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Heterotypic Sam-Sam association between Odin-Sam1 and Arap3-Sam: binding affinity and structural insights

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Abstract

Arap3 is a phosphatidylinositol 3 kinase effector protein that plays a role as GTP-ase activator (GAP) for Arf6 and RhoA. Arap3 contains a sterile alpha motif (Sam) domain that presents high sequence homology with the Sam domain of the EphA2-receptor (EphA2-Sam); both Arap3-Sam and EphA2-Sam are able to associate with the Sam domain of the lipid phosphatase Ship2 (Ship2-Sam).

Recently, we have reported on a novel interaction between the first Sam domain of Odin (Odin-Sam1), a protein belonging to the ANKS (ANKyrin repeat and Sam domain containing) family, and EphA2-Sam. In the current work we apply Nuclear Magnetic Resonance (NMR) spectroscopy, Surface Plasmon Resonance (SPR) and Isothermal Titration Calorimetry (ITC) to characterize the association between Arap3-Sam and Odin-Sam1. We show that these two Sam domains interact with low micromolar affinity. Moreover, by means of molecular docking techniques, supported by NMR data, we demonstrate that Odin-Sam1 and Arap3-Sam may bind with a topology that is common to several Sam-Sam complexes.

The unveiled structural details form the basis for the design of potential peptide-antagonists, that could be used as chemical tools to investigate functional aspects related to heterotypic Arap3-Sam associations.

Keywords

Sam Domains; Protein-Protein Interactions; NMR Spectroscopy; Surface Plasmon Resonance; Isothermal Titration Calorimetry

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Introduction

Arap3 (Arf GAP with Rho GAP domain, Ankyrin repeat and PH domain 3) is a phosphatidylinositol 3 kinase (PI3K) effector protein that has originally been discovered through a screening for novel binders of PtdIns(3,4,5)P3 (phosphatidylinositol (3,4,5)P3).[1] Arap3 works as a GTPase activating protein for Arf6 and RhoA; it plays roles in biological processes connected to formation of lamellipodia, cell adhesion and spreading, regulation of actin cytoskeleton.[2–5] In particular, recent reports have related Arap3 to developmental angiogenesis[6] and scirrhous gastric carcinoma.[7]

Arap3 primary sequence includes, among others, a sterile alpha motif (Sam) domain.[8] Two proteins containing Sam domains: Ship2 (Src homology 2 domain-containing phosphoinositide-5-phosphatase 2) and ANKS1 (ANKyrin repeat and Sam domain containing 1), have been identified in a yeast two-hybrid screen as possible Arap3 regulators.[4] The interaction between Arap3 and Ship2 is mediated by Sam-Sam heterodimerization and has been already well characterized.[4, 9] Arap3-Sam and Ship2-Sam bind to each other with a dissociation constant approximately 100 nM;[4] protein-protein association is mainly driven by specific electrostatic contacts.[9] A docking model of the Ship2-Sam/Arap3-Sam complex, assisted by NMR and mutagenesis data, shows that the two proteins adopt a head to tail topology of binding, that is close to that observed for many Sam-Sam associations, and is called Mid-Loop (ML)/End-Helix (EH) Model.[4, 10–11]

Similar analysis of the interactions between Arap3-Sam and Sam domains from the ANKS1 protein have not been reported so far. However, we have recently studied the heterotypic association between the first Sam domain of Odin (Odin-Sam1) and EphA2-Sam,[12] that represents an interaction possibly relevant for EphA2 receptor endocytosis.[13] Odin (also called ANKS1 and ANKS1A) is a protein belonging to the ANKS family that owns two tandem Sam domains (Sam1 and Sam2).[14] Due to the quite high sequence homology between EphA2-Sam and Arap3-Sam (~58%) we have investigated if Arap3-Sam could associate with Odin-Sam1. To verify and characterize binding between the two proteins we have followed an approach including several techniques (i.e., Nuclear Magnetic Resonance Spectroscopy (NMR), Isothermal Titration Calorimetry (ITC), Surface Plasmon Resonance (SPR), Molecular Docking and Mutagenesis). We show that Arap3-Sam interacts with Odin-Sam1 with a dissociation constant in the low micromolar range, a 1:1 stoichiometry and adopting a structural organization that closely resembles other ML/EH complexes such as Ship2-Sam/Arap3-Sam[9] and Odin-Sam1/EphA2-Sam.[12]

These studies provide novel and significant structural insights that should help the design of selective peptide/peptidomimetic molecules that could specifically antagonize Arap3-Sam heterotypic complexes. Such molecular probes could result very useful in cell-based assays to shed light on the functional implications of these interactions.

Results and Discussion

Sam domains represent small helical protein interaction modules that are made up of approximately 70 residues and are characterized, in spite of a very similar fold, by a high versatility as concerning binding preferences and function.[8, 15] Many Sam domain functions are mediated by formation of homo and heterotypic Sam-Sam interactions.[16] From a structural point of view Sam-Sam associations may adopt a head to head, tail to tail and even head to tail topology.[9, 17–21] This last binding mode is called Mid-Loop (ML)/ End-Helix (EH).

We previously characterized the associations between the Sam domains of the PI3K effector protein Arap3 (Arap3-Sam) and the lipid phosphatase Ship2 (Ship2-Sam)[9] and more

recently the interaction between the first Sam domain of Odin (Odin-Sam1) and the Sam domain of the EphA2 receptor (EphA2-Sam)[12] and showed that these associations may fit the head to tail topology. The current report focuses on the characterization of the Odin-Sam1/Arap3-Sam interaction.

