the global fit are dependent on the relative values of $k_1$ and $k_3$, which are poorly defined. The rate constants $k_1$ and $k_3$ agree well with the fit of double jump data in Fig. 6 insofar that their sum is 0.53 s$^{-1}$. Finally, $k_4$ and $k_5$ are fitted as 0.007-0.018 s$^{-1}$, but they are obviously very poorly defined, except that this step must be relatively slow relative to the other ones.

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**Binding kinetics of a 15-mer CRIPT peptide is monophasic.** To further investigate the conformational transition in the PSG supramodule, we performed binding experiments with an N-dansylated peptide corresponding to the 15 C-terminal residues of CRIPT, D-CRIPT$_{15}$. In contrast to D-CRIPT$_6$, we only observed single exponential kinetics with D-CRIPT$_{15}$, both in binding and displacement experiments (Fig. 8). The apparent rate constants ($k_{\text{on}}$ and $k_{\text{off}}$) for D-CRIPT$_{15}$, were more similar to $k_{\text{on1}}$ and $k_{\text{off1 app}}$, associated with the fast phase for D-CRIPT$_6$, and giving a similar $K_D$ (0.42 $\mu$M) as D-CRIPT$_6$. The $K_D$ determined by ITC for D-CRIPT$_{15}$ was 1.6±0.2 $\mu$M at a higher temperature (25°C) in fair agreement with $K_D$ from kinetics. One explanation, considering the close match of the $k_{\text{on}}$ values, would be that D-CRIPT$_{15}$ can only bind to PSG$_A$ and not to PSG$_B$. However, even with D-CRIPT$_6$, the kinetic amplitude of the
slow phase is low and for the longer $D$-CRIPT$_{15}$ it may not be visible. Whether or not $D$-CRIPT$_{15}$ binds to PSG$_{B}$, this conformation is present in equilibrium with PSG$_{A}$, as monitored by the smaller $D$-CRIPT$_{6}$, which can likely access a more narrow binding pocket present in PSG$_{B}$.

**DISCUSSION**

Much of our current knowledge on protein mediated interactions is based on the study of isolated domains in solution. Nevertheless, it is clear that since proteins are dynamic entities, the interaction between contiguous domains may play a role in their structure and function. A hallmark of these interactions is represented by hemoglobin, a system where it was soon established how the intricate interplay between the four subunits results in very complex binding schemes, that ultimately mediate the allosteric regulation and fine tune oxygen (un)binding to optimize its transport from lungs to tissues. While the dynamics of allosteric multisubunit proteins have a long history (28) much recent work has been focused on multidomain proteins in which several distinct protein domains occur in one single polypeptide as beads on a string. It is becoming clear that such proteins often display supertertiary structures (4) where supramodules could form from two or several domains, which may then cooperate, positively or negatively in ligand binding or other function. Similarly to hemoglobin, simplified models must be used to account for the behavior of such multidomain proteins.

The MAGUK family of proteins, to which PSD-95 belongs, is one class of multi-domain proteins that has been well studied, in particular from the point of view of single domains but also from the perspective of its two supramodules. We have previously determined binding rate constants for the isolated PDZ3 domain and established that its interaction with CRIPT is a one-step process (16, 24). In the present study we extend these studies to the PSG supramodule, which consists of PDZ3 and two more domains, SH3 and GK, and find that a model with two conformations, denoted PSG$_{A}$ and PSG$_{B}$, can satisfactorily describe the data. While PSG$_{A}$ binds CRIPT with similar rate constants as PDZ3, PSG$_{B}$ appears to display a more restricted binding pocket.

