Conformational flexibility within the nascent polypeptide–associated complex enables its interactions with structurally diverse client proteins

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ABSTRACT

As newly synthesized polypeptides emerge from the ribosome, it is crucial that they fold correctly. To prevent premature aggregation, nascent chains interact with chaperones that facilitate folding or prevent misfolding until protein synthesis is complete. Nascent polypeptide–associated complex (NAC) is a ribosome-associated chaperone important for protein homeostasis. However, how NAC binds its substrates remains unclear. Using native electrospray ionization MS (ESI MS), limited proteolysis, NMR and cross-linking, we analysed the conformational properties of NAC from Caenorhabditis elegans and studied its ability to bind proteins in different conformational states. Our results revealed that NAC adopts an array of compact and expanded conformations and binds weakly to client proteins that are unfolded, folded, or intrinsically disordered, suggestive of broad substrate compatibility. Of note, we found that this weak binding retards aggregation of the intrinsically disordered protein α-synuclein both in vitro and in vivo. These findings provide critical insights into the structure and function of NAC. Specifically, they reveal the ability of NAC to exploit its conformational plasticity to bind a repertoire of substrates having unrelated sequences and structures independently of actively translating ribosomes.

Upon emerging from the ribosome, most proteins have to fold into a unique three-dimensional structure in order to become biologically active. Finding the correct fold and avoiding misfolding and aggregation are major problems for polypeptide chains in the crowded cellular environment. This problem is exacerbated by the slow rate of synthesis compared with the rapid rate of folding of most proteins, which provides an opportunity for partially synthesised proteins to become trapped in misfolded or aggregation-prone states (1-3). Help is at hand by molecular chaperones: essential proteins found across all kingdoms of life which facilitate protein folding, unfolding and degradation, forming a network of interactions necessary for maintaining a healthy proteome (1). Some chaperones have the specific role of interacting with newly synthesised polypeptides to prevent intermolecular interactions that could result in aggregation (4-6). Two general groups of chaperones exist: those that interact with the ribosome and the nascent chain simultaneously, thereby controlling the early stages of folding (7), and those that are not involved until the complete
polypeptide has emerged from the ribosome exit tunnel (8).

Ribosome-associated chaperones vary across species. Bacteria possess the well-characterised trigger factor, a chaperone with a molecular cradle structure that captures polypeptide chains as they emerge from the ribosome (9,10). Its interior structure is lined with hydrophobic residues that provide multiple contact points for its unfolded protein substrates (11). Within eukaryotes, the chaperone network is more complex and consists of two systems: the ribosome-associated complex (RAC) (12,13) and the nascent polypeptide associated complex (NAC) (14,15).

Whereas NAC is highly conserved across eukaryotes, archaea possess a NAC homodimer consisting of two α-subunits, while yeast and mammalia possess a heterodimer formed of α- and β-NAC subunits (16). The overall sequence homology between the 215 residue α-NAC and the 206 residue β-NAC subunits is only 26 %. However, both subunits contain a highly conserved (41 % sequence identity) central NAC domain of approximately 61 residues (Figure 1a). X-ray crystal structures of truncated human NAC (Figure 1b) show that the α- and β-NAC subunits associate through their six-stranded β-barrel-like central NAC domains, although the structure, function and interactions made by the N- and C-terminal domains of the α-NAC and β-NAC subunits remain unknown (17,18). Human heterodimeric NAC has been shown to be more stable than its homodimeric assemblies and, importantly, homodimers of α-NAC do not form in the presence of β-NAC, suggestive of a specific heterodimeric complex despite the close sequence homology of the central NAC domains (17).

α-NAC differs from β-NAC in that it contains a single ubiquitin-associated (UBA) domain that comprises the C-terminal 40 amino acids of the polypeptide chain (Figure 1a) (19). Although the role of this domain remains unclear, a NAC variant which lacks the UBA domain has been shown to be a more potent suppressor of protein aggregation in vivo suggesting a regulatory role for the UBA domain in the chaperone activity of the complex (20). Previous reports have also shown that NAC interacts with translating ribosomes reversibly in a 1:1 stoichiometry and, while α-NAC forms contacts with both the ribosome and the nascent chain, it is the β-NAC subunit that mediates the dynamic interaction with the ribosome via the RRKKK- motif (residues 71-75 (Figure 1a)) in its N-terminal region (21-23). Previous studies have also shown that NAC is able to protect the emerging nascent chain from proteolysis, confirming its role in guarding nascent chains during their synthesis (24). Multiple functions have been suggested for NAC in vivo, including protection of nascent chains from proteolysis and regulation of apoptosis, and there is also evidence of a homodimer of α-NAC bound to DNA and RNA, implying that NAC can take the role of a transcription factor (16). Recently, NAC depletion in C. elegans was shown to cause mistargeting of translating ribosomes to the endoplasmic reticulum (ER) membrane and mistranslocation of mitochondrial proteins into the ER. Loss of NAC activity also reduced the median lifespan of C. elegans by 10 days by means of inducing ER and mitochondrial stress (25). Despite its ubiquity and central importance in protein synthesis and folding, how NAC binds and chaperones its substrate proteins, however, remained unknown.

Over the past twenty years, native mass spectrometry has become a powerful tool for the interrogation of non-covalent protein complexes and their interactions (26-31). Complimentary information can be obtained using hydrogen exchange MS (32), fast photochemical-oxidation of proteins (FPOP) (33), each combined with identification of the sites of modification using proteolysis followed by LC-MS/MS. Furthermore, native MS methods enable the structure, stability and conformation of proteins and their complexes to be investigated directly upon their ionisation into the gas phase (34-36). With the advancement of instruments and software it has also become possible to interrogate transient non-covalent interactions between proteins using MS-based techniques. For example, chemical cross-linking can be used to provide residue-specific information about transiently interacting partners using sequencing of cross-linked products using LC-MS/MS (37).

Here we use ESI-MS combined with ion mobility spectrometry-mass spectrometry (ESI-IMS-MS) to study the conformational properties of wild-type (WT) α/β-NAC (WT-NAC) from C. elegans for the first time. We also use MS-based techniques to provide information about the structure, dynamics and interactions of WT-NAC
with potential substrates in different conformational states. In addition, the conformations of WT-NAC and the heterodimer lacking the UBA domain (ΔUBA-NAC) are compared using ESI-IMS-MS to establish the effect of the UBA domain on conformational properties of NAC. It has been suggested that the UBA domain, which is attached to the α-NAC subunit via a highly flexible 34-residue linker, may regulate the chaperone activity of NAC (20). In parallel, chemical cross-linking of complexes formed between WT-NAC and α-synuclein (a 140-residue intrinsically disordered protein (IDP)) (38,39) and the 87-residue bacterial immunity protein Im7, in its four helical native state and unfolded by creating the triple mutant (TM-Im7) L18AL19AL37A were used to compare the interactions of WT-NAC with different protein substrates and to identify their binding sites. Combined with analysis of the NAC-α-synuclein complex using 1H-15N NMR, and assays of the effect of UAC binding on α-synuclein aggregation in vitro and in vivo, the results reveal that NAC bind substrates with very different sequence and structural properties, forming weak interactions in a dynamic complex independent of the presence of the UBA domain or actively translating ribosomes.