Odin-Sam1 binds Arap3-Sam: Chemical Shift perturbation Studies

To verify the association between Odin-Sam1 and Arap3-Sam we first conducted chemical shift perturbation studies with 2D [^{1}H, ^{15}N] HSQC experiments (Figure 1).[22] Thus, we acquired and compared NMR spectra of a ^{15}N uniformly labeled Odin-Sam1 sample in the free state and bound to unlabeled Arap3-Sam (Figure 1A). Association of the two Sam domains is clearly indicated by several changes that occur in the HSQC spectra of Odin-Sam1 recorded in presence of increasing amounts of Arap3-Sam. Once reached saturation conditions (i.e., no more changes could be revealed in the HSQC spectrum of Odin-Sam1 after further addition of Arap3-Sam), the equation $\Delta\delta = [(\Delta H_N)^2 + (0.17 * \Delta^{15}N)^2]^{1/2}$ (Figure 1B)[23] was applied to evaluate proton and nitrogen normalized chemical shift deviations and to identify the interaction surface of Odin-Sam1 (Figure 2, left panel). Largest chemical shift perturbations, $\Delta\delta \geq 0.2$ ppm, are observed in the central portion of Odin-Sam1, encompassing helices α3, α4 and the C-terminal portion of α2; changes also occur in the C-terminal tail, presumably because residues from this flexible portion come in proximity with the main binding interface (Figures 1B and 2 left panel).

The pattern of chemical shift variations *versus* residue number (Figure 1B), observed for Odin-Sam1 in complex with Arap3-Sam, closely resembles the one we have previously detected for the protein in complex with EphA2-Sam.[12]

NMR experiments with ^{15}N labeled Arap3-Sam and unlabeled Odin-Sam1 were also conducted to map the binding surface of Arap3-Sam for Odin-Sam1 (Figures 1C, 1D). Largest chemical shift changes ($\Delta\delta \geq 0.1$ ppm) are localized in the residues of α5 helix and the close α1α2 and α4α5 loop areas (Figures 1D, 2 right panel). Interestingly, these are the same regions participating in the binding of Arap3-Sam to Ship2-Sam.[9]

NMR perturbation data suggest that Odin-Sam1 and Arap3-Sam bind with an head to tail architecture where the central region of Odin-Sam1 and the α5 helix of Arap3-Sam along with the adjacent loop regions, mainly provide the binding interfaces.

To get insights into the Odin-Sam1 and Arap3-Sam binding stoichiometry, we carried out ^{15}N longitudinal (R1) and transversal (R2) nuclear spin relaxation rates measurements, evaluated the R2/R1 average values and thus estimated the correlation time τ_c of both Sam domains bound to each other.[24]

The τ_c of Arap3-Sam and Odin-Sam1 in their associated forms result 10 ±1 ns and 9.9 ± 0.8 ns respectively, these values are of course higher than those evaluated for the proteins in their free states (i.e., 7.3 ± 0.6 ns for Odin-Sam1 and 8.2 ± 0.4 ns for Arap3-Sam)[9, 12] because of the increased dimension of the complex and the consequent slower tumbling. Moreover, the τ_c estimates, ~10 ns, are also comparable to those evaluated for Odin-Sam1 in complex with EphA2-Sam,[12] Ship2-Sam bound to Arap3-Sam (i.e., 11 ns)[9] as well as the AIDA1-Sam1/Sam2 tandem (9.1 ns)[25] and point to a 1:1 binding stoichiometry for the Odin-Sam1/Arap3-Sam interaction.

SPR and ITC studies

To further quantify the Odin-Sam1/Arap3-Sam binding affinity, we performed SPR (Figure 3) and ITC experiments (Figure 4).

SPR binding assays were carried out by immobilizing Arap3-Sam on the chip surface and using Odin-Sam1 as analyte. The fitting of data obtained by plotting RUmax values as function of Odin-Sam1 concentration together with kinetic experiments, both implemented with a 1:1 binding model, gave a dissociation constant value in the low micromolar range (Figure 3).

Indeed a K_D=2.9 ± 0.4 μM was obtained with a non-linear regression analysis of experimental data (Figure 3).

In the ITC experiments (Figure 4), the exothermic heat peaks exhibited a monotonic decrease with addition of Arap3-Sam until saturation was reached. The data could be best fitted by a nonlinear least squares approach to the “one set of sites” binding model (in agreement with ^{15}N relaxation measurements, See previous paragraph), which yielded a dissociation constant K_D=0.37 ± 0.08 μM (Figure 4 and Supplemental Table S1). However, the estimated K_D values are similar to those determined in our previous binding assays for the Odin-Sam1/EphA2-Sam complex (i.e., 5.5 ± 0.9 μM and 0.62 ± 0.04 μM by SPR and ITC techniques respectively)[12] thus reflecting comparable binding affinities of Arap3-Sam and EphA2-Sam for Odin-Sam1.

Structural features of the Odin-Sam1/Arap3-Sam complex: Haddock models

To shed light on the pattern of possible intermolecular contacts at the basis of the Odin-Sam1/Arap3-Sam interactions we carried out molecular docking studies using Haddock.[26] These studies aimed at obtaining speculative models of the complex by using information from chemical shift perturbation studies and analysis of experimentally determined structures of other heterotypic Sam-Sam associations (Figure 5 lower panel and Supportive Section S2). Our models present a Mid-Loop (ML)/End-Helix (EH) topology[12, 25] in which the Mid-Loop interface is provided by Odin-Sam1 while the End-helix surface resides on Arap3-Sam (Figures 5 lower panel and Section S2).

Arap3-Sam binding region is predominantly rich in positively charged residues, whereas Odin-Sam1 surface contains many negatively charged residues thus, electrostatic interactions appear important for this association (Figure 5 lower panel).