Previous work on PSG from the PSD-95 family or other homologs have not assessed the mechanism with direct kinetic methods. However, multiple complementary methods including crystallography, NMR, SAXS, single-molecule FRET, binding experiments and modelling have demonstrated that the three domains form a well defined and dynamic supertertiary structure (8–10, 29, 30). For example, single molecule FRET in PSD-95 showed that PDZ3 displays motional averaging relative to the SH3 domain (8). Furthermore, NMR, SAXS and simulations were consistent with distinct structural ensembles in free PSG and CRIPT-bound PSG, where the ensemble of bound conformations were shifted towards extended rather than compact species (9). In more detail, the peptide binding groove of PDZ3 was shown to interact weakly with the SH3 domain (or the linker between them) such that, in the ligand-free state, the SH3 domain restricts ligand binding to PDZ3. Binding of a ligand to PDZ3 would thus promote a more open conformation of the PSG where the SH3 is released from PDZ3. These conformational transitions could be the basis of the biphasic kinetics observed in the present study. From a functional perspective, PSD-95 forms oligomers in the post synaptic density and one way is via the SH3 and GK domains (6). This oligomerization is mediated by ligand binding to PDZ3 (21). Furthermore, ligand binding to PDZ3 promotes binding of GK to other proteins such as the G protein subunit Gnb5 (23), while restricting binding of another protein, GukHolder (29). These previous studies are consistent with our kinetic data insofar that the binding kinetics suggest that the PSG$_{B}$ conformation is less accessible than PSG$_{A}$, as mirrored by the differences in $k_{on1}$ (PSG$_{A}$) and $k_{on2}$ (PSG$_{B}$) upon binding to $D$-CRIPT$_{6}$, and that $D$-CRIPT$_{15}$ apparently only binds to PSG$_{A}$. A more restricted binding site provides fewer possibilities of productive initial encounters, thus reducing the apparent association rate constant. It is possible that the more open PSG$_{A}$ is the conformation allowing SH3-GK-mediated oligomerization and binding between GK and the G protein subunit Gnb5 (17, 21–23).

In conclusion, there is a firm structural basis for a dynamic supertertiary structure of the PSG from PSD-95 (8, 9). This dynamic behavior is
characterized by some distinctive kinetic signatures, as highlighted by our kinetic analysis. Furthermore, our experiments estimate a timescale of these transitions of about 0.5 \( s^{-1} \) at 10°C. We speculate that such conformational transitions in the PSG are related to the previously reported conformational changes and are most likely connected to its function, as previously demonstrated in several studies \((17, 21–23, 29)\). Future work based on site directed variants will further explore the functional role of these transitions.

**Experimental Procedures**

**Protein purification.** The PSD-95 PDZ3-SH3-GK (PSG) supramodule (residues 308 - 724) contained an engineered Trp in the same position (F337W) as in previous studies on the isolated PDZ3 domain \((16, 24)\). PSG was expressed from a modified pRSET plasmid (Invitrogen), which was transformed into *Escherichia coli* BL21(DE3) pLys cells. The *Escherichia coli* (Invitrogen), which was transformed into *Escherichia coli* BL21(DE3) pLys cells. 0.5 mM DTT, 100 mM NaCl, 10 % glycerol and stored at -20°C until purification. The pellet was thawed and sonicated 2 \( \times \) 4 min followed by centrifugation. The supernatant was filtered and loaded onto a Nickel Sepharose Fast Flow column (GE Healthcare) pre-equilibrated with 50 mM Tris, 100 mM NaCl, 10 % glycerol and 0.5 mM dithiothreitol (DTT). The column was washed with the equilibration buffer and bound protein eluted with 250 mM imidazole. The protein sample was dialysed overnight into 50 mM Tris, 2 mM DTT, 100 mM NaCl, 10 % glycerol. The PSG sample was concentrated and further purified using size exclusion chromatography (S-100, GE Healthcare). Protein purity was quantified by SDS-PAGE and identity by MALDI-TOF mass spectrometry. Concentration of protein samples were measured by the absorbance at 280 nm and theoretical extinction coefficients based on the amino acid composition. Far-UV circular dichroism spectra (200-260 nm) of the protein (10 \( \mu M \)) was recorded to ensure that it was folded. Circular dichroism experiments were performed in 50 mM sodium phosphate pH 7.45, 21 mM KCl \((I = 150)\) at 10°C on a JASCO J-1500 spectropolarimeter using the average of 5 scans.

