CONFORMATION-SPECIFIC BINDING OF ALPHA-SYNUCLEIN TO NOVEL PROTEIN PARTNERS DETECTED BY PHAGE DISPLAY AND NMR SPECTROSCOPY*

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Alpha-synuclein (AS) is an intrinsically unstructured protein in aqueous solution but is capable of forming β-sheet-rich fibrils that accumulate as intracytoplasmic inclusions in Parkinson’s disease and certain other neurological disorders. However, AS binding to phospholipid membranes leads to a distinct change in protein conformation, stabilizing an extended amphipathic α-helical domain reminiscent of the exchangeable apolipoproteins. To better understand the significance of this conformational change, we devised a novel bacteriophage display screen to identify protein binding partners of helical AS and have identified 20 proteins with roles in diverse cellular processes related to membrane trafficking, ion channel modulation, redox metabolism, and gene regulation. To verify that the screen identifies proteins with specificity for helical AS, we further characterized one of these candidates, endosulfine alpha (ENSA), a small cAMP-regulated phosphoprotein implicated in the regulation of insulin secretion, but also expressed abundantly in brain. We used solution NMR to probe the interaction between ENSA and AS on the surface of sodium dodecyl sulfate (SDS) micelles. Chemical shift perturbation mapping experiments indicate that ENSA interacts specifically with residues in the N-terminal helical domain of AS in the presence of SDS but not in aqueous buffer lacking SDS. The ENSA-related protein ARPP-19 (cAMP-regulated phosphoprotein 19) also displays specific interactions with helical AS. These results confirm that the helical N-terminus of AS can mediate specific interactions with other proteins, and suggest that membrane binding may regulate the physiological activity of AS in vivo.

Alpha-synuclein (AS) is an abundant constituent of healthy brain tissue, but its normal function is not well understood. In recent years, AS has gained notice due to its association with both inherited and sporadic Parkinson’s disease (PD). Mutations in the AS protein sequence are associated with PD (1-3), as are gene duplication events leading to increased expression of the protein (4, 5). AS is also the major fibrillar protein component of Lewy bodies (6), which are associated with both sporadic and inherited PD, as well as Alzheimer’s disease, Down’s syndrome, and multiple system atrophy (reviewed in (7)). AS was originally identified in three different contexts: as a putative synaptic vesicle protein (8), as a component of protein aggregates in Alzheimer brain tissue (9), and as a gene regulated in the zebra finch song control circuit during the critical period for song learning (10). More recent evidence suggests a role in modulating neurotransmission (11, 12).
AS displays remarkable structural plasticity. While the protein is intrinsically unstructured in aqueous solution, it can adopt multiple distinct stable conformations depending upon its environment. Upon prolonged incubation in aqueous solution, the protein forms amyloid fibrils rich in β-sheet secondary structure, reminiscent of the pathological fibrils found in Lewy bodies. However, in the presence of acidic phospholipid vesicles, the N-terminus of AS folds into an amphipathic helical conformation that mediates membrane binding (13-15). The hydrophilic face of this helix, which is presumably exposed to the neuronal cytosol (16), is highly conserved at the level of individual amino acids (10), suggesting that interactions with other binding partners have constrained its evolution.

Conventional methods for identifying protein binding partners in solution (e.g., immunoprecipitation, yeast two-hybrid screening) are unsuitable for study of interactions that depend on the helical conformation of AS. Under intracellular conditions, AS appears to be in dynamic equilibrium between membrane and cytosol, and there is thus no practical way to enrich for a particular conformation in vivo. We therefore developed a novel in vitro approach utilizing bacteriophage display to screen for proteins that bind selectively to AS in its lipid-associated helical conformation. Applying this method, we identified 20 human brain proteins that interact specifically with phospholipid-bound AS. One of these proteins, endosulfine alpha (ENSA), was chosen for further investigation because its small size (13 kDa) is compatible with solution NMR. To characterize the interaction between ENSA and AS in detail, we employed chemical shift perturbation mapping experiments that demonstrate a highly selective interaction between ENSA and specific residues in the N-terminal helical domain of AS. This observation was extended by demonstration of a similar interaction between AS and cAMP-regulated phosphoprotein 19 (ARPP-19), a protein highly related to ENSA that is also enriched in brain.

Experimental Procedures

Phage Display - N-terminally his-tagged human alpha-synuclein (AS) protein was expressed in E. coli via the vector pET28a (Novagen), and purified on Ni-NTA (Qiagen) according to manufacturer’s directions. Phospholipid vesicles (3:1 POPC/POPA) were prepared according to published protocols (14, 17); our previous studies indicate that stabilization of the AS helical domain requires acidic phospholipids, and that helix content is specifically maximized by the inclusion of phosphatidic acid (13, 14). 75 µg his-AS was incubated with 1.5 mg vesicles, and protein/vesicle complexes were isolated on a Superose 6 size exclusion column (GE Biosciences). Protein/vesicle complexes were incubated with T7 bacteriophage displaying cDNA from human brain (Novagen T7 Select). 1 X 10^7 pfu were incubated with 5 µg vesicle-bound AS for 1 hour at RT. Ni-NTA agarose beads (Qiagen) were added and the mixture was incubated end-over-end for 2 hours at 4°C. The suspension was poured into a column and the beads were washed with 20 mM imidazole to remove unbound phage, followed by phospholipid vesicles (protein-free) to elute phage with specificity for POPC/POPA vesicles. Finally, bacteriophage that bound to membrane-bound AS were eluted by addition of purified AS/vesicle complexes. The eluates were pooled and amplified in E. coli, then subjected to 3 more cycles of biopanning. After the final round, the eluates were applied to an affinity column prepared with his-tagged AS alone, and the flow-through was collected, to select for phage that did not bind to unstructured (lipid-free) AS. Individual bacteriophage clones were amplified and subjected to sequencing to identify the human brain protein sequences displayed by each.