To further investigate this point, we studied the binding of the Arap3-Sam (H37D, R77D, R80D) triple mutant[9] to Odin-Sam1. In this mutant we commuted the charge of three residues that are placed in the presumed Arap3-Sam EH binding site (Figure 2 right panel). NMR and ITC assays fail to show meaningful binding of the triple mutant to Odin-Sam1 (Supportive Figure S3) and let speculate that either the mutated residues are supplying pivotal interactions at the Sam-Sam interface and/or are causing conformational changes in the binding region, that void association. Intriguingly, the residue Arg77 in Arap3-Sam in most of the docking solutions is engaged in electrostatic interactions with either Asp54 and/or Asp55 in Odin-Sam1 (Figure 5 lower left panel).

Based on these models, Arap3-Sam residues His37 and Arg80 seem less important although, in a few models His37 makes a salt bridge with Odin-Sam1 residue Asp55 (Supportive Section S2), while residue Arg80 could form a cation-π interaction with Odin-Sam1 Phe58 or a salt bridge with Odin-Sam1 Asp68 (Supportive Section S2). The Arap3-Sam (H37D, R77D and R80D) mutant is also unable to bind the Sam domain of the lipid phosphatase Ship2 that instead can associate with wild-type Arap3-Sam by adopting a ML/EH binding model in which Arap3-Sam contributes the EH interface.[9]

Another interaction to be pointed out in the Odin-Sam1/Arap3-Sam docking models concerns with the residue Gly73, that is positioned on the EH surface of Arap3-Sam (Figure 5 lower right panel).

It is interesting noting that the peak corresponding to the $^{1}H^{N}$ of Gly73 can be only detected in the [^{1}H, ^{15}N] HSQC spectrum of Arap3-Sam in its bound form (Figure 1C) thus indicating that this Gly may become buried and possibly engaged in an intermolecular contact in the Odin-Sam1/Arap3-Sam complex. Indeed, in a few docking solutions, the $^{1}H^{N}$ of Gly73 forms an intermolecular H-bond (Supportive Information). In particular, in the Odin-Sam1/Arap3-Sam model, shown in the lower panel of Figure 5, the backbone H^{N} atom of Gly73 makes an H-bond with the backbone carbonyl oxygen atom of Odin-Sam1 residue Asn51. This kind of interaction between the backbone amide proton of a Gly, that is positioned at the bottom of the α5 helix on the EH interface, and the backbone carbonyl oxygen of another residue, that is located at the C-terminal end of the α2 helix in a ML surface, is present in several experimentally determined structures of Sam-Sam complexes (See pdb codes: 1PK1,[27] 2KIV,[25] 3BS5,[20] 3SEI,[10] 3SEN[10]).

Interestingly recent NMR studies of the EphA2-Sam/Ship2-Sam complex revealed intermolecular NOEs in between the backbone H^{N} proton of Gly74 at the N-terminal end of α5 helix in the EphA2 receptor and the Hβ protons of an Asn located at the C-terminal end of α2 helix in Ship2-Sam (pdb code: 2KSO);[28] thus further confirming our hypotheses.

It has been proposed that a Gly residue in that position of an EH interface is highly desirable since can facilitate the approach towards the ML binding region.[25]

Next, we compared our Odin-Sam1/Arap3-Sam model with the NMR structure of the AIDA1-Sam1/Sam2 tandem (pdb code: 2KIV,[25] Figure 5 upper left panel) and found strict homology.

AIDA1[29] belongs, like Odin,[14] to the ANKS family; in the 3D structure of the AIDA1 Sam tandem, Sam1 and Sam2 bind by providing the ML and EH sites respectively[25] and interestingly, a Gly residue at the beginning of the α5 helix on the binding interface of Sam2 is likely providing an intermolecular H-bond with an Asn from the Sam1 interaction surface (Figure 5 upper left panel). Moreover, our Odin-Sam1/Arap3-Sam models resemble the docking structures we recently proposed for the Odin-Sam1/EphA2-Sam complex (Figure 5 upper right panel) in which EphA2-Sam is providing the EH interaction surface, several intermolecular salt bridges can take place at the Sam-Sam interface and a Gly at the N-terminal end of the α5 helix, on the EH side, may be involved in an intermolecular H-bond (Figure 5 upper right panel).[12]

A displacement assay with NMR techniques, indicates actually that Odin-Sam1 employs the same ML interaction site to bind both EphA2-Sam and Arap3-Sam (Supportive Figure S4), and thus behaves like the Sam domain of the lipid phosphatase Ship2 (Ship2-Sam).[9] In fact, Ship2-Sam binds with its ML site both Arap3-Sam and EphA2-Sam.[9, 19] The structural similarities between heterotypic associations of Arap3-Sam and EphA2-Sam reflect probably the high sequence homology in between these two Sam domains (Supportive Figure S5) that appears to dictate a shared protein interactions network. Since heterotypic Sam-Sam interactions of EphA2-Sam with either Odin and Ship2 are connected to EphA2 receptor endocytosis,[13, 30] we cannot exclude that Arap3-Sam may play a role in this process by sequestering two crucial regulators, but to date the intricate machinery that governs these heterotypic associations is not completely comprehended.

Conclusion

A yeast two-hybrid screen previously identified the ANKS1 protein as a binding partner of the PI3K effector Arap3.[4] ANKS family includes Odin (also known as ANKS1 and ANKS1A) and AIDA-1 (ANKS1B) which own two Sam domains in tandem.[14] The NMR structure of the tandem Sam domains of AIDA-1 reveals a characteristic Sam-Sam head to tail topology in which Sam1 and Sam2 bind with a Mid-Loop (ML)/End-Helix (EH) Model.[10, 25] The structure of Odin-Sam1 was solved by us with solution NMR techniques[12] whereas, the experimental structure of the tandem Sam domains of Odin is not yet available in the Protein Data Bank (PDB). However, due to the high sequence similarity with AIDA-1, it's likely that the Odin Sam1-Sam2 tandem adopts a similar ML/EH architecture.