**Results**

**ESI-MS reveals both compact and extended conformations of NAC**

The native ESI-mass spectrum of purified *C. elegans* NAC obtained in 100 mM ammonium acetate buffer at pH 6.9 is shown in Figure 2a. The spectrum confirms the presence of a heterodimer of α-NAC and β-NAC with a molecular weight of 39,306 Da (theoretical mass = 39,304 Da). The spectrum shows the presence of at least two distinct protein conformations for the αβ-NAC dimer: the most abundant species has 11+ to 13+ charges and is likely the most native-like conformer, with a second population being more highly charged (14+ to 22+), suggestive of more expanded conformations. NAC dimers (α2β2) and a small amount of the free α-subunit (measured mass = 21,803 Da; theoretical mass = 21,802 Da) and β-subunit (measured mass = 17,504 Da; theoretical mass = 17,502 Da) were also visible in the spectrum. Control experiments using native PAGE showed only a single band (inset of Figure 2a) suggesting that some dissociation of the heterodimer alongside self-association to larger species occurs in the gas-phase but does not occur in solution. Interestingly, native ESI-MS of the NAC construct lacking its UBA domain (ΔUBA-NAC, measured mass = 35,091 Da; theoretical mass = 35,091 Da) showed the same distribution of charge states as WT-NAC, with the 12+ charge state ions being the most abundant species (Figure 2b), ruling out major conformational changes of the protein upon deletion of this domain.

Native ESI-IMS-MS confirmed that species which have a range of compact and extended conformers of both WT-NAC and ΔUBA-NAC exist in the gas-phase (Figure 2c, Supporting Information Table S1). The measured collision cross-sections (CCS) for the lowest observed (11+) charge states were 2962 ± 88 Å² and 2761 ± 83 Å² for WT-NAC and ΔUBA-NAC, respectively (Figure 2c). The function of the UBA domain within NAC has yet to be ascertained, but we hypothesised that the extended highly charged conformers of WT-NAC observed here using ESI-MS could result from the attachment of the UBA domain to the NAC core via a flexible linker (Figure 1a). The ESI-IMS-MS results presented here rule out such a hypothesis, and show instead that the extended conformation observed for WT-NAC is also observed for the protein lacking the UBA domain. Importantly, the finding that the difference in CCS of WT-NAC and ΔUBA-NAC is only ~200 Å², consistent with the expected CCS of the folded UBA domain, confirms that NAC remains folded in the gas-phase despite the lack of bulk solvent water (42,43).

CD spectroscopy showed that WT-NAC contains 33% disorder, 27% helical and 17% β-stranded structure (Figure 2d and Supporting Information Table S1). Deletion of the 35-residue UBA domain resulted in a shift in the major peak from 206 nm to 204 nm and the overall α-helical content was reduced to ~15%. This indicates the UBA domain makes a large contribution to the total α-helical content of NAC, consistent with the known predominantly helical structure of the UBA domain (44). The proportion of unstructured protein was increased to 36% in this variant compared with WT-NAC (Supporting Information Table S2).

**CIU indicates ΔUBA-NAC is more susceptible to unfolding than WT-NAC**
To compare the stability of the WT-NAC and ΔUBA-NAC heterodimers in more detail, the proteins were each examined using collision induced unfolding (CIU). The 12⁺ charge state ions for WT-NAC (Figure 3a) and ΔUBA-NAC (Figure 3b) were each isolated in the first (quadrupole) analyzer of the ESI-MS-MS mass spectrometer and the collision energy increased step-wise in the trap cell prior to the IMS cell and second (time-of-flight) analyzer. No dissociation of the heterodimers was observed at 10 V with drift times of 8.7 and 7.4 ms, respectively (Figure 3, lower panels). Clear differences in the unfolding patterns of the two proteins were observed, however, as the collision voltage was increased. For WT-NAC, a single conformation persisted at 25 V, with a second, more unfolded conformer (drift time 10.3 ms) becoming populated at 35 V. By contrast, ΔUBA-NAC showed evidence for substantial unfolding at 25 V, with the appearance of a second population (~40% of molecules) with an arrival time of 9.1 ms in the IMS drift-time plot. Finally, at 35 V ΔUBA-NAC shows significant conformational rearrangements, with little, if any, residual population of native-like species with a drift time 7.8 ms. Instead a broad arrival time distribution (ATD) containing more highly expanded species results (Figure 3, top panel). The results presented here using far UV CD, ESI-IMS-MS and CIU together show that deletion of the UBA domain does not perturb the structure of the NAC heterodimer, but results in a complex that is more susceptible to unfolding in the dimeric state. This may result in a complex more able to bind its substrates, consistent with in vivo observations of an increased chaperone capacity of NAC upon deletion of the UBA domain (20).

Limited proteolysis of WT-NAC and ΔUBA-NAC Limited proteolysis followed by MS analysis of WT-NAC and ΔUBA-NAC was next carried out to investigate whether the presence of the UBA domain influences the accessibility of the α- and β-domains to protease (Figure 4a,b). After incubation of NAC with trypsin (1:500 (w/w) trypsin:NAC) for 15 min at 20 °C, a cleaved protein with a mass 2462 Da less than the native protein was observed (Figure 4a, lower). The population of this species became more intense relative to uncleaved NAC over time (data not shown). This mass loss from the native protein is consistent with possible loss of MTGSTETRQKEVK (α-NAC residues 1-13), ADEQ (α-NAC residues 158-161) and MDSK (β-NAC residues 1-4) or MTGSTETRQKEVK (α-NAC residues 1-13) and NETKADEQ (β-NAC residues 153-161). Notably the same mass loss was also detected in the ΔUBA-NAC sample (Figure 4a, upper), indicating that these sequences are similarly accessible to protease in both heterodimers. In the low m/z range of the spectrum (m/z 800-1600), fragments corresponding to peptides from the N-terminal region of α-NAC (e.g. 1-38, 9-38, 9-57 in WT α-NAC and 1-38, 1-54 and 2-57 of ΔUBA-NAC) and the C-terminal region of β-NAC (130-161 and 123-161 in WT-NAC and ΔUBA-NAC, respectively) were most abundant (Figure 4b). This indicates that these regions of the protein are most accessible to protease, while the core NAC regions, which form the dimer interface, remain uncleaved, as expected from their folded structure. These observations are consistent with the known domain architecture of the NAC complex (Figure 1a) and show that the UBA domain does not alter the accessibility of the protein complex to trypsin, although deletion of this domain decreases the stability of the complex to collision induced unfolding.

Mapping NAC substrate interactions In order to identify residues required for substrate binding, WT-NAC and ΔUBA-NAC were each mixed in a 1:1 molar ratio with α-synuclein (a 140-amino acid IDP) (39) which was used as a model for unfolded polypeptide substrates as they may emerge from the ribosome. Despite exploring a wide range of instrumental conditions (increased backing pressure, increased trap cell pressure, decreased activation voltages) a complex was not observed using native ESI-MS. Nor was a complex observed via native PAGE (Figure 5a). However, incubating α-synuclein in conditions in which it is aggregation-prone (125 µM protein, Dulbecco’s phosphate-buffered saline, 600 rpm agitation) with an equimolar concentration of WT-NAC prevented α-synuclein aggregation, at least over a timescale of 70 h (Figure 5b), suggesting that the proteins form a weak complex that cannot be maintained in the gas-phase, or on a native PAGE gel, but is sufficient to have a dramatic, protective effect on protein aggregation. To probe this NAC-client interaction further WT-NAC and α-synuclein were mixed and cross-linked using the homobifunctional amine cross-linker, BS3 (see Experimental
To identify residues involved in forming the NAC-α-synuclein complex, an in-gel tryptic digest of the band arising from the putative complex was performed and LC-MS/MS was used to identify lysine residues that form intra- or intermolecular cross-links (Figure 5d and Supporting Information Tables S3-S5). Many intermolecular cross-links were observed between α-NAC and β-NAC (Supporting Information Table S3), especially involving their N-terminal domains (Figure 5d), suggestive of many, possibly transient, interactions between these regions. Only one cross-link was observed between the N-terminal and C-terminal domains of α-NAC, consistent with this complex being extended in nature with few interactions with the UBA domain (Figure 5d). Importantly, of the 8 lysines present in the αβ-NAC core, only one intra-molecular cross link was observed between K84/K86 in α-NAC, consistent with the known crystal structure of the αβ-NAC core (PDB 3LKK) (18). K72 within α-NAC cross linked to the N-terminus of α-NAC, consistent with the known dynamics in this region. Furthermore, K82 within β-NAC reacted with BS3 but did not form cross-links, suggesting that this amino acid is solvent accessible but does not interact with client proteins or the adjacent lysine-rich ribosome binding motif within β-NAC (Supporting Information Table S3 & S6). These data are consistent with the view that the cross-links observed reflect specific interactions formed within the αβ-NAC complex (Figure 5d). Interestingly, several cross-links were observed between the N- and C-terminal domains of β-NAC, suggesting that this subunit is more compact than the α-subunit in the NAC:substrate complex. An array of cross-links were observed between the N- and C-terminal domains of NAC and residues 1-102 of α-synuclein (Supporting Information Table S5). This interaction must also involve the acidic C-terminal region of α-synuclein revealed by NMR chemical shift perturbation (Figure 6b), although since this region lacks lysines no cross-links are observed. The absence of chemical shift perturbation in other regions of α-synuclein that do form cross-links to NAC is consistent with a diffuse binding interface in which the sidechain ε-NH$_2$ of lysine residues form transient, presumably electrostatic interactions with NAC, without perturbation of the chemical environment of the main-chain,
reminiscent of the binding of other ATP-independent chaperones with their clients (9,45). Together, the results highlight the synergy of the MS and NMR approaches taken and confirm that NAC binds weakly to α-synuclein, forming transient interactions that are able to suppress its aggregation.