Recombinant protein expression - Human endosulfine alpha (ENSA) was cloned from the full-length phage clone into the vector pDEST17 (Invitrogen), which encodes the N-terminal his-tagged linker MSYYHHHHHHHLESTLYKKA GSAAAPFT. The ARPP-19 cDNA (Origene) was also cloned into pDEST17. Recombinant protein expression was induced with 0.2% arabinose at 30°C for 3-4 hours (ENSA) and 5 hours (ARPP-19) and purified on Ni-NTA
agarose (Qiagen) according to manufacturer's directions.

An untagged ENSA construct was made in the vector pET21 (Novagen). Protein was expressed by induction with 1 mM IPTG at 30°C, followed by alkaline lysis, boiling, and precipitation with 60% ammonium sulfate. Precipitates were purified by hydrophobic interaction chromatography on a HiPrep 16/10 Butyl Fast Flow Sepharose column (GE Biosciences), and eluted with decreasing salt. ENSA-containing fractions were pooled and concentrated, then subjected to high-resolution gel filtration chromatography (HiPrep 16/10 Sephacryl S-200, GE Biosciences). Purity of recombinant ENSA, his-tagged ENSA, and his-tagged ARPP-19 proteins were confirmed by Coomassie staining. All proteins were shown to be immunoreactive with a chicken polyclonal antibody raised against full-length human ENSA (Abcam ab14297) (Fig. 1). In addition, predicted molecular weights of recombinant proteins were verified by mass spectroscopy (data not shown).

Full-length, untagged AS protein was produced for NMR experiments according to published procedures (18). For isotopically labeled protein, cultures were grown in M9 minimal media supplemented with 2 g [13C]-glucose, 1 g [15N]-ammonium chloride, 10 mg biotin, 10 mg thiamine and 10 mL [13C, 15N]-Bioexpress (Cambridge Isotopes Laboratories, Inc., Andover, MA). The final steps of the purification (size-exclusion chromatography and filtration (0.2 µm)) were performed immediately prior to NMR experiments, in order to avoid the presence of oligomeric species of AS.

**NMR spectroscopy** - Solution NMR spectra were acquired at the VOICE NMR Facility on a Varian INOVA 600 MHz spectrometer equipped with a 5 mm, triple resonance (1H-13C-15N) triaxial gradient probe, using VNMRJ version 2.1B with the BioPack suite of pulse programs released in early 2006. 2D 1H-15N HSQC spectra were measured typically for two hours per spectrum, digitizing 512 complex points in the indirect 15N dimension (t1max = 232 ms). Further details specific to each spectrum are provided in the figure captions. Spectra were processed with NMRPipe (19) and analyzed in Sparky (20), using the previously established assignments of 15N and 1H chemical shifts of AS in the free (21) and micelle-bound states (15, 22). The free state consisted of 0.2 to 0.5 mM 15N-labeled-AS in 50 mM phosphate buffer, pH 7.4, 10% D2O, and 0.1% DSS as an internal chemical shift reference. The micelle-bound state of AS was prepared by addition of sodium dodecyl sulfate (SDS) directly to the NMR tube, achieving a final concentration of at least 28 mM, i.e., a 140:1 molar ratio of SDS to AS, with the concentration of SDS significantly above its critical micelle concentration. The critical micelle concentration in 50 mM phosphate buffer at 25°C was determined to be ~2 mM using isothermal titration calorimetry (ITC) (data not shown). In all NMR experiments, the 15N-AS sample was examined in the unfolded state to confirm sample integrity; subsequently, SDS was added to prepare the micelle-bound state, and natural abundance partner proteins were titrated immediately thereafter. Control spectra of AS were highly reproducible and showed no evidence of aggregation, such as peak broadening, changes in chemical shifts as a function of time, or signal intensity losses.

**Partner protein titrations** - For each partner protein, two series of titration experiments were performed. First, free 15N-AS was prepared as described above and the control HSQC spectrum acquired. To this 0.2 mM AS solution (400 µL), ~40 µL aliquots of natural abundance partner protein (0.2 mM) were added. HSQC spectra were acquired at each step, up to the point at which AS and the partner protein had equal concentrations. Second, a fresh sample of 15N-AS was prepared in the micelle-bound state, the control HSQC spectrum acquired, and titrations of the same partner protein (without additional SDS) performed over a range of AS to partner protein ratios of 1:0 to 1:1. In a subset of the experiments, the order of addition was changed by first mixing the AS and partner protein in their free states and then adding an excess of SDS, and in yet another case the partner protein (ENSA) was first bound to excess SDS before addition to the AS-SDS solution. These procedures all resulted in HSQC spectra corresponding to the final step of the partner protein titration, which were identical to within the limit of statistical significance (~0.01 ppm). The chemical shift perturbation integrating both
changes in $^{1}H$ and $^{15}N$ chemical shifts was defined as $\delta = ([0.1 \delta_{n}]^2 + \delta_{H}^2)^{1/2}$, considering that the $^{15}N$ shift covers a range ~10 times that of $^{1}H$.

RESULTS

Identification of binding partners of helical alpha-synuclein by phage display. Vesicle-bound AS was used to pan a human brain bacteriophage display library as described in Experimental Procedures. After 3-5 rounds of selection, individual clones from the pool were amplified, and their inserts were determined by sequencing with primers flanking the insertion site in the gene encoding coat protein 10B. 338 bacteriophage clones were sequenced, of which 38 yielded readable in-frame sequences of identified human brain proteins. These 38 sequences represented 20 unique proteins, seven of which were isolated more than once and 13 that were single isolates.