**Kinetic methods.** All kinetic experiments were performed in 50 mM sodium phosphate pH 7.45, 21 mM KCl \((I = 150 \mathrm{mM})\), 0.5 mM tris(2-carboxyethyl)phosphine (TCEP) at 10°C. Kinetic binding and dissociation experiments were performed in an upgraded SX-17 MV stopped flow spectrophotometer (Applied Photophysics, Leatherhead, UK) and carried out as described previously \((16)\). Briefly, the association rate constant \((k_{on})\) was obtained from binding experiments approaching pseudo first order conditions \([\text{PSG}][D-\text{CRIPT}]). PSG \((1 \mu M)\) was rapidly mixed with different concentrations of N-terminally dansyl-labeled CRIPT. The shorter peptide dansyl-YKQTSV is denoted \(D-\text{CRIPT}_6\) and the longer one, dansyl-GKKVLDTKYNKQTSV is denoted \(D-\text{CRIPT}_{15}\). The binding reaction was monitored by fluorescence where the Trp at position 337 in PDZ3 is quenched by the dansyl group of the CRIPT peptide. Trp 337 was excited at 280 nm and emission was followed at 330 nm (using a 330 \( \pm \) 25 nm interference filter). Kinetic traces were recorded and averaged (a minimum of 5 individual traces were used at each concentration) and fitted to single or double exponential functions to obtain observed rate constant(s) \((k_{obs})\) for each CRIPT concentration. To estimate \(k_{on}\) values, \(k_{obs1}\) and \(k_{obs2}\) (for PSG with \(D-\text{CRIPT}_6\)) or \(k_{obs}\) (for PSG with \(D-\text{CRIPT}_{15}\)) were plotted versus CRIPT concentration and fitted to a linear function, where the slope corresponds to \(k_{on}\). Due to slow dissociation rates, \(k_{off}\) could not be accurately determined by extrapolation of \(k_{obs}\) to zero CRIPT in the binding experiments. Thus, displacement experiments were performed to obtain more accurate estimates of \(k_{off}\). In these experiments PSG \((2 \mu M)\) was pre-incubated with \(D-\text{CRIPT}_6\) \((10 \mu M)\). \(D-\text{CRIPT}_6\) was then displaced from PSG by rapidly mixing the complex with an excess of unlabeled CRIPT \((100, 150, 200 \mu M)\). The high excess of unlabeled peptide ensured an irreversible dissociation reaction, allowing \(k_{off}\) to be estimated from the average of three experiments, in which \(k_{obs} (= k_{off})\) did not change.
with CRIPT\textsubscript{6} concentration. Similarly to the binding, all displacement experiments performed with PSG and D-CRIPT\textsubscript{6} were fitted to a double exponential function yielding two distinct observed dissociation rate constants.

Double jump mixing experiments were performed on an Applied Photophysics SX18-MV stopped-flow instrument according to the manufacturer’s instructions with regard to mixing volumes. In particular, a solution containing 4 \(\mu\)M PSG in the presence of 50 mM phosphate buffer pH 7.5 was first mixed 1:1 with D-CRIPT\textsubscript{6} peptide at an initial concentration of 8 \(\mu\)M. Then, after a controlled delay time (between 10 ms-200 s), the solution was challenged with a high concentration of unlabeled CRIPT\textsubscript{6} in a second mixing event (initial concentration of PSG was 100 \(\mu\)M. Thus, final concentrations in the displacement reaction was 1 \(\mu\)M PSG, 2 \(\mu\)M D-CRIPT\textsubscript{6} and 50 \(\mu\)M unlabeled CRIPT. In analogy to single mix stopped-flow experiments, the double jump experiment was carried out at 10°C and analyzed as described in the Results section.

Global fitting of kinetic data. Kinetic transients from single and double jump stopped flow experiments were fitted globally to a minimal model (Fig. 5) using KinTek Explorer (26, 27). This software utilizes numerical integration, and thus allows for fitting of raw kinetic data directly to a mechanistic model. The interrupted binding experiments, with delay times varying from 24 ms to 200 s were included in the global fit together with binding experiments in which [D-CRIPT\textsubscript{6}] was varied between 2-14 \(\mu\)M. For practical reasons, the lower boundary of \(k_{d}\) and \(k_{a}\) was set to \(10^{-5}\) s\(^{-1}\) in the fitting procedure. \(k_{\text{on}}\) for the CRIPT\textsubscript{6} peptide used in dissociation experiments (with no dansyl group) was locked to be the same as for D-CRIPT\textsubscript{6} and \(k_{\text{off}}\) was locked to 0 (irreversible binding is practically achieved at concentrations where CRIPT\textsubscript{6} >> D-CRIPT\textsubscript{6}). The remaining parameters in the model were fitted without constrains. To account for baseline shifts in the double jump kinetic experiments, constants were added to the output expressions. A scaling factor was used to account for the difference in total fluorescence output between the single jump and double jump stopped flow experiments.