Herein, we investigated the interaction between Odin-Sam1 and Arap3-Sam and demonstrated that the two domains bind with a dissociation constant in the low micromolar range by forming a ML/EH hetero-dimer, that is seemingly stabilized by electrostatic interactions. In our model, the central part of Odin-Sam1 and the C-terminal helix together with close loop regions of Arap3-Sam supply the ML and EH binding sites respectively. Taken together, our studies suggest that the interactions between Odin-Sam1 and Arap3-Sam may require separation of Odin-Sam2 from the Odin-Sam1-Sam2 tandem in the intact protein, similarly to what we have previously hypothesized for the association between EphA2-Sam and Odin-Sam1.[12] Moreover, our observations, in conjunction with earlier data, suggest that Arap3-Sam adopts interaction patterns to Odin-Sam1 that highly resemble those between EphA2-Sam and Ship2-Sam. Since Odin and Ship2 are both regulators of EphA2 receptor endocytosis,[13, 30] the revealed analogies may indicate a possible involvement of Arap3-Sam in this process. However, to date, the functional consequences of the association between Arap3-Sam and Odin-Sam1 are unknown thus, our immediate goals will be to generate, based on our structural models, new peptide/peptidomimetic molecules that can interfere selectively with Arap3 Sam-Sam interactions, and analyse the eventual phenotypic alterations that such molecular probes induce in a cellular environment.

Experimental Section

Protein expression

Sam domains were expressed as recombinant proteins in *E. coli*. PET15B-constructs encoding human Arap3-Sam wild-type (residues 1–80, UniprotKB/TrEMBL code: Q8WWN8),[9] Arap3-Sam triple (H37D, R77D, R80D) mutant,[9] Odin-Sam1 (residues 691–770 of human Odin, UniprotKB/TrEMBL code: Q92625),[12] EphA2-Sam (residues 901–976 of human EphA2, UniprotKB/TrEMBL code: P29317)[12] were purchased from Celtek Bioscience (Nashville, TN).

Genes were transformed using BL21-Gold (DE3) competent cells (Stratagene). Protein expression and purification procedures were conducted as previously described.[9, 12] In particular, to express unlabeled proteins, bacteria were grown in LB medium; $^{15}N/^{13}C$ doubly labeled and ^{15}N labeled proteins were expressed in M9 minimal media containing ^{13}C-Glucose and/or $^{15}NH_4Cl$ respectively. Bacteria were grown at 37°C; β-D-thiogalactopyranoside (IPTG) (1 mM) was used to induce protein over-expression at a cell optical density OD_{600}=0.6 nm (T= 25 °C, induction time: overnight). Purification of His-tag provided proteins was performed on a nickel column with an AKTA Purifier apparatus (GE Healthcare, Milan, Italy).

Backbone resonance assignments of the Odin-Sam1/Arap3-Sam complex

NMR experiments were performed at 25 °C on a Varian Unity Inova 600 MHz spectrometer equipped with a cold probe. HNCA and 3D ^{15}N resolved [^{1}H, ^{1}H] NOESY (100 ms mixing time) spectra, acquired with samples containing either ^{15}N/^{13}C double labeled Odin-Sam1 (620 μM) and unlabeled Arap3-Sam (~3 mM), or doubly labeled Arap3-Sam (600 μM) and unlabeled Odin-Sam1 (1.2 mM), were analyzed to get resonance assignments for the backbone H, N and Cα atoms of the Odin-Sam1/Arap3-Sam complex. NMR samples (600 μL volumes) were prepared in phosphate buffer saline (PBS, 10 mM phosphates, 138 mM NaCl, 2.7 mM KCl) (Fisher) at pH=7.7 with 0.2% NaN_3 and 5% D_2O. Spectra were processed with the Varian software (Vnmrj version 1.1D) and analyzed with NEASY[31] (http://www.nmr.ch/).

Relaxation measurements

NMR experiments to evaluate backbone ^{15}N longitudinal (R1) and transverse (R2) relaxation rates, were performed at 25 °C on a Varian Unity Inova 600 MHz spectrometer provided with a cold probe. Measurements were carried out with two samples of Sam-Sam complexes made up of either ^{15}N/^{13}C double labeled Odin-Sam1 (620 μM) plus unlabeled Arap3-Sam (3 mM) and ^{15}N/^{13}C double labeled Arap3-Sam (100 μM) plus unlabeled Odin-Sam1 (about 300 μM).

R1 and R2 relaxation data were collected as 1D spectra (4 K data points and 1–4 K transients). R1 data sets were recorded with the following relaxation delays: 0.01, 0.1, 0.3, 0.6, 1.0 s; R2 data sets were acquired with relaxation delays: 0.01, 0.03, 0.05, 0.07, 0.09, 0.11, 0.15, 0.19 s.

Average R1 and R2 were estimated by monitoring the decrease of signal intensity as function of the relaxation delays. The software tmest (A. G. Palmer III, Columbia University) was implemented to calculate the rotational correlation time from average R2/R1 ratios.[32]

NMR Binding studies

NMR titration experiments with 2D [^{1}H, ^{15}N] HSQC spectra were carried out to study the protein-protein interaction. Odin-Sam1 binding interface for Arap3-Sam was identified by analysis of 2D [^{1}H, ^{15}N] HSQC spectra of ^{15}N labeled Odin-Sam1 (80 μM) in the unbound-form and after addition of unlabeled Arap3-Sam (120, 370, 620 μM). To recognize the binding site of Arap3-Sam for Odin-Sam1, 2D [^{1}H, ^{15}N] HSQC spectra of a ^{15}N labeled Arap3-Sam sample (90 μM) were recorded in absence and presence of unlabeled Odin-Sam1 (100, 300 and 800 μM).