In Table 1, the candidates are sorted based upon the functional data available in the literature. Seven of these proteins have been reported previously to interact physically or functionally with AS under various conditions. These proteins include alpha-tubulin, the sequence isolated with the greatest frequency overall (6/38 bacteriophage clones). Our laboratory has previously reported that AS binds monomers of alpha- and beta-tubulin (23), and it was recently reported that AS binds monomeric tubulin and stimulates microtubule polymerization (24). Like alpha-tubulin, septin 4 (also known as CDCrel-2) is a filament-forming G protein thought to function as a scaffolding protein for membrane/cytoskeleton interactions (reviewed in (25)); septin 4 was shown to bind AS when the two proteins are co-expressed in cultured cells, and to co-associate with AS in Lewy bodies (26). Kinesin light chain 1S and dynein heavy chain I are components of microtubule motors critical for vesicular and organellar trafficking in neurons and other cells. Our identification of these proteins confirms a previous report by Utton et al. that AS interacts with complexes containing either dynein or kinesin-1 in neurons (27). As previously reported by Elkon et al. (28), we find that AS binds to cytochrome C oxidase, the mitochondrial complex IV enzyme. FK506 binding proteins, members of the immunophilin protein family, were recently reported to stimulate fibril formation by alpha-synuclein (29); one of these family members, FKBP52, was recovered in our screen. Of the remaining proteins in Table 1, only G protein-coupled receptor kinase 2 (GRK2) has been reported to interact with AS in prior studies. GRK2 phosphorylates AS at serine 129, a modification that enhances AS inclusion formation in cell culture (30) and in a Drosophila model of PD (31).

The sizes of the cDNA inserts in the bacteriophage inserts in the bacteriophage library vary, but generally encode no more than 200 amino acids of the corresponding human brain proteins. This means that in many cases a protein domain sufficient for interaction with AS can be inferred from the sequence of the phage insert. These preliminary “AS-binding domains” are mapped in Figure 2, along with known functional domains when available.

For our present purpose, of greatest interest are the 13 interacting proteins identified for the first time in this screen. These may represent interactions that depend specifically on assumption of a lipid-dependent helical structure. Each of these interactions is worthy of specific study; here we focus on detailed characterization of the interaction with endosulfine alpha (ENSA), the smallest protein in the set. ENSA was originally described as an endogenous ligand for the sulfonylurea receptor SUR1 (32), a subunit of the ATP-sensitive K+ channel. ENSA is abundant in the brain, where its expression is downregulated in Alzheimer’s disease (33) and Down’s syndrome (34).

NMR characterization of AS-ENSA interactions. NMR chemical shift perturbation mapping experiments were undertaken to determine the conformation dependence and structural specificity of the interactions between AS and ENSA. First, we tested for interactions between non-helical AS and ENSA. $^{15}N$-AS was prepared in aqueous buffer at $\sim$0.5 mM concentration. The $^{1}H$-$^{15}N$ HSQC spectrum was acquired and confirmed to be consistent with the unfolded state of AS as previously reported by Eliezer (15, 35) (Fig. 3A). Superimposed on the spectrum of pure $^{15}N$-AS is the spectrum of $^{15}N$-
AS with an equimolar quantity of ENSA. The peak positions throughout the spectrum remain essentially unchanged, with the exception of a portion of the C-terminal domain of AS, where some changes on the order of 0.01 ppm were observed. Elsewhere, changes in the spectra were within the random error of the chemical shift measurements (δ ~0.01 ppm). Sources of uncertainty in the chemical shifts include slight changes in pH or buffer concentration; therefore all the protein solutions were prepared by dialysis against a common buffer solution to minimize such effects.

Next, we evaluated the interaction between ENSA and helical AS. A fresh $^{15}$N-AS sample was prepared and SDS was added to create an excess number of detergent micelles, in the presence of which AS forms a stable, folded hairpin helical structure throughout the N-terminal ~100 residues (36). The stoichiometric excess of SDS, as previously determined by Eliezer (15) and utilized in studies by Chandra (22) and Ulmer (22, 36), was confirmed in our experiments by titrating SDS into a solution of free AS. We confirmed that the molar ratio of approximately 70:1 SDS:AS was required to ensure that all AS molecules were micelle-bound. Addition of SDS beyond the 70:1 molar ratio causes only small changes, and in the range studied here for protein-protein interactions, from ~100:1 to 200:1 SDS:AS ratio, there are no chemical shift perturbations of significance (>0.01 ppm; see Supplemental Figure 1). All of the subsequent titrations involving helical AS were carried out in molar ratios ≥ 140:1 SDS:AS, which is more than sufficient for complete binding of AS. Moreover, a ~50% or greater excess of unpopulated micelles remains available in the solution for binding to the candidate protein. The spectrum of $^{15}$N-AS on a micelle (Figure 3B) agrees well with published chemical shifts for $^{15}$N and $^1$H.

When ENSA was then added, systematic and highly reproducible changes in the spectra occurred. Fig. 3C illustrates the beginning and end points of the ENSA titration, where the chemical shift perturbation is most easily identified. The signals fall into three categories. First, many signals show substantial changes in chemical shift, i.e., the peak positions change by significantly more than the total peak line width. For example, A19, E20, T44, G51, A69, V71, and V74 are residues that show such unambiguous changes. Second, many other resonances show smaller shifts in chemical shift, i.e., the peak positions throughout the spectrum remain systematically and highly reproducible changes in chemical shift, i.e., the peak positions near the beginning and end points of the ENSA titration, δ near the center of states 1 and 2 is observed. The exact peak positions were within the random error of the chemical shifts (~0.01 ppm). Sources of uncertainty in the chemical shifts include slight changes in pH or buffer concentration; therefore all the protein solutions were prepared by dialysis against a common buffer solution to minimize such effects.

Fig. 3D-F shows the chemical shift changes systematically as a function of ENSA concentration. The progression of peak positions from the initial to final state is continuous and approximately linear with the ENSA concentration. For example, the G67 (Fig. 3E) resonance moves down (downfield in $^{15}$N) and to the right (upfield in $^1$H) by more than 0.1 ppm δ_N. A69 (Fig. 3D) changes most significantly in the $^1$H dimension. In other regions of the spectrum, several resonances such as A27, G73 and V74 (Fig. 3D, E, F) show a similar pattern. Other resonances move by smaller total amounts, but typically in a linear manner with concentration.

