N-terminal domain of *Pyrococcus furiosus* L-asparaginase functions as a non-specific, stable, molecular chaperone

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The enzyme L-asparaginase of *Pyrococcus furiosus* (PfA) functions as a dimer with each monomer consisting of distinct N- and C-terminal domains (NPfA and CPfA, respectively), connected by a linker. Here we present data to show that NPfA functions as a non-specific molecular chaperone. Independently expressed NPfA refolded spontaneously whereas CPfA formed insoluble aggregates. However, when mixed and refolded together, NPfA augmented CPfA to fold with ~90% recovery. NPfA also protected a variety of substrate proteins from thermal and refolding-mediated aggregation as monitored by a reduction in light scattering. The co-appearance of substrate protein with NPfA in antibody pull-down assays as well as in eluted gel filtration peaks indicated direct protein–protein interaction. These interactions were hydrophobic in nature as determined by 8-anilino-1-naphthalene sulfonic acid fluorescence. NPfA inhibited polyglutamine-mediated amyloid formation and also facilitated disintegration of preformed amyloid fibrils of amyloid-β (1–42) as determined by reverse-phase HPLC-based sedimentation assay and thioflavin T binding assays, respectively. Dynamic light scattering experiments suggested that NPfA readily assembled into polydispersed oligomeric species. With no sequence similarity to α-crystallin or any known molecular chaperone, we present here NPfA as a novel molecular chaperone.

Structured digital abstract

• Aβ amyloid 1-42 and Aβ amyloid 1-42 bind by fluorescence technology (View interaction)
• alpha-amylase and alpha-amylase bind by light scattering (View interaction)
• Aβ amyloid 1-42 and Aβ amyloid 1-42 bind by transmission electron microscopy (View interaction)
• NPfA binds to BCA II by anti tag communoprecipitation (View interaction)
• MSG and MSG bind by light scattering (View interaction)
• BCA II and BCA II bind by light scattering (View interaction)

Introduction

*Pyrococcus furiosus* L-asparaginase (PfA) consists of two distinct α/β domains, a large (1–182) N-terminal domain (NPfA) connected by a linker region (183–200) to a small (201–326) C-terminal domain (CPfA) [1]. Previously we reported active-site mutants of PfA with promising therapeutic and industrial usage. A structure–function relationship was presented to describe the mode of action of this enzyme [1]. With

Abbreviations
ANS, 8-anilino-1-naphthalene sulfonic acid; BCA II, bovine carbonic anhydrase II; CPfA, C-terminal domain of PfA; MSG, malate synthase G; NPfA, N-terminal domain of PfA; PfA, *Pyrococcus furiosus* L-asparaginase; polyQ, polyglutamine; sHSP, small heat shock protein; ThT, thioflavin T.
the intention of studying the effect of inter-domain interactions and domain stability in facilitating the function of PfA, we studied individual domains and their folding propensities. In the process we found that NPfA not only acts as an intramolecular chaperone but also functions as a general small molecular chaperone.

Small molecular chaperones are different from intramolecular chaperones by being non-specific to their substrates [2]. Most of these belong to a group of proteins called the small heat shock proteins (sHSPs). sHSPs are ubiquitous proteins having monomer size ranging from 12 to 43 kDa [3,4]. They form large oligomeric complexes of 9–50 subunits, ranging in molecular weight from 125 kDa to 2 MDa [5]. sHSPs and other small molecular chaperones display significant polydispersity, limiting the availability of structural information [6–8]. To date, a large number of small molecular chaperones/sHSPs have been found with aggregation inhibitory properties against thermally or chemically denatured substrate proteins [9,10]. For example, bovine α-crystallin, murine HSP25 and human HSP27 have been shown to prevent the thermal aggregation of a variety of model proteins [11–14]. Similarly, chaperones like artemin, tubulin, clusterin etc. have been shown to prevent the refolding-mediated aggregation of a variety of proteins [15–17]. A large number of sHSPs have also been studied from hyperthermophilic archaea, e.g. the sHSP20 from Sulfolobus solfataricus P2, HSP 16.5 from Methanococcus jannaschii and sHSP from Pyrococcus furiosus [18–20]. The involvement of sHSPs in disease and their potential for therapeutic intervention have also been explored recently [21,22].

The chaperoning efficiency of small molecular chaperones/sHSPs depends upon the type of substrate proteins, the type of chaperone and their mass ratios [23]. Sometimes specific physicochemical conditions are required for chaperones to interact with their substrates [24,25]. Irrespective of this, all substrate–small molecular chaperone interactions lead to large oligomeric complexes formed primarily by hydrophobic interactions. Protein aggregation is a concentration-dependent phenomenon, where refolding/unfolding intermediates undergo intermolecular interactions through exposed hydrophobic surfaces [26,27]. Small molecular chaperones prevent aggregation by mimicking these surfaces. Since hydrophobic interactions are non-specific, the interactions of chaperones with substrates are also primarily non-specific. The non-specificity is advantageous as any hydrophobic small molecule may be used to inhibit aggregation of partially denatured proteins, as has been shown earlier [28].