Binding of Arap3-Sam (H37D, R77D, R80D) mutant to Odin-Sam1 was investigated by analogous NMR chemical shift perturbation experiments that were conducted with either ^{15}N labeled Arap3-Sam triple mutant (200 μM) and unlabeled Odin-Sam1 (200 and 300 μM) and ^{15}N labeled Odin-Sam1 (144 μM) and unlabeled Arap3-Sam mutant (285 and 625 μM) (See Supportive Data).

For the NMR displacement experiment, 2D [^{1}H, ^{15}N] HSQC spectra were recorded for a ^{15}N labeled Arap3 protein sample at the concentration of 50 μM in its unbound form, after addition of Odin-Sam1 (150 μM), and in concurrent presence of Odin-Sam1 and EphA2-Sam (protein ratios after final dilutions: 1 (Arap3-Sam), 3 (Odin-Sam1), ~40 (EphA2-Sam)). Analysis of titration experiments and overlays of 2D spectra were generated with the program Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

Surface Plasmon Resonance

Arap3-Sam was immobilized in acetate buffer (10 mM) pH=5.0 (flow rate: 5 μL/min, injection time: 7 min) on a CM5 Biacore sensor chip, using EDC/NHS chemistry.[33] Residual reactive groups were deactivated with ethanolamine hydrochloride (1 M), pH=8.5; the reference channel was prepared by activating with EDC/NHS and deactivating with ethanolamine. The immobilization level for Arap3-Sam was 1840 RU. Experiments were conducted at 25 °C and a constant 20 μL/min flow rate using as running buffer a solution of Hepes (10 mM), pH=7.4, NaCl (150 mM), surfactant P20 (0.05% v/v and 90 μL injections for each experiment). Binding assays were conducted by using Odin-Sam1 at concentrations ranging from 0.20 to 40 μM. The BIA evaluation analysis package (version 4.1, GE Healthcare, Milano, Italy) was used to subtract the signal of the reference channel and to estimate K_D values. The software GraphPad Prism, version 4.00 (GraphPad Software, San Diego, California) was implemented to fit RUmax data *versus* protein concentrations by non-linear regression analysis.[34]

ITC studies

ITC experiments were carried out with an iTC200 calorimeter (Microcal/GE Healthcare, Milan, Italy). Arap3-Sam (250 μM concentration in PBS buffer pH=7.7) was titrated into a solution of Odin-Sam1 (10 μM concentration in PBS buffer pH=7.7). Data were fit to a single binding-site model with the Origin software provided by GE Healthcare (GE Healthcare, Milan, Italy).

Similar ITC studies were conducted with a solution of Arap3-Sam (H37D, R77D, R80D) triple mutant (250 μM in PBS pH=7.7) and Odin-Sam1 (10 μM in PBS pH=7.7).

Molecular Modeling

Docking studies were conducted with the Haddock web server.[26] Models of the Odin-Sam1/Arap3-Sam complex were generated starting from NMR structures (i.e., first conformer of Odin-Sam1: pdb code 2LMR,[12] and first five structures of Arap3-Sam NMR ensemble: pdb code 2KG5).[9] For Arap3-Sam the flexible C-terminal tail (encompassing residues 90–100) was omitted from the docking procedure. Ambiguous interaction restraints were generated from chemical shift perturbation data. For both Odin-Sam1 and Arap3-Sam active and passive residues were represented by a subset of the ones with higher normalized Δδ, filtered by evaluating either their solvent exposure and/or the possible involvement into intermolecular interactions, as suggested by analysis of experimental structures of Sam-Sam complexes (for example the AIDA-1 tandem Sam domain, pdb code: 2KIV).[25] For Odin-Sam1, residues L50, N51, F53, D54, D55, F58, E65, E66, D68, D71 were set as active whereas, residues L49, G52, V56, H57, S61, N62, V63, M64, Q67, R70 were considered passive; the region from L50 to E66 was set as semi-flexible interface finally, the N and C-terminal tails (segments from M21 to G24 and from A95 to N101 respectively) were put as fully flexible during all the docking stages. For Arap3-Sam, the docking procedure included G73, H74, K76, R77 as active residues; R75, L79 as passive residues; T72-R80 and V36-L38 as semi-flexible tails; H20-D26 as fully flexible region.

The solvated docking mode was implemented.[35] One thousand structures were generated during the first phase of the docking protocol that consists of the rigid body energy minimization; next, a semi-flexible simulated annealing of the best 200 solutions was conducted; the last stage consisted of a refinement in water.

The final 200 Haddock solutions were visually analyzed and wrong models, in disagreement with experimental NMR data or presenting non canonical Sam-Sam orientations, were discarded. After this first selection strategy, 106 Haddock models were further inspected

with the software MOLMOL[36] and compared with experimental structures of other heterotypic Sam-Sam complexes (reference pdb code 2KIV), [25] to recognize relevant structural aspects. In the end, twenty-three models were considered representative of the possible conformations of the Arap3-Sam/Odin-Sam1 complex. Solutions were clusterized using a pairwise RMSD cutoff value of 2.26 Å and at least one structure *per* cluster. RMSD values were calculated with MOLMOL[36] by superimposing the models on the backbone atoms of the secondary structure elements of both Arap3-Sam and Odin-Sam1 (i.e., residues 29–34, 39–47, 61–66, 72–83 for Arap3-Sam and 32–39, 42–49, 57–60, 66–72, 77–88 for Odin-Sam1). Four clusters were obtained from the selected solutions (See Supportive Data, Section S2).