At no point in the titration of ENSA were multiple peaks observed for a given AS amide $^1$H-$^{15}$N site. This is consistent with a fast chemical exchange process, in which there is an interconversion between (1) the micelle-bound state of AS and (2) the micelle-bound state of AS in complex with the micelle-bound state of ENSA. (Several biophysical methods, including NMR (37), isothermal titration calorimetry (ITC), and circular dichroism (CD) spectroscopy (Boettcher et al., unpublished data) demonstrate strong binding of ENSA to SDS micelles, accompanied by a substantial change in secondary structure). If this process were slow (exchange rates of much less than 1,000 s$^{-1}$), each molecule would be either in state 1 or 2 during the course of the NMR acquisition, and two signals would be observed for each residue. Instead, characteristic of the fast exchange process (rates much greater than ~1,000 s$^{-1}$), the weighted average of the chemical shifts of the states 1 and 2 is observed. The exact peak position therefore depends upon the populations of states 1 and 2 (38, 39); the population of state 2 gradually increases throughout the titration,
and therefore the peak gradually moves in this direction.

In Fig. 4 the chemical shift perturbations are plotted as a function of residue number. Fig. 4A illustrates the very small effects of adding ENSA to a solution of AS in the absence of SDS. We interpret this as corresponding to no observable change in structure and minimal interaction between the unfolded proteins (primarily in the C-terminus of AS). Fig. 4B illustrates the corresponding shifts observed, for the end point of the titration, in the presence of SDS. In this case, large chemical shifts are observed in many positions throughout AS, including many prominent shifts throughout the N-terminal domain, which are not seen at all in the unfolded state. Analysis of $^{15}$N $T_2$ relaxation (Supplemental Table 1) is consistent with a population of SDS-AS-ENSA ternary complex that is larger than the SDS-AS binary complex.

**Common AS-binding domain in ARPP family proteins.** ENSA is a member of the cAMP-regulated phosphoprotein family, which also includes cAMP-regulated phosphoproteins ARPP-16 and ARPP-19. ARPP-16/19 are differentially spliced products of the same gene, found at the chromosomal locus 15q21.2 in humans (Unigene Hs.512908, (40)), and are identical except for their extreme N-termini. ENSA is the product of a distinct gene at the locus 1q21(41), which also differs from ARPP-16/19 primarily at the N-terminus. A central domain of 66 amino acids is highly conserved among ENSA and ARPP-16/19 (Fig. 5), suggesting that these proteins might interact similarly with helical AS. To test this hypothesis, recombinant his-tagged ARPP-19 was prepared, and its interactions with AS were assessed by solution NMR.

The NMR data illustrate the qualitatively similar behavior of AS when titrated with ARPP-19 as when titrated with ENSA. AS shows no substantial changes in the spectra when AS and ARPP-19 are combined in free aqueous solution; the spectra of $^{15}$N-AS, with and without a stoichiometric equivalent of ARPP-19 in the solution, are essentially identical within experimental error. No $^{15}$N chemical shifts vary by more than 0.02 ppm. Figure 6 illustrates changes from the starting point of the SDS micelle-bound AS sample, as ARPP-19 is added. Throughout the ARPP-19 titration, many of the same features in the spectra of $^{15}$N-labeled AS are observed as in the aforementioned ENSA titration experiments. Common observations include: (1) the chemical shift changes are systematically observed at many sites throughout the molecule; (2) the changes are approximately linear with partner protein concentration; (3) at no time are multiple resonances observed for an individual AS residue. Plotting the chemical shift changes as a function of residue number (Fig. 7) illustrates significant shifts throughout AS in the presence of SDS. As with the plot of ENSA data in the same format (Fig. 4), a qualitatively similar pattern of changes is observed in the N-terminal domain. The major difference observed for the titration of SDS micelle-bound AS with ARPP-19 (which bears an N-terminal his-tag) as compared to ENSA (which is untagged) is the presence of chemical shift perturbations >0.02 ppm in the C-terminal 45 amino acids. Similar C-terminal perturbations were observed in control experiments when SDS-bound AS was titrated with his-tagged ENSA (data not shown), suggesting that these perturbations result from a secondary electrostatic interaction between the basic his-tag and the acidic C-terminus of AS.

**AS and ENSA do not simply compete for binding to micelles.** The final NMR titration experiments were performed starting with stoichiometric equivalents of $^{15}$N-AS and natural abundance ENSA in aqueous buffer, titrating with increments of 35 equivalents of SDS per titration point (Fig. 8). The control spectrum (not shown) with no SDS reproduces the experiment shown in Fig. 3A. The first titration point, at a stoichiometry of 35:1:1 SDS:AS:ENSA, corresponds to one-half micelle for every two protein molecules. All signals corresponding to backbone amide sites in the first ~95 residues of AS are suppressed below the limit of detection sensitivity, due either to the long global correlation times of the micelle occupied by several protein molecules or chemical exchange among multiple micelle-populated species. Only signals in the C-terminal domain of AS (including A107, Q109, G111, D119, S129 and A140), as well as the sidechain Asx and Glx signals, are observed under these conditions; these same signals are
observed in solution NMR spectra of AS bound to small unilamellar vesicles (15), owing to the flexibility of this domain (i.e., its rapid domain motion on the NMR timescale). The N-terminal residues bound to the micelle are, however, broadened beyond detection and only begin to be observed at the ratio of 70:1:1 SDS:AS:ENSA (Fig. 8B). At this point, with approximately one micelle for every two proteins, a small population of micelles with only one AS is observed; these signals begin to emerge with significant sensitivity as the number of micelles with one bound protein increases (i.e., at 1.5 micelles, or 105 molecules of SDS, for each AS-ENSA pair, Fig. 8C). Signals that arise over this range of the titration include many readily assigned to the N-terminal domain of AS, such as K10, A19, K32, E46, G51, T44, A56, G67 and V74. As the number of micelles approaches the number of proteins (Fig. 8D), the intensity of the AS N-terminal domain signals reaches a plateau, corresponding to an equilibrium population in the limit of excess SDS (Fig. 8D). Addition of further SDS (Figs. 8E and 8F) yields no further changes in peak positions or (beyond the dilution factor) peak intensity.

DISCUSSION

Enrichment for conformation-dependent binding partners of alpha-synuclein. We conducted a screen for proteins that interact specifically with the helical conformation of AS, and identified 20 candidate proteins, including 7 proteins previously reported to interact physically or functionally with AS, and 13 novel partners. Using solution NMR, we performed a rigorous validation of the conformation-specific interaction of AS with one of these novel proteins, ENSA. Our results provide further insight into the potential physiological function of AS and support the hypothesis that AS is sensitive to local environments within the cell and may modulate its interactions with diverse protein partners according to these environments.