To address the physiological relevance of our finding that NAC is able to suppress α-synuclein aggregation in vitro, we used a transgenic C. elegans strain expressing α-synuclein fused to YFP (Figure 5e). By day 3 of adulthood these animals showed α-synuclein::YFP puncta. Strikingly, depletion of NAC by RNAi increased the number of α-synuclein puncta, showing that the presence of NAC also ameliorates α-synuclein in vivo.

**Does NAC interact with folded proteins?**

As NAC was shown to interact with an IDP, we next used native ESI-MS and chemical cross-linking to determine whether NAC can also bind folded proteins. For this, the 87-residue, four-helical bacterial immunity protein Im7 was used (40), alongside its triple mutant (L18AL19AL37A; TM-Im7), which is trapped in an unfolded state (Figure 7a) (41). Im7 has been shown previously to fold in seconds (40) and hence could be considered as a mimic of a protein domain that folds rapidly upon emergence from the ribosome tunnel. By contrast, TM-Im7 was used as a model for an unfolded chain with an amino acid composition distinct from that of a highly charged and poorly hydrophobic IDP (46). Notably, both Im7 and TM-Im7 have been shown previously to bind the ATP-independent chaperone Spy (45).

Cross-linking WT Im7 or TM-Im7 to NAC resulted in a unique band at ~60 kDa when analysed using SDS-PAGE suggesting that NAC can bind to Im7 in both its folded and unfolded states (Figure 7b). No complex was observed using native ESI-MS (not shown) indicating that the complex is too lowly populated, or too weak to survive passage into the gas-phase, as was also observed when NAC was mixed with α-synuclein. In-gel tryptic digestion of the bands and subsequent LC-MS/MS analysis, enabled two unique cross-links to be identified for the NAC-WT Im7 complex (Figures 7c and Supporting Information Table S7). Two unique cross-links were also detected for the NAC-TM-Im7 complex (Figure 7d, Supporting Information Table S10). Again, the lack of higher order complexes, and the fact that only K79 of Im7 formed detectable cross-links, despite having 7 other solvent accessible lysine residues in each protein, supports the specificity of the cross-links observed. Interestingly, one of the cross-links identified, between the N-terminus of α-NAC and K79 of Im7, was the same in both samples. Additional cross-links from K79 of TM-Im7 to the C-terminal region of β-NAC were also observed. These data suggest, therefore, that NAC interacts with both folded and unfolded proteins using similar binding regions.

**Discussion**

Although many reports have suggested possible functions for NAC (16), much remains to be discovered about its structure and function. First identified in 1994 by Wiedmann et al. (47), NAC is now known to be crucial for protein folding and transport in the cell (25,48). Despite its importance as a chaperone, how NAC binds its client proteins, the role of different domains of NAC in client binding, and the nature of the substrate (whether folded, unfolded or intrinsically disordered) remained unknown. Here, we have used native ESI-MS, limited proteolysis and cross-linking mapped by MS/MS, and combined these experiments with CD, NMR and aggregation assays in vitro and in vivo to characterise the conformational dynamics of NAC and its ΔUBA variant and to probe the nature of substrate recognition of NAC for three different model protein substrates. Native ESI-MS showed two different charge state distributions for NAC, suggesting that the αβ-NAC heterodimer is dynamic in structure, visiting compact species as well as more extended states. Whilst care must be taken in interpreting the conformational properties of dynamic proteins by native MS, especially for unfolded chains and multi-domain proteins since collapse of the protein can occur in the gas phase (43), the observation that the termini of NAC are protease sensitive supports the view that these regions of the protein are dynamic and may unfold to give rise to the more extended species observed by ESI-MS. A similar array of charge states with similar drift times (taking the smaller mass of the complex into consideration) was also observed for the ΔUBA-NAC variant. Thus, the conformational dynamics observed cannot be attributed to the UBA
domain, which is known to be connected to the α-
NAC domain via a flexible linker (20). This
dynamic structure of NAC may be important for
imparting its ability to bind a range of protein
substrates, including both natively folded and
unfolded structures, as shown here using native
Im7, unfolded-TM Im7 and α-synuclein as
examples.

Collision cross-sections determined using
ESI-IMS-MS showed that ΔUBA-NAC has a
reduced CCS compared with WT-NAC (Figure 2c).
Based on the relationship between the molecular
mass and CCS for globular proteins (49,50), WT-
NAC would be expected to have a CCS of ~2700
Å². The measured value of 2962 Å² for WT-NAC
is larger than this value, consistent with NAC
containing flexible regions that contribute to the
protein being more expanded than globular proteins
of similar mass. A CCS of 2761 Å² was measured
for ΔUBA-NAC. As ΔUBA-NAC is 4.2 kDa lower
in molecular mass than WT-NAC, it would be
expected to have a CCS reduced by ~200 Å².
The experimentally determined difference between the
lowest charge state of WT-NAC and ΔUBA-NAC
was 201 Å² which indicates that the smaller mass is
the main factor underlying the reduced CCS and not
an altered conformation of the NAC heterodimer
upon deletion of the UBA domain.

Although both NAC and ΔUBA-NAC give
rise to native ESI mass spectra with similar charge
state distributions, these proteins have different
stability in the gas-phase as indicated using CIU
(Figure 3). These experiments showed that the
absence of the UBA domain results in a complex
that is more susceptible to unfolding in the dimeric
state. We were unable to detect ubiquitin binding to
NAC using native mass spectrometry (data not
shown), and hence the role of the UBA domain in
NAC remains unclear. Recently, Ott et al.
demonstrated that ΔUBA-NAC is a better inhibitor
of protein aggregation in nacAsb4 yeast cells,
suggesting that the ΔUBA-NAC heterodimer could
have improved chaperone activity compared with
the WT-NAC protein (20). The fact that ΔUBA-
NAC formed an extended conformation at a lower
collision energy than the WT-NAC suggests that
this variant is more susceptible to undergoing
conformational change which may, in part,
rationalise the in vivo observations of improved
chaperone activity.