We also performed docking calculations with the more conventional Haddock protocol[26] (Supportive Section S6). For these studies, we chose as active those exposed residues (solvent exposure evaluated with MOLMOL ≥ 24.6 %) of Odin-Sam1 and Arap3-Sam with chemical shift variations ≥ 0.2 ppm and 0.1 ppm respectively; passive residues were set automatically by Haddock; calculations were started from initial ten conformers of both Sam domains; semi flexible and fully flexible regions were chosen like in the previous run. The achieved results are discussed in the Supportive Info and are in good agreement with those obtained with the other described strategy in which we used instead a reduced set of active and passive residues. It's obvious that our models, built only on the basis of chemical shift perturbation data and analyzed by looking for structural analogies with other Sam-Sam hetero-complexes, need to be considered exploratory as, of course they do not have the precision of a structure calculated by using unambiguous restraints such as intermolecular NOEs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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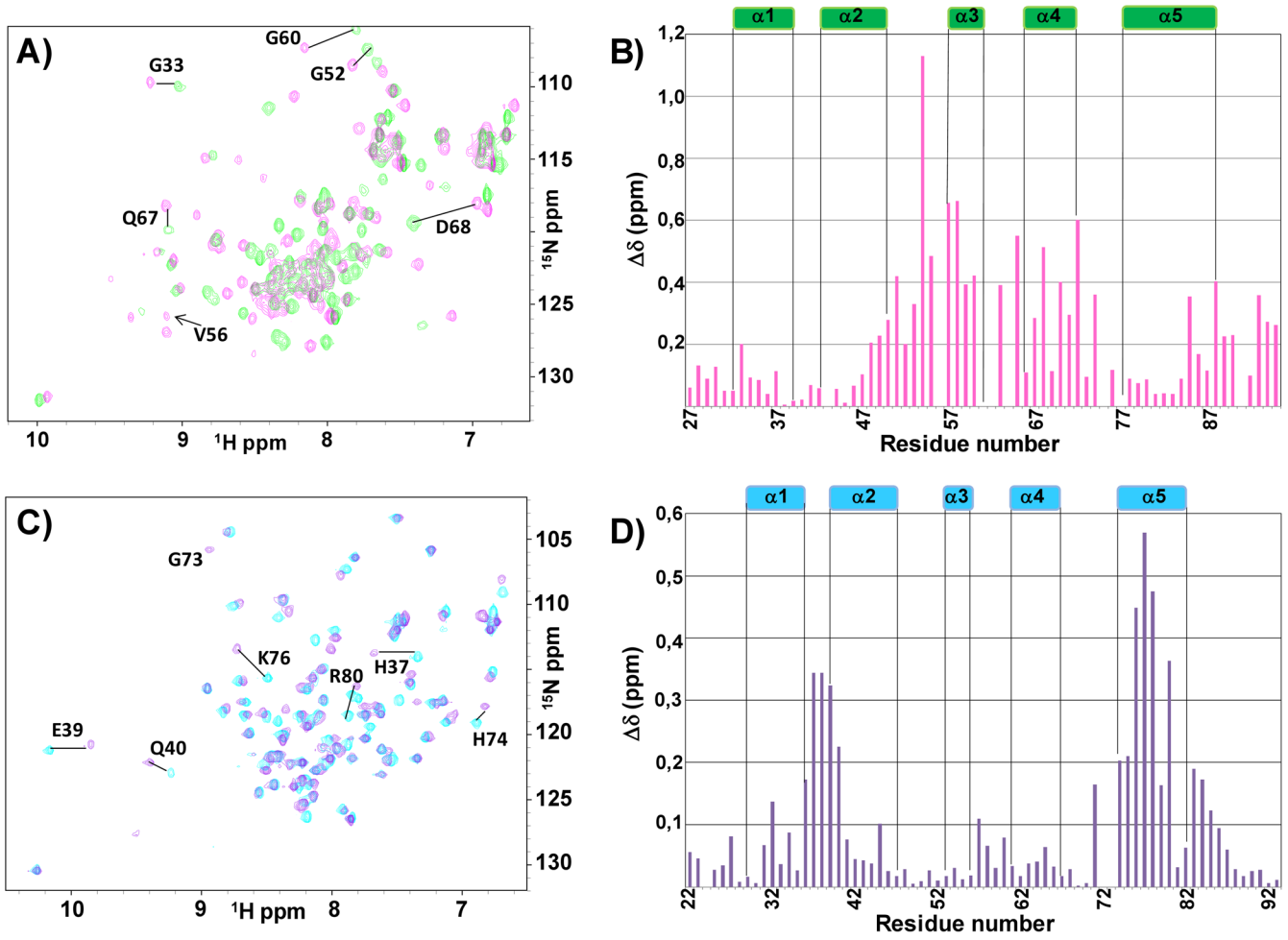

Figure 1.

A) Comparison of [^{1}H, ^{15}N] HSQC spectra of ^{15}N labeled Odin-Sam1 (80 μM) in the free state (green) and associated to Arap3-Sam (370 μM) (magenta). **B)** Diagram of chemical shift deviations ($\Delta\delta = [(\Delta H_N)^2 + (0.17 * \Delta^{15}N)^2]^{1/2}$) against Odin-Sam1 sequence numbers. Residues Q43, V56, N62 (their peaks can be revealed only in the spectrum of the Odin-Sam1/Arap3-Sam complex), S61, M64, S75 (unassigned), P77 and P91, were excluded from the analysis.

C) Superposition of [^{1}H, ^{15}N] HSQC spectra of ^{15}N labeled Arap3-Sam (90 μM) in absence (cyan) and presence (violet) of unlabeled Odin-Sam1 (300 μM). **D)** Graph reporting chemical shift deviations *versus* Arap3-Sam residue numbers. Data were set equal to 0 for residues T72, G73, that were identified only in the spectrum of the bound protein, and P24.