We applied a two-stage screening study to select for interactions that occur only in the presence of lipids. In the first phase, we selected for binding partners of membrane-bound alpha-synuclein, a complex including both the helical N-terminus and the relatively unstructured C-terminus. This was followed by a subtractive step to select against binding partners that bound to lipid-free synuclein. Thus our approach should have enriched for conformation-dependent binding partners, but might not have rigorously excluded proteins that interact with the C-terminus, depending on the efficacy of the subtractive step. Indeed, two of the proteins identified in this screen (α-tubulin and cytochrome C oxidase) have been previously reported to interact with lipid-free AS (23, 24, 28). To specifically validate the conformation-dependence of interactions we used solution NMR experiments, as discussed below.

Our results confirm previous reports that AS interacts with elements of the cytoskeleton, especially microtubules and their associated motor proteins (23, 24, 27). However, an interesting candidate emerged from this category: synapsin 1a, which to our knowledge has never been reported to bind AS. Synapsin and AS share intriguing similarities. Both associate reversibly with presynaptic vesicles, and are thought to influence the activity-dependent recruitment of a reserve pool of vesicles to the readily-releasable pool (12, 42). The specific sequence isolated from our bacteriophage screen corresponds to domain E of synapsin 1a (Fig. 2), a region specifically implicated in this process (43, 44).

Four candidates in Table I participate in various transcriptional complexes. The name “synuclein” is derived from the early report of its localization to both synapses and nuclei (8), and several recent studies confirm its nuclear localization (45, 46), where it is found to interact with histones and to inhibit histone acetylation. Indeed, another synuclein protein family member, gamma-synuclein, has been reported to function as a chaperone for estrogen receptor-alpha, stimulating ligand binding and activation of the receptor (47). While the conformation dependence of AS interactions with these partners remains to be determined, the potential association of AS with nuclear receptor complexes is intriguing.

Four additional candidates have functions related to redox metabolism. These
AS/partner protein interactions may be of particular interest in AS pathology, as PD pathogenesis is strongly linked to mitochondrial dysfunction and oxidative stress. As previously reported by Elkon et al. (28), we find that AS binds to cytochrome C oxidase, the mitochondrial complex IV enzyme. We also observe a novel interaction with a newly identified type 2 cytosolic R-β-hydroxybutyrate dehydrogenase (BDH2). Its substrate R-β-hydroxybutyrate is beneficial in alleviating mitochondrial inhibition and oxidative stress in several animal models of PD (48-50), so intracellular regulation of its metabolism by AS is potentially very significant.

Three of the candidate proteins identified in this screen, sorcin(51), annexin A6(52), and endosulfine alpha(53), have been reported to directly modulate the activities of ion channels. These observations suggest a role for AS in modulation of neuronal excitability, interesting because AS has been reported to negatively regulate neurotransmission (11, 12). Endosulfine alpha (ENSA) was originally identified as an endogenous regulator of K-ATP channels (32), although this potential function remains controversial (54, 55). We chose ENSA as a specific focus for additional validation by chemical shift perturbation mapping experiments because 1) its relatively small size makes it especially amenable to study by solution NMR, and 2) it was previously reported to be downregulated in Alzheimer’s disease (33), Down’s Syndrome (34), and several models of learning and stress (56).

Structural analysis of AS-ENSA interaction. NMR chemical shift perturbation mapping experiments were carried out both to demonstrate site-specific effects and to provide a structural context for data interpretation. AS in aqueous solution is unfolded, with only a small degree of secondary structure; in this state, only non-specific interactions between AS and the partner proteins are observed, involving residues in the acidic C-terminus of AS with ENSA, which has a net positive charge at pH 7.5. The amplitude of these effects is in most cases less than 0.003 ppm, with changes on the order of 0.01 ppm for only a few residues. With ARPP-19, even the C-terminus of AS shows only extremely small changes.

In contrast, the micelle-bound state of AS, which has a distinct helical hairpin fold as recently determined by Ulmer et al. (36), gives rise to a consistent and systematic pattern of chemical shift perturbations. These chemical shift patterns observed for $^{15}$N-AS in the presence of ENSA are appreciated in the context of the 3D structure of AS (Fig. 9). The AS structure (PDB entry 1XQ8) as determined by Ulmer et al. (36) is illustrated with residues color-coded according to the magnitude of the chemical shift perturbation observed at the highest concentration of ENSA used here. Blue residues have less than 0.015 ppm shifts, which is likely of no significance. White and pink range from 0.015 ppm to ~0.035 ppm, significantly greater than the noise or uncertainty of peak positions. Darker red sites have greater than 0.035 ppm shifts, which are highly significant, an order of magnitude greater than the random measurement error. The ribbon diagram (Fig. 9A) is labeled with specific amino acid locations, and the van der Waals radii depiction (Fig. 9B) emphasizes the strong correlation of perturbed sites with 3D structure.

Using this structure, the pattern of chemical shift perturbations illustrates the regiospecific interaction. The C-terminal domain (far right unfolded region in Fig. 9A) of AS has no significant perturbations, but the N-terminal domain of SDS-bound AS has a number of prominent perturbations upon addition of ENSA. Residues V3, A11, K12, A19, E20, T22 and V26 all change by more than 0.03 ppm, more than 0.06 ppm in the case of A19 and E20 (as summarized in Fig. 7B), and additional changes of significance (>0.015 ppm) are observed throughout the subsequent two turns of the helix, with residues Q24, G25, A27, E28, A29 and A30, the latter being one of the early-onset PD mutation sites. These residues in the N-terminal helix (helix A) are close in 3D space to a second set of residues in helix B, starting at approximately K58 (across from E28) and extending to V74 (across from A11). All resolved signals in this K58 to V74 stretch show changes of >0.02 ppm K60, V63, V66, A69, V71 and V74 change by more than 0.05 ppm, an order of magnitude greater than the threshold of significance. (Signals for residues T59 and N65 are not sufficiently well resolved to determine
the perturbation values.) Both micelle-facing residues (V52, Q62, V77, T81) as well as solvent-exposed residues (E61, T72, A76), are perturbed significantly, indicating subtle but significant structural effects on both sides of the helix. The locations of these interactions both on helices A and B, close in the folded structure but distant in the primary sequence, imply that ENSA is likely to have a specific interaction with this domain of the folded AS molecule on the micelle.