Isothermal titration calorimetry. Isothermal titration calorimetry (ITC) experiments were performed in 50 mM sodium phosphate, pH 7.45, 21 mM KCl (\(I = 150\)), 0.5 mM TCEP at 20, 25, 30 and 35°C in an iTC200 instrument (Malvern). D-CRIPT\textsubscript{6} and PSG were dialysed overnight in the same buffer to minimize artifacts from buffer mismatch. D-CRIPT\textsubscript{6} (220 \(\mu\)M) was titrated (16-19 injections) into the cell containing 22 \(\mu\)M PSG and saturation was obtained by having approximately two times excess of D-CRIPT\textsubscript{6}. Baseline correction was performed manually to minimize the chi-value in the curve fitting using the software provided with the iTC200 instrument.

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Figure 1. Structure of the PSG supramodule from PSD-95. Schematic supertertiary structure of the PSG supramodule from PSD-95 together with crystal structures of PDZ3 with a bound CRIPT peptide (pdb code: 1BE9) and of the SH3-GK tandem (pdb code: 1JXO).
Figure 2. Binding kinetics of the PSG supramodule and CRIPT. (A) Example of kinetic transient for the association of PSG with D-CRIPT<sub>6</sub>. PSG (1 µM final concentration) was mixed rapidly in the stopped flow with D-CRIPT<sub>6</sub> (10 µM final concentration). The residuals are from fits to single and double exponential functions, respectively. The double exponential fit yielded the following parameters: \( k_{\text{obs}1} = 64 \text{ s}^{-1} \) (amplitude = 4.4) and \( k_{\text{obs}2} = 16 \text{ s}^{-1} \) (amplitude = 0.27). (B) The experiment was repeated over a range of D-CRIPT<sub>6</sub> concentrations and \( k_{\text{obs}} \) values obtained from the fit to a double exponential function were plotted versus [D-CRIPT<sub>6</sub>]. \( k_{\text{obs}} \) values > 150 s<sup>-1</sup> were omitted since they very slightly but progressively deviate from a straight line due to instrumental limitations in the mixing. (C) Kinetic amplitudes associated with the respective \( k_{\text{obs}} \) value.
Figure 3. Dissociation kinetics of the PSG supramodule and D-CRIPT<sub>6</sub>. (A) Example of kinetic trace from an experiment where a complex of PSG and D-CRIPT<sub>6</sub> (2+10 µM) was rapidly mixed with an excess of unlabeled CRIPT (150 µM). The resulting trace was fitted to a single or a double exponential and the residuals from the respective fit are presented below the trace. The double exponential fit yielded the following parameters: \( k_{\text{off1}}^{\text{app}} = 1.35 \text{ s}^{-1} \) (amplitude = 0.32) and \( k_{\text{off2}}^{\text{app}} = 0.16 \text{ s}^{-1} \) (amplitude = 1.4) (B-C) This experiment was repeated six times, each time at three different concentrations of unlabeled CRIPT (100, 150, 200 µM). The double exponential fit yielded (B) \( k_{\text{off}}^{\text{app}} \) values and their associated amplitudes (C, normalized), which were plotted versus CRIPT concentration. The large scatter in the points, in particular for the amplitudes, reflects the co-variation of parameters in the curve fitting. The average of all 18 \( k_{\text{off}}^{\text{app}} \) values for each kinetic phase yielded the following parameters (standard deviation in parenthesis): \( k_{\text{off1}}^{\text{app}} = 1.35 \pm 0.42 \text{ s}^{-1} \) and \( k_{\text{off2}}^{\text{app}} = 0.17 \pm 0.08 \text{ s}^{-1} \) (indicated by horizontal lines in panel B). The average of all 18 amplitudes yielded the following values (standard deviation in parenthesis): \( \text{Amp}_1 = 0.33 \pm 0.24 \) and \( \text{Amp}_2 = 0.67 \pm 0.24 \) (indicated by horizontal lines in panel C).
Fig. 4. Isothermal titration calorimetry experiments of PSG and CRIPT. ITC experiments were performed at four different temperatures (20-35°C). The parameters shown in the respective panel were obtained by fitting a 1:1 binding model using the software provided with the instrument. The $K_d$ values from these experiments agreed well with those calculated from the kinetic experiments at 10°C ($k_{off_1}^{app}/k_{on_1}$ or $k_{off_2}^{app}/k_{on_2}$).
Figure 5. A kinetic scheme consistent with the binding kinetics of PSG and CRIPT. A scenario with two conformations, PSGA and PSGB, which are in equilibrium and which can both bind CRIPT is consistent with the kinetic and equilibrium data. Access to the binding groove is more restricted in the PSGB conformation.