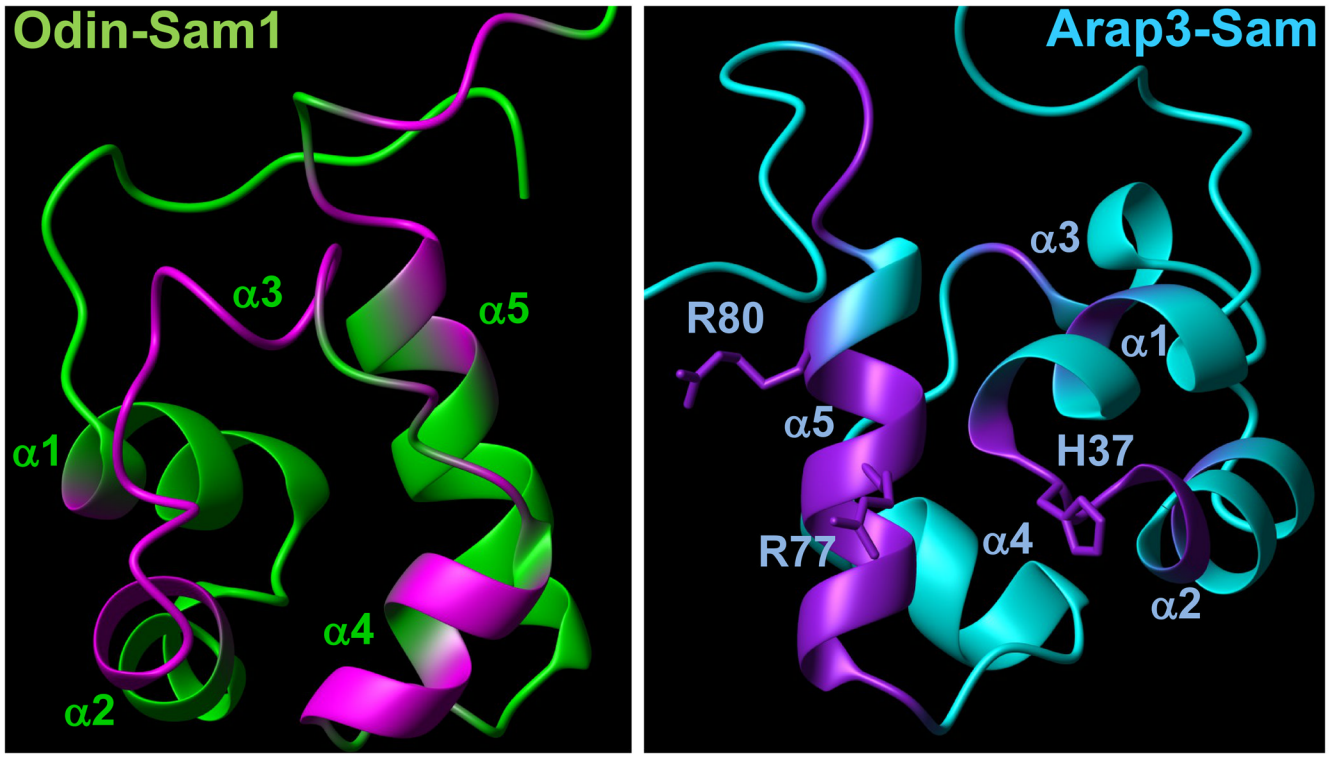

Figure 2.

(Left side) The first conformer of Odin-Sam1 NMR ensemble (pdb code: 2LMR)[12] is reported in a ribbon representation where the backbone of residues with chemical shift deviations, $\Delta\delta \geq 0.2$ ppm (i.e., G33, L48, L49, L50, N51, G52, F53, D54, D55, V56, H57, F58, L59, G60, V63, E65, Q67, D68, R70, D71, I72, I74, Q85, R88, S89, L90, V93, K94, A95), are highlighted in magenta. **(Right side)** Ribbon drawing of Arap3-Sam (NMR conformer number 1, pdb code: 2KG5);[9] residues presenting $\Delta\delta \geq 0.1$ ppm (i.e., W32, V36, H37, L38, E39, Q40, F45, R57, A71, T72, G73, H74, R75, K76, R77, I78, L79, R80, Q83, T84, G85) are colored purple. Side chains of amino acids related to mutagenesis studies are shown.

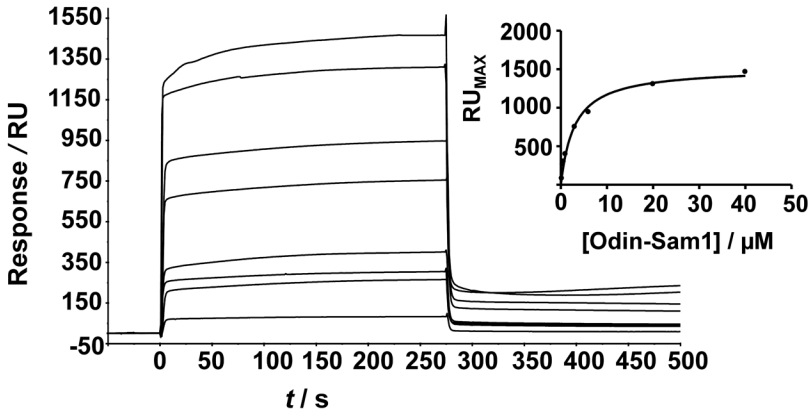

Figure 3.

SPR Experiments. Superposition of sensorgrams corresponding to the interaction of Odin-Sam1 with immobilized Arap3-Sam (concentration range 0.20–40 μM). A plot of RU_{max} from each binding as function of Odin-Sam1 concentration is reported in the upper panel; data were fit by non-linear regression analysis.