A third region that is significantly perturbed includes the loop between helices, from V37 to G47, and the start of helix B. Residues in the loop demonstrating significant chemical shift perturbations include T44, E46, H50, V52, A53 and T54; among these are two of the early-onset mutation sites in AS, as labeled on Fig. 9. A third mutation site, A30P, is near the region of significant perturbations in helix A. Reorientation of this helix or substantial differences in the dynamics, as observed in recent studies (36, 57), may alter binding affinity with ENSA.

One trivial explanation for the observed perturbations is that they arise from competition for micelle surface area. For example, some AS signals with perturbed chemical shift were facing the micelle. This raised the question of whether AS and ENSA were competing for a common micelle, so that the chemical shift perturbations on the solvent-exposed surface of AS were an indirect consequence of this competition. Several lines of evidence rule out this possibility.

First, the chemical shift perturbations upon addition of ENSA to the SDS-bound AS sample are on average only a few percent of the chemical shift changes observed upon AS folding. Second, if ENSA were displacing AS from the micelle, the chemical shifts would move systematically back in the direction of unfolded AS. This also is not the case; rather, chemical shifts are perturbed in directions that are not correlated to the free state of AS, but a third state (i.e., the molecular complex consisting of AS-SDS and ENSA-SDS). Third, a competition for the micelle would result primarily in changes of chemical shifts corresponding to those sites with the strongest interactions with the micelle, such as the Lys residues whose sidechains specifically coordinate with the phosphate headgroups, or the other aliphatic residues that have sidechains buried into the detergent. Although some micelle-facing residues are perturbed upon addition of ENSA to the SDS-bound AS sample, these are by no means the only residues demonstrating shifts, and indeed many of the largest shifts are observed for sites that face the aqueous environment. The observations in all three cases suggest a model of each protein (AS and ENSA) bound to its own micelle and interacting at the interface.

We tested this hypothesis directly by performing an additional series of experiments in which SDS was titrated into a solution of $^{15}$N-AS and natural abundance ENSA in equimolar quantities (Fig. 8). These spectra show that in the limit of excess protein (e.g., SDS ratios of 35:1:1, 70:1:1 or 105:1:1, where there would be respectively 0.5, 1.0 or 1.5 micelles for every 2 protein molecules), high molecular weight complexes and/or species in intermediate chemical exchange are present. Signals from the N-terminal domain of AS are broadened beyond detection (as observed by Eliezer with small unilamellar vesicles (15)). As the number of micelles approaches and then exceeds the number of protein molecules, the signals from AS increase in intensity substantially. Once a sufficient excess of SDS is achieved, no further changes occur. For example, in Fig. 8C, signals corresponding to the micelle-bound states of E46, A56, T64, G73, V74, T92 and G93 appear, and these signals increase in strength as the SDS-to-protein ratio is further increased to 140:1:1 (Fig. 8D) and 170:1:1 (Fig. 8E). Only a few subtle changes are observed between these two spectra, and addition of a further large excess of SDS (to a final ratio of 280:1:1, or 4.0 micelles per 2 protein molecules, Fig. 8F) causes no further changes.

This experiment demonstrated that the order of addition of reagents did not influence the result and the result does not depend on the precise amount of SDS once in sufficient excess. Based on this result, we performed all the partner protein titration experiments with sufficient excess of SDS (at least 170 molecules of SDS per AS molecule).
Functional significance of AS interactions with ENSA and ARPP-16/19. The precise cellular functions of the CAMP-regulated phosphoproteins ENSA and ARPP-19 are still unclear. Despite their high degree of primary sequence similarity, these two proteins have been characterized independently and in quite varying contexts. ENSA was originally described as an endogenous ligand for the sulfonfonylurea receptor SUR1 (32), a subunit of the ATP-sensitive K⁺ (K\(_{\text{ATP}}\)) channel. K\(_{\text{ATP}}\) channels are well known for their role in regulating insulin secretion (58), but were also recently implicated in Parkinson’s disease (59).

ARPP-16 and -19, along with ARPP-21 and DARPP-32, were originally described as robust phosphorylation substrates for PKA in brain (60). It is speculated that ARPP-16 may be involved in dopamine signaling, as it is enriched in basal ganglia, phosphorylated as a result of D1 dopamine receptor activation, and dephosphorylated in response to D2 activation (61). Its precise cellular function has not been defined, however. Similar to ENSA, expression of ARPP-19 is downregulated in Down’s syndrome and Alzheimer’s disease; this is accompanied by a downregulation of PKA (62).

While our data do not immediately reveal a specific function for AS, or for the interaction between AS and ARPP family members, it is suggestive of participation by these proteins in a protein signaling network associated with G protein-coupled receptors.

We have mapped the domain of AS interaction with ENSA to residues in the N-terminal helical domain of AS, a region that is structured only in the presence of membrane lipids or lipid mimetics. We have determined the K\(_d\) of the AS-ENSA interaction to be ~61±12 μM in the presence of SDS micelles, based on analysis of the chemical shift changes as a function of ENSA concentration (63, 64). This value was determined by titrating SDS-bound ENSA into SDS-bound AS. No interaction is detectable between the proteins in aqueous solution.

Relatively few proteins have been shown to interact specifically with the N-terminal domain of AS; our search of the research literature revealed only calmodulin (65), hDAT (66), synphilin (67), Aβ peptide (68), and PLD1 (69). Interactions with Aβ and PLD1 were lipid-independent. Of the other 3 proteins, only calmodulin has been characterized with respect to affinity and lipiddpendence. Calmodulin binds AS with K\(_d\) ~ 1 μM, and binds either free or membrane-bound AS. Interestingly, calmodulin binding causes AS to dissociate from the membrane.