To determine whether NAC substrates are
required to be unfolded such as those that remain
unstructured as they emerge from the ribosome exit
tunnel (22), or whether NAC can recognise
structured domains such as those that fold rapidly
upon emergence from the ribosome (51), we used
chemical cross-linking coupled with LC-MS/MS to
map the binding of NAC to two model proteins (α-
synuclein and Im7/TM-Im7) as potential substrates.
The results showed that NAC binds weakly to the
IDP α-synuclein, as well as to Im7 in both its native
and unfolded states. A weak interaction was
supported by the small, but significant NMR
chemical shift perturbations specifically involving
the acidic C-terminal region of α-synuclein upon
NAC binding. That this interaction is functionally
relevant was demonstrated by the finding that NAC
suppresses α-synuclein aggregation both in vitro
and in vivo in C. elegans (Figure 5b, e). A recent
study showed that although ribosome-tethered α-
synuclein is a weak substrate for trigger factor, this
chaperone was observed to interact with the first
110 N-terminal residues of the protein (52)
Similarly, binding of α-synuclein and Im7/TM-Im7
to NAC could not be detected by use of native ESI-
MS or native PAGE, consistent with a weak
interaction. Such weak binding may be required for
ATP-independent chaperones such as NAC, which
rely on relatively rapid dissociation to enable
substrate folding upon release (45,52-54). Overall,
therefore, the results presented demonstrate that
NAC can interact with both structured and
disordered polypeptides, forming weak and/or
transient interactions that predominantly involve
the terminal domains of both the α- and β-subunits,
at least for the model proteins used here. Whether
these binding sites are specific for Im7/TM-Im7/α-
synuclein, or are utilised ubiquitously for other
polypeptide chains remains to be seen. Moreover,
the results presented indicate that attachment of
NAC to the ribosome is not required for binding to
the substrates used in this study. Ribosome binding
could alter binding affinity and/or the kinetics of
binding, and may alter the regions of NAC involved
in substrate recognition. Indeed, the N-terminal
domain of β-NAC may not be involved in substrate
binding at the ribosome exit tunnel given that the
RRKKK- motif in this domain is required for
ribosome binding (Figure 1a) (21-23). Future work
exploiting detailed NMR studies of these
Structure and function of NAC complexes, combined with other experiments able to probe weak and transient complexes in residue-specific detail (52) will now be needed to study the interactions of NAC with its clients in atomic detail. Similar studies of the same sequences when nascent on the ribosome (51, 55) will then be able to reveal how the chaperone activity of NAC differs when on- and off- the ribosome.

**Experimental Procedures**

**Experimental design and statistical rationale**

For CD experiments duplicate samples were analysed to ensure reproducibility. ESI-IMS-MS, cross-linking and proteolysis experiments were performed at least in triplicate in separate experiments using freshly prepared samples. Controls included comparing NAC with and without substrate bound so as to reveal differences in conformation upon interaction. Details regarding search parameters and acceptance criteria for MS/MS are given below.

**NAC expression and purification**

BL21(DE3) Rosetta cells (Novagen, Merck (UK) Ltd., Watford, UK) were transformed with a plasmid encoding a His$_6$-SUMO-NAC construct (25, 48) which contains tandem hexahistidine and SUMO tags at the N-terminus of $\alpha$-NAC. Bacteria were grown overnight on LB-agar plates supplemented with 100 $\mu$g/mL ampicillin and 25 $\mu$g/mL chloramphenicol. A single colony was used to inoculate a 200 mL culture of LB medium containing 100 $\mu$g/mL ampicillin and 25 $\mu$g/mL chloramphenicol and incubated overnight at 30 °C with shaking at 120 rpm. The overnight culture was used to inoculate 6x1 L flasks of sterile LB containing 100 $\mu$g/mL ampicillin and 25 $\mu$g/mL chloramphenicol to an OD$_{600}$ of 0.1. The cultures were incubated at 30 °C and 120 rpm shaking until an OD$_{600}$ of 0.6-0.8 was reached (~3 h). Protein expression was induced by addition of 1 mM IPTG and incubation continued for 5 h after which time cells were harvested at 4,400 rpm and 4 °C for 20 min (Beckman Avanti J-26 XP centrifuge, JLA 8.1 rotor; Beckman Coulter (UK) Ltd., High Wycombe, Bucks, UK). Cells were resuspended in cold Buffer B1 (50 mM sodium phosphate buffer, 300 mM NaCl, 6 mM MgCl$_2$, 2 mM DTT, 2 mM PMSF, 10 % (v/v) glycerol, pH 8.0), harvested and the pellet stored at -80 °C.

Cells were lysed using a French press (1100 psi) in Buffer B1 containing protease inhibitors and DNase (20 $\mu$g/mL) and then centrifuged at 16,000 rpm and 4 °C for 20 min (Sorvall SS34 rotor). 2.5 g of Protino Ni-IDA matrix (Macherey Nagel GmbH, Duren, Germany) was added to the lysate and incubated with agitation for 1 h at 4 °C. The matrix was then washed with 50 mL of Buffer B1 containing 750 mM NaCl, followed by 50 mL of Buffer B1 where the concentration of NaCl was reduced to 25 mM. NAC was eluted from the matrix using Buffer B1 supplemented with 250 mM imidazole and fractions containing NAC (as determined by SDS-PAGE) were pooled and the concentration assessed using a Bradford assay. Ulp1(25, 48) was added for SUMO-cleavage (8 $\mu$g enzyme per mg of substrate) and NAC was dialysed into Buffer B2 (20 mM sodium phosphate buffer, 25 mM NaCl, 6 mM MgCl$_2$, 2 mM DTT, 5 % (v/v) glycerol, pH 7.4). The next day the NAC/Ulp1 mixture was loaded onto a Resource Q anion exchange column (6 mL column volume, GE Healthcare) that had been equilibrated with Buffer B2. Proteins were eluted using an increasing gradient of high salt buffer (Buffer B3; 20 mM sodium phosphate buffer, 650 mM NaCl, 6 mM MgCl$_2$, 2 mM DTT, 5 % (v/v) glycerol, pH 7.4) over 25 column volumes and fractions of 1 mL were collected. Fractions containing both cleaved $\alpha$-NAC (21.8 kDa) and $\beta$-NAC (17.5 kDa), as evaluated by SDS-PAGE, were pooled and dialysed overnight at 4 °C into Buffer B2. Aliquots were frozen in liquid N$_2$ and stored at -80 °C.

$\alpha$-Synuclein expression and purification

Unlabelled and $^{15}$N-labelled $\alpha$-synuclein were expressed recombinantly in E. coli BL21 (DE3) cells and the protein purified as described previously (56). Cell pellets were resuspended in lysis buffer (25 mM Tris-HCl, pH 8.0, 100 $\mu$g/mL lysozyme, 50 $\mu$g/mL PMSF and 20 $\mu$g/mL DNase), homogenised and then heated to 80 °C for 10 min. The homogenate was then centrifuged (35,000 x g, 4 °C, 30 min) and the protein, isolated in the soluble fraction, was twice precipitated with 50 % (w/v) ammonium sulphate at 4 °C, 30 min. The pellet was resuspended in 20 mM Tris-HCl, pH 8.0 and loaded onto an anion exchange column (Q-Sepharose, GE Healthcare, Amersham, Bucks., UK), protein was eluted with a salt gradient. Final salt concentration...
was 500 mM NaCl in 20 mM Tris-HCl, pH 8. Gel filtration (HiLoadTM 26/60 Superdex 75 preparative grade gel filtration column using 20 mM sodium phosphate, pH 7.5) was then used as a final purification step. Pure protein was dialysed against 50 mM ammonium bicarbonate and lyophilised.

**Im7 expression and purification**

Im7 constructs were over-expressed in *E. coli* and purified as described previously (40,41). Briefly, BL21 (DE3) cells transformed with chosen plasmid (containing WT Im7 or TM Im7 (L18AL19AL37A) were cultured in LB medium with carbenicillin (100 µg/mL) selection at 37°C, 200 rpm. Protein expression was induced using 1 mM IPTG at an OD 600= 0.6 and cells grown for a further 5 h. The bacteria were then harvested and lysed using sonication. Im7 proteins were then purified using Ni²⁺-affinity chromatography (Ni²⁺-Sepharose column (5 mL volume) (GE Healthcare, Amersham, Bucks., UK)) using a gradient of 10-500 mM imidazole in 50 mM Tris-HCl, 0.3 M NaCl, pH 8.0. Fractions containing Im7 were pooled, dialysed into 50 mM sodium phosphate buffer, pH 6.0 and purified further using ion-exchange chromatography (Source15Q resin, 7 mL column (GE Healthcare, Amersham, Bucks., UK)) and gel filtration (300 mL Superdex-75 GL column) in the same buffer. Pure proteins were dialysed into 18 MΩ water and lyophilised.