Figure 6. Interrupted binding experiments. (A) Schematic figure of the double jump setup. In the first jump, 4 μM PSG was mixed with 8 μM D-CRIPT (i.e., the final concentrations after mixing were 2 μM and 4 μM, respectively) and incubated for a certain delay time in an ageing loop. Following the set delay time any formed complex was dissociated by a large excess of unlabeled CRIP in a second jump (final concentrations were 1 μM PSG, 2 μM D-CRIPT, and 50 μM unlabeled CRIP). (B) The dissociation kinetics were monitored in the flow cell. The kinetic traces were fitted simultaneously to a double exponential function with either locked $k_{\text{off}}$ values (the two average $k_{\text{off}}$ values from single jump experiments, 1.35 s$^{-1}$ and 0.17 s$^{-1}$, respectively) or shared and free fitted $k_{\text{off}}$ values ($k_{\text{off,1}} = 2.1$ s$^{-1}$ and $k_{\text{off,2}} = 0.35$ s$^{-1}$). The best fit curves in the figure correspond to the latter fit. (C-D) The kinetic amplitudes from the respective fits in panel B were plotted against delay time. This reflects the build up of PSGA: D-CRIPT and PSGB: D-CRIPT, respectively, with time. These amplitude data were in turn fitted to a double exponential function to obtain two "observed double jump rate constants", for the build up of PSGA and PSGB, respectively. From the fit, $k_{\text{obs,1}}$ values of around 20-40 s$^{-1}$ and 0.1-0.8 s$^{-1}$, respectively, were obtained. While the parameters are underdetermined it is clear that there is an initial increase in the respective population followed by a decrease in PSGA: D-CRIPT. The expected concomitant increase in PSGB: D-CRIPT is lost in the experimental noise.
Figure 7. Global fitting to a minimal scheme describing PSG:CRIPT binding. Data from binding and dissociation kinetic experiments were fitted globally with KinTek Explorer. (A) The scheme shows the fitted square model with the best fit parameters and their standard errors. The amplitude factors for PSGA:D-CRIPT$_6$ and PSGB:D-CRIPT$_6$ were fitted to $0.9 \pm 0.1$ and $0.13 \pm 0.03$, respectively. It should be noted that the reported standard errors for all parameters are likely underestimated. We are using the global fit to qualitatively test our suggested minimal model. Thus, less emphasis should be put on the estimates of the microscopic rate constants, which are in some cases poorly constrained. $k_{on2}$ is likely overestimated since we could only use lower D-CRIPT$_6$ concentrations in the global fit to capture the fast phase (See Fig. 2). (B) Fit of interrupted binding stopped flow data to the minimal model in panel A. Five kinetic traces are shown for clarity. (C) Fit of the single jump stopped flow data to the model, with residuals shown below the curves. The fits to kinetic traces of three different concentrations of D-CRIPT$_6$ are shown for clarity.
Figure 8. Binding kinetics of the PSG supramodule and D-CRIPT_{15}. (A) PSG (1 μM final concentration) was mixed rapidly in the stopped flow with D-CRIPT_{15} (10 μM final concentration). A single exponential function describes well the experimental transient, as shown by the comparison of residuals from fit to single and double exponential functions. (B) The experiment was repeated over a range of D-CRIPT_{15} concentrations and observed rate constants ($k_{\text{obs}}$) obtained from the fit to a single exponential function was plotted versus [D-CRIPT_{15}] to obtain $k_{\text{on}}^{\text{app}} = (8.4\pm0.2) \times 10^6$ M$^{-1}$s$^{-1}$ (fitting error). (C) Dissociation of the complex; PSG:D-CRIPT_{15} (2+10 μM) was rapidly mixed with an excess of unlabeled CRIPT (150 μM). The resulting trace was fitted to both single and double exponential functions. The residuals from the fits show that the dissociation kinetics follow single exponential kinetics. The average of three experiments using 100, 150 and 200 μM CRIPT, respectively, for displacement yielded a $k_{\text{off}}^{\text{app}} = 3.53\pm0.011$ s$^{-1}$ (± standard deviation).
Functional interplay between protein domains in a supramodular structure involving the postsynaptic density protein PSD-95
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