The affinity of the AS-ENSA interaction (~61μM) is typical for regulatory interactions among proteins in signaling networks. Like AS, ENSA and ARPP-19 are intrinsically unstructured proteins (IUP’s) (37). One characteristic of such proteins is that they can achieve highly specific binding with partner proteins via low affinity interactions, which is advantageous in functional contexts requiring multiple interacting partners on a timescale of coordinated biological processes (71). Such specific yet reversible binding events are critical for the proper functioning of protein signaling networks, which has been proposed to account for the association of IUP’s with such networks. For example, several low-affinity binding interactions have been identified in the endocytic pathway (72); many of these interactions have K\(_d\) values on the order of 5 to 500 μM (71, 73). We envision a similar complexity of the interactions of AS with multiple partner proteins in accomplishing its biological functions.

Conclusion. We present an important proof-of-principle that the N-terminus of AS can function as a membrane-dependent scaffold for interactions with other proteins. It is well established that membrane binding by AS is mediated by a series of imperfect 11-mer repeats at its N-terminus, spanning ~100 of a total of 140 residues (14, 36). The domain encompassing residues 1-102 is 92% identical among birds and mammals. In the presence of phospholipid vesicles, these repeats fold into an amphipathic helix that lies parallel to the membrane surface. In the absence of lipid, the repeat-containing domain has no stable structure (13).

The molecular significance of the extended, structurally plastic helical domain has so far been a mystery. A similar structural mechanism mediates lipid interactions by the
exchangeable apolipoproteins, but these proteins are not as well conserved at the primary sequence level as AS (74, 75). We show here that the helical N-terminus of AS is capable of mediating specific interactions with other proteins. Because this helical structure is itself membrane-dependent and readily reversible, any protein complexes assembled around it should also be transient and sensitive to changes in the local membrane environment (e.g. via action of phospholipases or phospholipid kinases).

We therefore propose that the unique, plastic structure of AS subserves its function as a lipid-dependent protein scaffold. One consequence of this structural plasticity is that AS may continually cycle through unfolded or partially folded states, which could explain its particular propensity to self-associate into oligomers or fibrils, as observed in Parkinson’s disease. In this context, lipid-dependent protein interactions might stabilize the folded structure of AS, decreasing its in vivo oligomeric potential. In such a scenario, PD-associated point mutations in the N-terminal domain (A30P, A53T, E46K) might promote misfolding of AS by disrupting interactions with partner proteins. Further analysis of this novel class of AS-interacting proteins is warranted to assess the potential for dynamic participation of AS in signaling complexes at the membrane surface.

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FOOTNOTES

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1The abbreviations used are: ARPP, cAMP-regulated phosphoprotein; AS, alpha-synuclein; cAMP, 3'-5'-cyclic adenosine monophosphate; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; ENSA, endosulfine alpha; HSQC, heteronuclear single quantum correlation; IUP, intrinsically unstructured protein; K-ATP channel, ATP-sensitive K⁺ channel; MPTP, 1-methyl 4-phenyl 1,2,3,6, tetrahydropyridine; NMR, nuclear magnetic resonance; PD, Parkinson’s disease; PKA, cAMP-dependent protein kinase; POPA, 1-palmitoyl-2-oleoyl phosphatidic acid; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; SDS; sodium dodecyl sulfate; SUR, sulfonylurea receptor.

FIGURE LEGENDS

Figure. 1. Characterization of purified recombinant ENSA and ARPP-19 proteins. Purified proteins (histagged ARPP-19, his-tagged ENSA, and untagged ENSA) were separated by SDS-PAGE, followed by (A) Coomassie staining for total protein or (B) immunoblot with a chicken polyclonal antibody raised against full-length human ENSA. As expected from the similarity of these sequences, the antibody cross-reacts with ARPP-19.

Figure. 2. Alignment of phage-displayed sequences with full-length human proteins. Sequences of bacteriophage inserts (solid rectangles) are aligned with the corresponding full-length human sequences (black lines). Known functional domains in the full-length proteins are also depicted for comparison (open rectangles). Sequence lengths are drawn to scale, except as indicated by broken lines.

Figure. 3. ^15^N-HSQC NMR spectra of ENSA interacting with AS. This interaction requires an anionic micelle. (A) Spectrum of 0.5 mM free AS (red) overlaid with the spectrum of the sample after addition of ENSA in a 1:1 molar ratio (blue). Both spectra were acquired at 10°C. (B) Spectrum of a 0.5 mM AS sample with excess SDS (83 mM). The spectrum was acquired at 25°C. (C) The same spectrum in (B) (red) overlaid with a spectrum of the sample with the addition of ENSA in a 1:1 molar ratio (blue). All spectra were acquired with 4 scans per row and 512 complex points in the ^15^N dimension, and processed with sine bell apodization and zero filling prior to Fourier transformation. (D-F) Expanded regions of the spectrum demonstrating chemical shift perturbations of specific residues upon titration of SDS-bound AS with ENSA. Ratios of AS to ENSA are 1:0 (red), 1:0.4, (green) and 1:1.0 (blue).

Figure. 4. Chemical shift perturbations upon titration of AS with ENSA. The perturbations (δ = ([0.1δ_H]^2 + δ_N^2)^1/2) were calculated for well-resolved sites in the 2D HSQC spectra. (A) Perturbations of free AS by the addition of ENSA at 1:1 molar ratio. (B) Perturbations of SDS-bound AS by the addition of ENSA at 1:1 ratio.
Figure 5. Alignment of the amino acid sequence of endosulfine alpha with ARPP-16 and 19. ARPP-16 and –19 are differentially spliced products of the same gene, and differ only at their N-termini. Boxed residues are identical among the sequences. The conserved PKA phosphorylation site is indicated (*).

Figure 6. 15N-HSQC NMR spectra of AS interacting with ARPP-19. Spectrum of 0.5 mM 15N-AS sample with excess SDS (83 mM) (red) is overlaid with the spectrum of the sample after the addition of ARPP-19 in a 1:1 molar ratio (blue). The spectra were acquired at 25° C, with 4 scans per row and 512 complex points in the 15N dimension, and processed with sine bell apodization and a single zero fill.