**Native PAGE**

10 % native gels were prepared in-house by adding 17 mL of dH₂O to 10 mL of 30 % acrylamide (w/v) / 0.8 % (w/v) bisacrylamide and 3 mL of 10x Tris/glycine buffer (250 mM Tris-Cl, 1.94 M glycine, pH 8.5). Gels were polymerised by addition of 150 µL 10 % (w/v) ammonium persulphate and 20 µL TEMED. Samples were diluted 2-fold with loading buffer (50 mM Tris-Cl pH 8.6, 10 % (w/v) glycerol, 0.1 % (w/v) bromophenol blue) and 20 µL loaded onto the gel alongside NativeMark™ unstained protein standard (Thermo Fisher Scientific). Gels were run at 250 V for 40 min, stained with 0.25% (w/v) Coomassie Blue/ 40 % (v/v) MeOH / 10 % (v/v) acetic acid for 3 h and then destained in 40% (v/v) methanol / 10 % (v/v) acetic acid overnight.

**Circular dichroism spectroscopy (CD)**

Far-UV circular dichroism (CD) spectra were recorded on a Chirascan CD spectrophotometer (Applied Photophysics, Leatherhead, Surrey, UK) using a 1 mm path length cuvette. Proteins were buffer exchanged into 10 mM sodium phosphate buffer, pH 7.0 and measured at a protein concentration of 10 µM. Three scans were acquired over the range 190–260 nm with a bandwidth of 2.5 nm and a scan speed of 1 nm/s. The three datasets were averaged and the buffer contribution subtracted to produce the final spectrum. Secondary structure content was estimated by uploading the data into DichroWeb (57) and using the CONTIN (58) algorithm.
**Limited proteolysis**

NAC proteins were buffer exchanged into 100 mM ammonium acetate (pH 6.9) and diluted to 10 µM before adding trypsin at a 1:500 (w/w) protease:protein ratio (Sequencing Grade Modified Trypsin, Promega, UK Ltd., Southampton, UK). Mass spectra were measured after 15 min, 30 min and 1 h under the same conditions as described above for the native MS experiments. Fragments were assigned manually by comparison with theoretical digest peak lists obtained using the MS-Digest tool in ProteinProspector v5.20.0 (University of California, San Francisco, USA). The mass tolerance for precursor ions was 20 ppm and for fragment ions 10 ppm was employed. Raw mass spectrometry data is available at the following DOI: [archive.researchdata.leeds.ac.uk/291/](http://archive.researchdata.leeds.ac.uk/291/)

**Chemical cross-linking**

Substrate proteins were exchanged into 10 mM sodium phosphate buffer, pH 7.0 and added to NAC at a 1:1 molar ratio (20 µM NAC + 20 µM substrate). 1 mg BS3-d⁰ and 1 mg BS3-d⁴ (bis-(sulfosuccinimidyl) suberate) (Thermo Fisher Scientific, Altrincham, Cheshire, UK) were dissolved in 277 µL sodium phosphate buffer to produce a stock solution of 12.5 mM. The crosslinker was then added to the proteins at 20-fold or 50-fold molar excess and the reaction allowed to proceed at room temperature for 1 h before quenching by the addition of 50 mM Tris-HCl, pH 7.5. Samples were analysed by Tris-tricine gels (15 % (w/v) acrylamide, 0.4 % (w/v) bisacrylamide) followed by staining with InstantBlue™ (Expedeon, San Diego, CA, USA). Gel bands were excised from the gel, cut into 1 mm x 1 mm pieces and washed in 500 µL 25 mM ammonium bicarbonate, pH 7.8 for 1 h with shaking. The solution was removed and the pieces destained with 100 µL 525 mM ammonium bicarbonate in 60 % (v/v) acetonitrile. This step was repeated three times. Gel pieces were then dehydrated with 100 % acetonitrile (v/v) for 10 min and left to air-dry in a laminar flow hood for 1 h. Rehydration of the gel pieces was achieved by adding 0.1 mg/mL trypsin in 25 mM ammonium bicarbonate and incubating the samples on ice for 30 min. Excess trypsin was then removed, 25 mM ammonium bicarbonate added to cover the gel pieces and the samples incubated at 37 °C with shaking (1000 rpm) overnight. Peptides were extracted from the gel using three washes with 60 % (v/v) acetonitrile / 5 % (v/v) formic acid. The extracts were pooled and concentrated using a SpeedVac before being analysed using a ACQUITY UPLC M-Class coupled to a Synapt HDMS G2Si mass spectrometer (Waters UK Ltd., Wilmslow, UK). Peptides were injected onto a C18 column equilibrated with 0.1 % formic acid (v/v) in water and eluted using an increasing gradient of 0.1 % (v/v) formic acid in acetonitrile over 60 min at a flow rate of 0.3 µL/min. The Synapt HDMS G2Si was operated in positive mode using a capillary voltage of 3.0 kV, cone voltage of 40 V, backing pressure of 3.6 mbar and a trap bias of 2.0 V. The source temperature was 80 °C and the trap pressure was 8.70 × 10⁻³ mBar. Glu-fibrinogen and leucine enkephalin were infused as lock mass calibrants. Data acquisition was achieved using Data Dependent Analysis (DDA) with a one second MS scan over a m/z range of 250–3000 being followed by three 1 s MS/MS scans taken from the five most intense ions in the MS spectrum over a m/z range of 50–2000. Data were acquired using MassLynx v4.1 and processed using PEAKS Studio 7 (Bioinformatics Solutions, Ontario, Canada). Cross-links were identified using StravroX software (59) and verified manually. Raw mass spectrometry data is available at the following DOI: [archive.researchdata.leeds.ac.uk/291/](http://archive.researchdata.leeds.ac.uk/291/)

**Analysis of cross-linking data**

Raw data files were acquired on a ACQUITY M-Class LC-MS coupled to a Synapt G2Si mass spectrometer. Data files (.raw) were imported into PEAKS Studio v8.0 for peptide identification from MS/MS data. An FDR of 1 % was applied. Data were exported as a Mascot Generic File (mgf) to be imported into StravroX3.6.0. FASTA files of each sequence were imported into StravroX and used to search for theoretical crosslinked peptides that were then compared to the experimental dataset. Threshold score/expectation value for accepting individual spectra was in line with StavroX guidelines. Trypsin was selected as the protease and crosslinking at K, S, T and Y residues were used as the search parameters. The decoy dataset was used to determine the score threshold above which crosslinked peptides had been assigned with confidence.
NMR spectroscopy

$^{1}H$-$^{15}N$ HSQC spectra were obtained using 50 μM $^{15}N$-labelled α-synuclein in 10 mM sodium phosphate buffer, pH 7.2 containing 10 % (v/v) D$_2$O. Spectra were acquired in the absence or presence of equimolar (unlabelled) NAC using a 600 MHz NMR magnet (Oxford Instruments Plc, Abingdon, UK) with a room temperature probe and an Avance III HD console (Bruker UK Ltd., Coventry, UK). Data were processed and visualised using NMRPipe and CcpNMR analysis software (60). Assignments of $^1$H and HN atoms were transferred from the deposited chemical shifts in the Biological Magnetic Resonance Bank (ID:16543) (38). In cases where transferring assignments was difficult due to resonance overlap, the assignments were confirmed using HNCACB and HNCBCACO triple resonance spectra. Triple resonance spectra were acquired using a sample of 400 μM uniformly $^{15}N$ $^{13}C$ labelled α-synuclein using a Varian Inova spectrometer performing at 600 MHz. Chemical shift differences were calculated using the formula:

$$\Delta \delta = \sqrt{(5 + \delta^1 H)^2 + (\delta^{15} N)^2}$$

ThT aggregation kinetics

25 μL samples containing 125 μM α-synuclein in Dulbecco’s phosphate buffered saline (Sigma-Aldrich, Irvine, UK, D8537), 0.02 % (w/v) sodium azide and 10 μM ThT were incubated at 37 °C in sealed 384 well plates (Greiner bio-one Ltd, England) in a BMG Clariostar (BMG Labtech Ltd, England) plate reader using an excitation wavelength of 440±10 nm and emission wavelength of 475±10 nm, with agitation at 600 rpm.