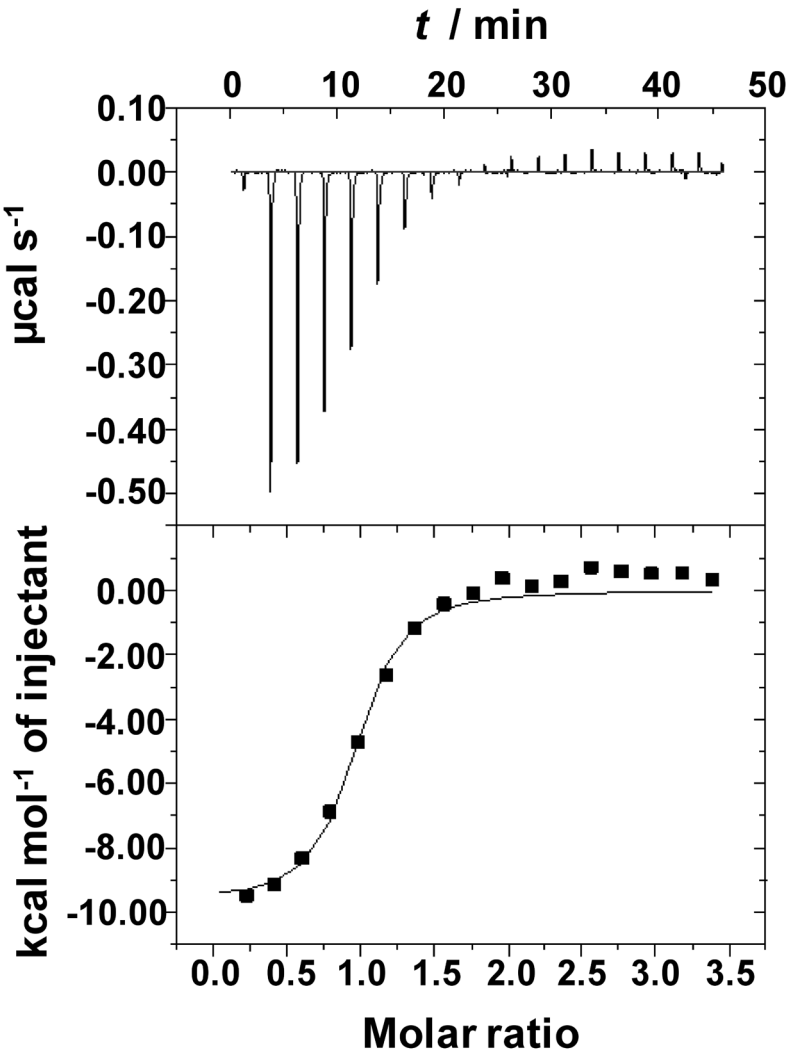

Figure 4.

ITC Studies. Calorimetric curve relative to Odin-Sam1 (10 μM) titration with Arap3-Sam (250 μM). Raw and integrated data are shown in the upper and lower panels respectively. In the lower section data fitting was achieved with a single binding site model.

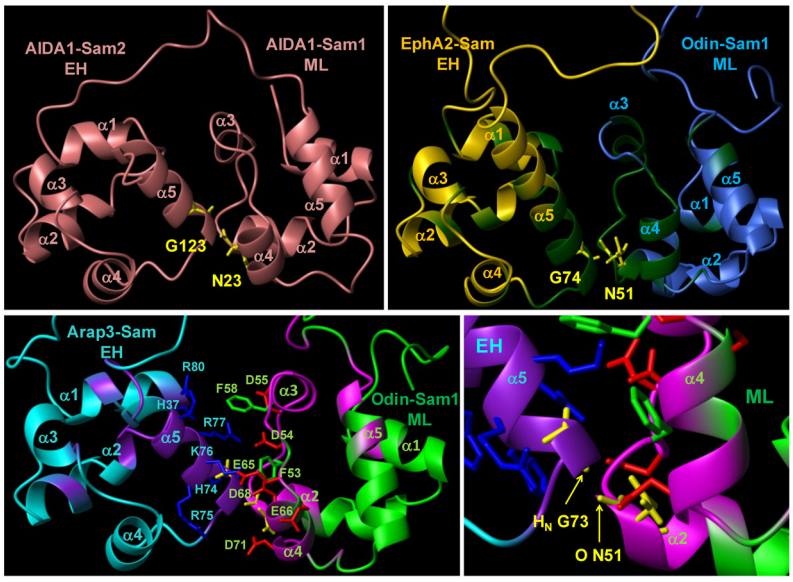

Figure 5.

(Upper left panel) NMR structure of the AIDA-1 Sam domain tandem (pdb id: 2KIV,[25] structure n.1). **(Upper right panel)** One representative Haddock[26] model (number 2) of the Odin-Sam1/EphA2-Sam complex.[12] The backbone of residues participating to the interaction, according to NMR chemical shift perturbation data, are colored green on the ribbon representations of Odin-Sam1 and EphA2-Sam.[12] **(Lower left panel)** Haddock model (number 57) of Odin-Sam1/Arap3-Sam complex; residues, that following association undergo major chemical shift variations, are colored magenta and violet respectively on the structures of Odin-Sam1 (green) and Arap3-Sam (cyan). Side chains of a few amino acids that could contribute to the interaction interface are shown. **(Lower right panel)** Detail of the Odin-Sam1/Arap3-Sam Haddock model that better illustrates the H-bond between the backbone amide proton of Gly73 on the Arap3-Sam EH site and the backbone carbonyl oxygen of Asn51 (Odin-Sam1 ML binding region).