Figure 7. Chemical shift perturbations of AS upon titration with ARPP-19. (A) Perturbations with the absence of SDS. (B) Perturbations in the presence of excess SDS. The perturbations were calculated ($\delta = (\sqrt{0.1\delta_N^2 + \delta_H^2})^{1/2}$) for well-resolved sites in the 2D HSQC spectra.

Figure 8. 15N-HSQC NMR spectra of labeled AS in a 1:1 molar ratio with unlabeled ENSA titrated with SDS. SDS:AS:ENSA ratio is (A) 35:1:1, (B) 70:1:1, (C) 105:1:1, (D) 140:1:1, (E) 170:1:1, (F) 280:1:1. All spectra were acquired at 25° C, with 4 scans per row and 1024 complex points in the 15N dimension, processed with sine bell apodization, 3 Hz of Gaussian line broadening in the indirect dimension, a single zero-fill for both dimensions.

Figure 9. Chemical shift perturbation mapping of SDS micelle-bound AS upon interaction with ENSA. (A) Ribbon diagram of SDS micelle-bound AS structure (PDB entry 1XQ8, Ulmer et al.) in ribbon format, with key positions in the sequence labeled by amino acid residue. Sites of PD-associated mutations are indicated in bold. (B) The same structure and point of view in van der Waals representation. The chemical shift perturbation was defined as $\delta = (\sqrt{\delta_N^2 + \delta_H^2})^{1/2}$. Perturbations greater than 0.015 ppm are significant, and greater than 0.03 ppm highly significant.
Table I: Candidate human brain proteins that interact with helical alpha-synuclein, as identified by bacteriophage display.

| Protein (synonyms) | Uniprot accession # | Function(s) | # Isolates | Prior report of AS interaction |
|--------------------|----------------------|-------------|------------|--------------------------------|
| **Cytoskeleton/vesicle trafficking** | | | | |
| α-Tubulin | P68363 | microtubule subunit | 6 | (23, 24) |
| Septin 4 (Sept 4, cdc-rel2) | O43236 | cytoskeletal component | 2 | (26) |
| Kinesin light chain 1S (KLC1S) | Q07866-8 | microtubule motor | 3 | (27) |
| Dynein heavy chain 1 (DHC1) | Q14204 | microtubule motor | 2 | (27) |
| Synapsin 1a | P17600 | actin binding, regulates presynaptic vesicle pools | 1 | |
| Ectodermal neural cortex 1 (ENC1NRP/B) | O14682 | actin binding, regulates neuronal process formation | 4 | |
| **Ion channel modulation** | | | | |
| Endosulfine-α (ENSA) | 043768 | modulates K-ATP channels, L-type Ca** channels | 1 | |
| Sorcin (SORCN) | P30626 | modulates K-ATP channels, L-type Ca** channels | 1 | |
| Annexin A6 | P08133 | modulates L-type Ca** channels | 1 | |
| **Redox metabolism** | | | | |
| R-β-hydroxybutyrate dehydrogenase, type 2 cytosolic (BDH2) | Q9BUT1 | converts R-β-hydroxybutyrate to acetoacetate | 1 | |
| Multifunctional enzyme 2 (MFE2) | P51659 | fatty acid beta-oxidation, also reduces estradiol to estrone | 1 | |
| Cytochrome C oxidase, subunit 1 (COX1) | Q6RR13 | mitochondrial electron transport | 1 | (28) |
| Peroxiredoxin 1 (PRDX1) | Q06830 | peroxidase, chaperone | 1 | |
| **Transcriptional complexes** | | | | |
| Proline-, glutamic acid-, and leucine-rich protein 1 (PELP1, MNAR) | Q8IZL8 | co-activator of estrogen receptor alpha, transcriptional co-repressor | 1 | |
| SAP30 binding protein (SAP30BP, HCNGP, HTRP) | Q9UHR5 | transcriptional co-repressor, possible component of histone deacetylase complex | 1 | |
| FK506 binding protein 52 (FKBP52) | Q02790 | chaperone, component of steroid receptor complexes | 4 | (29) |
| Heat shock cognate 70 (Hsc70, HspA8) | Q96IS6 | chaperone, component of steroid receptor complexes, clathrin-uncoating ATPase | 4 | |
| **Other** | | | | |
| Prefoldin 2 (PFDN2) | Q9UHV9 | folding chaperone | 1 | |
| Ubiquitin specific protease 47 (USP47) | Q96K76 | ubiquitin specific protease | 1 | |
| G protein-coupled receptor kinase 2 (GRK2) | P25098 | G protein-coupled receptor desensitization | 1 | (31, 76) |
Figure 1
Figure 2
Figure 3
Figure 4

[Graph A: Chemical Shift Perturbation, δ (ppm) vs. Alpha-Synuclein Residue Number]

[Graph B: Chemical Shift Perturbation, δ (ppm) vs. Alpha-Synuclein Residue Number]
| ENSA | MSQKQEENPAETG EEPDTEGEGILPE KAAAEKLKARYPFSLG QKPGGSDFLRKRIQK |
|------|---------------------------------------------------------------|
| ARPP_19 | -----MSAEVFRAAS AEEEMEDKVTSPE KAAAEKLKARYPFSLG QKPGGSDFLRKRIQK |
| ARPP_16 | MEEKVTSPE KAAAEKLKARYPFSLG QKPGGSDFLRKRIQK |

| ENSA | GQYTFSGTDYNNAKA KMKKQLPSPAIPKN LVTGDHIPTPQDLPLQ RKPSLVASKLAGCQV E |
|------|-------------------------------------------------------------------|
| ARPP_19 | GQYTFSGTDYNNAKA KMKKQLPTAAPSIKT EVTGDHIPTPQDLPLQ RKPSLVASKLAG--- E |
| ARPP_16 | GQYTFSGTDYNNAKA KMKKQLPTAAPSIKT EVTGDHIPTPQDLPLQ RKPSLVASKLAG--- E |

**Figure 5**
Figure 7
Figure 8
Conformation-specific binding of alpha-synuclein to novel protein partners detected by phage display and NMR spectroscopy

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