Electron microscopy

Transmission electron micrographs were acquired from the ThT aggregation assay samples using a JEM-1400 (JEOL Ltd) transmission electron microscope. Protein samples were pipetted onto carbon coated copper grids and stained with 1 % (w/v) uranyl acetate solution.

C. elegans strains and RNAi treatment

C. elegans was cultured according to standard techniques (61). Strain NL5901 (pkIs2386 [unc-54p::alpha-synuclein::YFP + unc-119(+)]) was obtained from the Caenorhabditis Genetics Center. RNAi was performed by feeding the worms with E. coli HT115(DE3) harbouring the vector L4440 to express dsRNA of the respective genes. Simultaneous knockdown of icd-1 and icd-2 was achieved as described previously (62).

Immunoblot analysis and antibodies

Protein samples were applied to bisTris–PAGE and electro-botted onto a nitrocellulose membrane according to standard protocols. Polyclonal antibody against C. elegans NAC (1:5000) was described previously (48). As a loading control, anti-actin (1:5000, Santa Cruz Biotechnology) was used.

Explanation of terms reported in cross-linking data tables

Score = best score calculated for the crosslink within the two peptides. Crosslinked peptides with a score higher than the scores calculated from a decoy dataset (inverted sequences) are more probable.

Abbreviation used: $m/z$ = mass-to-charge ratio, $z$ = peptide charge, M$+H^+$ = mass of singly charged precursor, Calculated = theoretical single charged mass of crosslinked peptide, Deviation = deviation of theoretical and experimental mass in ppm, Peptide 1/2 = Peptide sequence, Protein 1/2 = Protein that peptide is derived from, From/To = defines the start and stop position of peptide within the protein.

Site = indicates the residue in the cross-linked peptide that gave the best score

Accession Codes

α-NAC; >sp|Q86S66-2|1-195. β-NAC; >sp|Q18885|1-161. α-Synuclein; >sp|P37840|1-140. Im7; >sp|Q03708|1-87. Im7 contains an additional His-tag (MEH₆) for purification purposes which is not observed in Uniprot database.
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Author contributions: E.M.M. performed all mass spectrometry and crosslinking experiments. M.P.J provided α-synuclein and made figures. M.G provided NAC protein and helped devise experiments. K.G. performed all C. elegans experiments. J.R.H. provided Im7 protein and made figures. T.K.K performed NMR experiments. E.D, S.E.R. and A.E.A. devised the experimental plan and supervised the work. All authors wrote the manuscript.
References

1. Hartl, F. U., Bracher, A., and Hayer-Hartl, M. (2011) Molecular chaperones in protein folding and proteostasis. *Nature* **475**, 324-332

2. Knowles, T. P. J., Vendruscolo, M., and Dobson, C. M. (2014) The amyloid state and its association with protein misfolding diseases. *Nat. Rev. Mol. Cell. Biol.* **15**, 384-396

3. Radwan, M., Wood, R. J., Sui, X., and Hatters, D. M. (2017) When proteostasis goes bad: Protein aggregation in the cell. *IUBMB Life* **69**, 49-54

4. Wegrzyn, R. D., and Deuerling, E. (2005) Molecular guardians for newborn proteins: ribosome-associated chaperones and their role in protein folding. *Cell. Mol. Life Sci.* **62**, 2727-2738

5. Sharma, S. K., and Priya, S. (2017) Expanding role of molecular chaperones in regulating α-synuclein misfolding; implications in Parkinson’s disease. *Cell. Mol. Life Sci.* **74**, 617-629

6. Balchin, D., Hayer-Hartl, M., and Hartl, F. U. (2016) *In vivo* aspects of protein folding and quality control. *Science* **353**

7. Kramer, G., Boehringer, D., Ban, N., and Bukau, B. (2009) The ribosome as a platform for cotranslational processing, folding and targeting of newly synthesized proteins. *Nat. Struct. Mol. Biol.* **16**, 589-597

8. Preissler, S., and Deuerling, E. (2012) Ribosome-associated chaperones as key players in proteostasis. *Trends Biochem. Sci.* **37**, 274-283

9. Hoffmann, A., Bukau, B., and Kramer, G. (2010) Structure and function of the molecular chaperone trigger factor. *Mol. Cell. Res.* **1803**, 650-661

10. Kaiser, C. M., Chang, H.-C., Agashe, V. R., Lakshmipathy, S. K., Etchells, S. A., Hayer-Hartl, M., Hartl, F. U., and Barral, J. M. (2006) Real-time observation of trigger factor function on translating ribosomes. *Nature* **444**, 455-460

11. Saio, T., Guan, X., Rossi, P., Economou, A., and Kalodimos, C. G. (2014) Structural basis for protein antiaggregation activity of the trigger factor chaperone. *Science* **344**, 1250494

12. Gautschi, M., Lilie, H., Fünfschilling, U., Mun, A., Ross, S., Lithgow, T., Rücknagel, P., and Rospert, S. (2001) RAC, a stable ribosome-associated complex in yeast formed by the DnaK-DnaJ homologs Ssz1p and zuotin. *Proc. Nat. Acad. Sci.* USA **98**, 3762-3767

13. Zhang, Y., Ma, C., Yuan, Y., Zhu, J., Li, N., Chen, C., Wu, S., Yu, L., Lei, J., and Gao, N. (2014) Structural basis for interaction of a cotranslational chaperone with the eukaryotic ribosome. *Nat. Struct. Mol. Biol.* **21**, 1042-1046

14. Koplin, A., Preissler, S., Ilina, Y., Koch, M., Scior, A., Erhardt, M., and Deuerling, E. (2010) A dual function for chaperones SSB–RAC and the NAC nascent polypeptide–associated complex on ribosomes. *J. Cell. Biol.* **189**, 57-68

15. Rospert, S., Dubaquié, Y., and Gautschi, M. (2002) Nascent-polypeptide-associated complex. *Cell. Mol. Life Sci.* **59**, 1632-1639

16. Kogan, G. L., and Gvozdev, V. A. (2014) Multifunctional nascent polypeptide-associated complex (NAC). *Mol. Biol.* **48**, 189-196

17. Wang, L., Zhang, W., Wang, L., Zhang, X. C., Li, X., and Rao, Z. (2010) Crystal structures of NAC domains of human nascent polypeptide-associated complex (NAC) and its αNAC subunit. *Protein & Cell* **1**, 406-416
18. Liu, Y., Hu, Y., Li, X., Niu, L., and Teng, M. (2010) The crystal structure of the human nascent polypeptide-associated complex domain reveals a nucleic acid-binding region on the NACA subunit. Biochem. 49, 2890-2896

19. Spreter, T., Pech, M., and Beatrix, B. (2005) The crystal structure of archaeal nascent polypeptide-associated complex (NAC) reveals a unique fold and the presence of a ubiquitin-associated domain. J. Biol. Chem. 280, 15849-15854

20. Ott, A.-K., Locher, L., Koch, M., and Deuerling, E. (2015) Functional dissection of the nascent polypeptide-associated complex in Saccharomyces cerevisiae. PLoS ONE 10, e0143457

21. Grallath, S., Schwarz, J. P., Böttcher, U. M. K., Bracher, A., Hartl, F. U., and Siegers, K. (2006) L25 functions as a conserved ribosomal docking site shared by nascent chain-associated complex and signal-recognition particle. EMBO Reports 7, 78-84

22. Beatrix, B., Sakai, H., and Wiedmann, M. (2000) The α and β subunit of the nascent polypeptide-associated complex have distinct functions. J. Biol. Chem. 275, 37838-37845

23. Wegrzyn, R. D., Hofmann, D., Merz, F., Nikolay, R., Rauch, T., Graf, C., and Deuerling, E. (2006) A conserved motif is prerequisite for the interaction of NAC with ribosomal protein L23 and nascent chains. J. Biol. Chem. 281, 2847-2857

24. Wang, S., Sakai, H., and Wiedmann, M. (1995) NAC covers ribosome-associated nascent chains thereby forming a protective environment for regions of nascent chains just emerging from the peptidyl transferase center. J. Cell Biol. 130, 519-528

25. Gamerdinger, M., Hanebuth, M. A., Frickey, T., and Deuerling, E. (2015) The principle of antagonism ensures protein targeting specificity at the endoplasmic reticulum. Science 348, 201-207

26. Konijnenberg, A., Butterer, A., and Sobott, F. (2013) Native ion mobility-mass spectrometry and related methods in structural biology. Proteins and Proteomics 1834, 1239-1256

27. Heck, A. J. R. (2008) Native mass spectrometry: a bridge between interactomics and structural biology. Nat. Meth. 5, 927-933

28. Ruotolo, B. T., Benesch, J. L. P., Sandercock, A. M., Hyung, S.-J., and Robinson, C. V. (2008) Ion mobility-mass spectrometry analysis of large protein complexes. Nat. Protocols 3, 1139-1152

29. Smith, D. P., Radford, S. E., and Ashcroft, A. E. (2010) Elongated oligomers in β2-microglobulin amyloid assembly revealed by ion mobility spectrometry-mass spectrometry. Proc. Natl. Acad. Sci. USA 107, 6794-6798

30. Schiffrin, B., Calabrese, A. N., Devine, P. W. A., Harris, S. A., Ashcroft, A. E., Brockwell, D. J., and Radford, S. E. (2016) Skp is a multivalent chaperone of outer-membrane proteins. Nat. Struct. Mol. Biol. 23, 786-793

31. Young, L. M., Saunders, J. C., Mahood, R. A., Revill, C. H., Foster, R. J., Tu, L. H., Raleigh, D. P., Radford, S. E., and Ashcroft, A. E. (2015) Screening and classifying small-molecule inhibitors of amyloid formation using ion mobility spectrometry-mass spectrometry. Nat. Chem. 7, 73-81

32. Rand, K. D., Pringle, S. D., Murphy, J. P., Fadgen, K. E., Brown, J., and Engen, J. R. (2009) Gas-phase hydrogen/deuterium exchange in a traveling wave ion guide for the examination of protein conformation. Anal. Chem. 81, 10019-10028

33. Xu, G. H., and Chance, M. R. (2007) Hydroxyl radical-mediated modification of proteins as probes for structural proteomics. Chem Rev 107, 3514-3543
34. Calabrese, A. N., and Radford, S. E. (2018) Mass spectrometry-enabled structural biology of membrane proteins. *Methods*, in press (doi 10.1016/j.ymeth.2018.1002.1020)
35. Calabrese, A. N., Ault, J. R., Radford, S. E., and Ashcroft, A. E. (2015) Using hydroxyl radical footprinting to explore the free energy landscape of protein folding. *Methods* **89**, 38-44
36. Sinz, A., Arlt, C., Chorev, D., and Sharon, M. (2015) Chemical cross-linking and native mass spectrometry: A fruitful combination for structural biology. *Prot. Sci.* **24**, 1193-1209
37. Leitner, A., Walzthoeni, T., Kahraman, A., Herzog, F., Rinner, O., Beck, M., and Aebersold, R. (2010) Probing native protein structures by chemical cross-linking, mass spectrometry, and bioinformatics. *Mol. Cell. Prot.* **9**, 1634-1649
38. Woods, W. S., Boettcher, J. M., Zhou, D. H., Kloepper, K. D., Hartman, K. L., Ladror, D. T., Qi, Z., Rienstra, C. M., and George, J. M. (2007) Conformation-specific binding of α-synuclein to novel protein partners detected by phage display and NMR spectroscopy. *J. Biol. Chem.* **282**, 34555-34567
39. Ulmer, T. S., Bax, A., Cole, N. B., and Nussbaum, R. L. (2005) Structure and dynamics of micelle-bound human α-synuclein. *J. Biol. Chem.* **280**, 9595-9603
40. Friel, C. T., Smith, D. A., Vendruscolo, M., Gsponer, J., and Radford, S. E. (2009) The mechanism of folding of Im7 reveals competition between functional and kinetic evolutionary constraints. *Nat. Struct. Mol. Biol.* **16**, 318-324
41. Pashley, C. L., Morgan, G. J., Kalverda, A. P., Thompson, G. S., Kleanthous, C., and Radford, S. E. (2012) Conformational properties of the unfolded state of Im7 in nondenaturing conditions. *J. Mol. Biol.* **416**, 300-318
42. Jurneczko, E., and Barran, P. E. (2011) How useful is ion mobility mass spectrometry for structural biology? The relationship between protein crystal structures and their collision cross sections in the gas phase. *Analyst* **136**, 20-28
43. Devine, P. W. A., Fisher, H. C., Calabrese, A. N., Whelan, F., Higazi, D. R., Potts, J. R., Lowe, D. C., Radford, S. E., and Ashcroft, A. E. (2017) Investigating the structural compaction of biomolecules upon transition to the gas-phase using ESI-TWIMS-MS. *J. Am. Soc. Mass Spectrom.* **28**, 1855-1862
44. Marklund, E. G., Degiacomi, M. T., Robinson, C. V., and Wiedmann, M. (2015) Collision cross sections for structural proteomics. *Structure* **23**, 791-799
51. Cabrita, L. D., Cassaignau, A. M. E., Launay, H. M. M., Waudby, C. A., Wlodarski, T., Camilloni, C., Karyadi, M. E., Robertson, A. L., Wang, X., Wentink, A. S., Goodsell, L., Woolhead, C. A., Vendruscolo, M., Dobson, C. M., and Christodoulou, J. (2016) A structural ensemble of a ribosome-nascent chain complex during cotranslational protein folding. Nat. Struct. Mol. Biol. 23, 278-285

52. Deckert, A., Waudby, C. A., Wlodarski, T., Wentink, A. S., Wang, X., Kirkpatrick, J. P., Paton, J. F. S., Camilloni, C., Kukic, P., Dobson, C. M., Vendruscolo, M., Cabrita, L. D., and Christodoulou, J. (2016) Structural characterization of the interaction of α-synuclein nascent chains with the ribosomal surface and trigger factor. Proc. Natl. Acad. Sci. USA 113, 5012-5017

53. Suss, O., and Reichmann, D. (2015) Protein plasticity underlines activation and function of ATP-independent chaperones. Front. Mol. Biosci. 2, 43

54. Lambert, W., Rutsdottir, G., Hussein, R., Bernfur, K., Kjellstrom, S., and Emanuelsson, C. (2013) Probing the transient interaction between the small heat-shock protein Hsp21 and a model substrate protein using crosslinking mass spectrometry. Cell Stress Chap. 18, 75-85

55. Cabrita, L. D., Hsu, S.-T. D., Launay, H., Dobson, C. M., and Christodoulou, J. (2009) Probing ribosome-nascent chain complexes produced in vivo by NMR spectroscopy. Proc. Natl. Acad. Sci. USA 106, 22239-22244

56. Kang, L., Moriarty, G. M., Woods, L. A., Ashcroft, A. E., Radford, S. E., and Baum, J. (2012) N-terminal acetylation of α-synuclein induces increased transient helical propensity and decreased aggregation rates in the intrinsically disordered monomer. Prot. Sci. 21, 911-917

57. Whitmore, L., and Wallace, B. A. (2004) DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Res. 32, W668-W673

58. Sreerama, N., and Woody, R. W. (2000) Estimation of protein secondary structure from circular dichroism spectra: Comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. Anal. Biochem. 287, 252-260

59. Götzte, M., Pettelkau, J., Schaks, S., Bosse, K., Ihling, C. H., Krauth, F., Fritzshe, R., Kühn, U., and Sinz, A. (2012) StavroX—A software for analyzing crosslinked products in protein interaction studies. J. Am. Soc. Mass Spec. 23, 76-87

60. Vranken, W. F., Boucher, W., Stevens, T. J., Fogh, R. H., Pajon, A., Llinas, M., Ulrich, E. L., Markley, J. L., Ionides, J., and Laue, E. D. (2005) The CCPN data model for NMR spectroscopy: Development of a software pipeline. Prot. Struct. Funct. Bioinfo. 59, 687-696

61. Brenner, S. (1974) The genetics of Caenorhabditis elegans. Genetics 77, 71-94

62. Min, K., Kang, J., and Lee, J. (2010) A modified feeding RNAi method for simultaneous knockdown of more than one gene in Caenorhabditis elegans. Biotechniques 48, 229-232
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The abbreviations used are: ATD, arrival time distribution; BS3, bis-(Sulfosuccinimidyl) suberate; ESI-MS, electrospray ionisation-mass spectrometry; IDP, intrinsically disordered protein; NAC, nascent polypeptide associated complex; TM Im7, the triple mutant of the bacterial immunity protein Im7 that contains the amino acid substitutions L18AL19AL37A; UBA, ubiquitin-associated domain; WT, wild-type; ΔUBA-NAC, the NAC heterodimer lacking the UBA domain from the α-subunit; IMS-MS, ion mobility spectrometry-mass spectrometry; CIU, collision induced unfolding; ThT, thioflavin T.
Figure 1: Schematic representation of the nascent polypeptide associated complex (NAC). a) Domain structure of human NAC highlighting the UBA domain on α-NAC and the location of the ribosome binding motif (RRK-(X)nKK) on β-NAC. b) Dimerisation of the NAC domains, with α-NAC shown in blue and β-NAC shown in purple (PDB 3LKX) (18).
Figure 2: Comparison of native ESI-mass spectra of WT-NAC and ΔUBA-NAC. Native ESI-mass spectra of a) WT-NAC and b) ΔUBA-NAC. Each spectrum shows two charge state distributions for each heterodimer (green and red for WT-NAC and ΔUBA-NAC, respectively), with low populations of αβ2-NAC dimers as indicated. In addition, low populations of dissociated α-NAC and β-NAC subunits are observed (blue). The insets show the proteins analysed by native PAGE which reveal a single band of the heterodimer and no evidence of dissociation. c) Estimated collision cross-sections (CCS) from ESI-IMS-MS experiments for WT-NAC (green circles) and ΔUBA-NAC (red triangles) show that compact and extended forms co-exist for both species (see also Supporting Information Table S1). d) Far UV CD spectra of WT-NAC (green) and ΔUBA-NAC (red). The secondary structure content obtained using CONTIN (58) is given in Supporting Information Table S2.
Figure 3: Collision induced unfolding (CIU)-IMS-MS for the 12+ charge state ions of WT-NAC and ΔUBA-NAC. The quadrupole selected 12+ charge state ions are shown at a trap collision energy of 25 V for a) WT-NAC and b) ΔUBA-NAC (left). Extracted ATDs for these charge states at 10 V, 25 V and 35 V are shown alongside.
Figure 4: Limited proteolysis of WT-NAC and ΔUBA-NAC followed by ESI-MS analysis. Each protein was treated with 1:500 (w/w) trypsin:substrate for 15 min at room temperature. a) Native ESI-MS shows a truncated complex with a reduced mass of 2,462 Da (blue peaks) and which remains assembled, is the first cleavage product for both WT-NAC and ΔUBA-NAC. b) Tryptic fragments observed in the low m/z range reveal a range of peptides released from the N-terminal domain of α-NAC (blue) and from the C-terminal domain of β-NAC (orange). The same fragments are observed for WT-NAC and ΔUBA-NAC. Numbers denote the fragments within each NAC domain. Charge states resulting from the intact (undigested) NAC are shown in black.
Figure 5: Interactions of α-synuclein with WT-NAC and ΔUBA-NAC. a) Coomassie blue stained native polyacrylamide gel of α-synuclein alone and mixed with an equimolar concentration of WT-NAC or ΔUBA-NAC, showing that a stable complex is not detected. b) Kinetic aggregation assays of α-synuclein (125 µM, black), a 1:1 molar ratio of α-synuclein with WT-NAC (purple), and WT-NAC alone (green) measured using ThT fluorescence. An equimolar concentration of WT-NAC to α-synuclein inhibits its aggregation over a 70 h timescale. Images alongside show negative stain transmission electron micrographs of the reaction endpoint (at 70 h) for each sample. Scale bar = 500 nm. c) SDS-PAGE analysis of cross-linked WT-NAC and ΔUBA-NAC mixed with equimolar α-synuclein (a 50-fold excess of BS3 was used (Experimental Procedures)). The arrows highlight the covalent complex between NAC and α-synuclein. d) Map of cross-links between WT-NAC and α-synuclein identified following in-gel tryptic digestion of the
band arrowed in (c). Intra-NAC or intra-α-synuclein cross-links (purple), inter-NAC cross-links (green) and NAC-α-synuclein cross-links (red). Peptides identified are listed in Supporting Information Tables 3-5. e) RNAi-mediated NAC depletion leads to increased α-synuclein puncta formation in vivo. Fluorescence microscope images of transgenic worms (head regions are shown) expressing α-synuclein::YFP in body-wall muscle cells. Worms were grown on empty vector control (ev) or αβ-NAC RNAi, respectively. Images were taken at day 1 and day 3 of adulthood. Scale bar = 50 μm. Inset western blot shows NAC protein expression levels, at indicated time points, by immunoblotting. Immunoblot against actin served as loading control.
Figure 6: $^1$H-$^{15}$N HSQC spectra of α-synuclein following addition of WT-NAC. a) Spectra are overlaid of 50 μM $^{15}$N-labelled α-synuclein alone (black) and in the presence of 1 mol. equivalent of WT-NAC (red). Resonances which shift upon the addition of NAC are indicated with an arrow. b) Chemical shift perturbations in α-synuclein upon NAC binding. Residues exhibiting a significant chemical shift difference (> 1 standard deviation over the mean (dashed line)) are highlighted in red.

Figure 7: NAC binds WT Im7 and TM Im7. a) Far UV CD spectra of WT Im7 (blue) and TM-Im7 (orange) under the conditions used for the cross-linking experiments (see Experimental Procedures). b) SDS-PAGE analysis of NAC cross-linked alone (green) or to WT Im7 (blue) or TM-Im7 (orange) using BS3. Lanes show the addition of a 20x or 50x excess of BS3. The red arrow highlights the putative complex between NAC and the protein substrates. c) Map of the cross-links identified following in-gel tryptic digest of the putative NAC-Im7 complex: intra-NAC cross-links (purple), inter-NAC cross-links (green) and NAC-WT Im7 cross-links (red). d) Map of the cross-links identified following in-gel tryptic digest of the putative NAC-TM-Im7 complex band coloured as in (c). Peptides identified are listed in Supporting Information Tables S6-S10.
Conformational flexibility within the nascent polypeptide–associated complex enables its interactions with structurally diverse client proteins

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