Here we present data to show that isolated NPfA functions as a small molecular chaperone. During purification of NPfA and CPfA domains (individually cloned and expressed) both appeared as inclusion bodies. Refolding from inclusion bodies yielded only NPfA in the soluble form, whereas CPfA failed to refold and formed insoluble aggregates. Interestingly, when refolded together, both the domains appeared in the soluble fraction suggesting that NPfA probably acts as an intramolecular chaperone for CPfA. Intramolecular chaperones are specific sequences within a polypeptide that are essential for attaining native structure by the polypeptide but may not be necessary for its function. These sequences, also known as propeptide or prosquences, are generally removed autocatalytically or by proteases as soon as the protein attains its native form as has been reported in the case of subtilisin [29]. Intramolecular chaperones are categorized as type I or type II depending on the location of the sequence (at either the N- or the C-terminus) and the assistance they provide in attaining functional form. Regardless of type, the sequences are very specific for their partner proteins [30]. In our case, NPfA does not fall into any of these categories of intramolecular chaperone as it is neither an autocatalytic product, nor the CPfA (which was assisted by NPfA) exists as an independent functional unit. We found that refolded NPfA assembles into polydispersed, large oligomeric complexes. These complexes showed significant surface hydrophobicity, high β-sheet content and remarkable thermal stability. These characteristics are typical of any other small molecular chaperone [31–33]. Small molecular chaperones are independently folded entities that non-specifically assist in the folding of substrate proteins [2]. NPfA when checked for aggregation prevention properties on a variety of proteins qualified well as a typical non-specific molecular chaperone. NPfA prevented thermal aggregation of bovine carbonic anhydrase II (BCA II), α-amylase and malate synthase G (MSG) as well as refolding-induced aggregation of these proteins. The observed chaperoning activity was not due to a bulk solvent effect but rather to direct protein–protein interaction. This is indicated by co-immunoprecipitation of substrate with NPfA and co-elution as a major peak in size exclusion chromatography. Binding of NPfA with substrate proteins was through hydrophobic interactions as observed by 8-anilino-1-naphthalene sulfonic acid (ANS) fluorescence. The chaperoning effect was also reflected in NPfA’s capacity to dissociate fibrillar aggregates of amyloid-β protein and inhibit polyglutamine (polyQ) conversion to amyloids. Therefore, NPfA appears to be functionally similar to sHSPs.
However, NPfA does not show sequence similarity to any known sHSP and hence may be categorized as a separate class of molecular chaperone. To the best of our knowledge, no Pyrococcus furiosus proteins have so far been shown to have domain-specific chaperone activity.

Results

Protein purification

After affinity chromatography, refolding and centrifugation, the majority of the NPfA appeared in the soluble fraction which showed a single 22 kDa band on SDS/PAGE. In contrast, CPfA appeared mainly in the pellet fraction and showed a 16 kDa band. However, when a mixture of unfolded domains (NPfA + CPfA) was subjected to refolding together and centrifuged, no pellet was observed. The refolded mixture when analyzed on SDS/PAGE showed bands corresponding to both the domains. This indicates that NPfA interacts with CPfA and helps the latter to fold (Fig. 1A).

Molecular mass determination

The molecular weight of refolded NPfA was confirmed by MALDI-TOF mass spectrometry. A single 22.102 kDa peak was observed corresponding to the monomeric molecular weight of NPfA (Fig. S1). The nature of the subunit association in refolded proteins was determined by dynamic light scattering experiments. In the case of NPfA, the size distribution profile in terms of intensity data showed three prominent peaks representing a predominance of large sized particles of polydisperse nature. The intensity profile of co-refolded NPfA + CPfA primarily showed a single peak indicating a monodispersed nature (Fig. 1B,C).

Thermal stability of NPfA

The far-UV CD spectrum of NPfA heated to 90 °C overlapped with the spectrum obtained after cooling to 25 °C, suggesting that NPfA is thermally stable within this temperature range (Fig. 1D). The signature peak at 218 nm in the CD spectrum also indicates the predominance of β-sheet structure in NPfA.

Fig. 1. Molecular weight, subunit association and thermal stability of proteins. (A) SDS/PAGE of samples obtained after refolding: lane 1, molecular weight marker; lane 2, soluble NPfA; lane 3, pellet showing CPfA; lane 4, soluble NPfA + CPfA mixture. (B), (C) Dynamic light scattering data showing size distribution profile of NPfA and co-refolded NPfA + CPfA mixture, respectively. (D) Far-UV CD spectra of unheated (●), heated (■) and cooled (△) NPfA.
Prevention of thermal and refolding-mediated aggregation of substrate proteins by NPfA

Substrate proteins such as BCA II, porcine pancreas \( \alpha \)-amylase and *Escherichia coli* MSG were subjected to aggregation conditions both in the presence and in the absence of NPfA. Aggregation was induced either by heating or by refolding from a chemically denatured state. Each substrate protein when heated alone showed increased scattering indicating increased aggregation. However, aggregation was suppressed substantially when substrate proteins were incubated in the presence of increasing concentrations of NPfA (Fig. 2A–C). The effect was most dramatic for BCA II, where in the presence of a 1 : 1 molar ratio of BCA : NPfA almost complete prevention of aggregation was observed (Fig. 2A, trace 5). In the presence of a 1 : 1 molar ratio of \( \alpha \)-amylase : NPfA, a nearly 2.5-fold reduction in \( \alpha \)-amylase thermal aggregation was achieved (Fig. 2B, trace 4). For MSG, however, a similar fold reduction in aggregation was observed only when the molar ratio of MSG : NPfA was raised to 1 : 8 (Fig. 2C, trace 3). In these cases, a further increase in NPfA concentration did not show any change in the aggregation profile. In the case of refolding-mediated aggregation, a reduction in aggregation was seen in the presence of a narrow molar concentration range of NPfA with respect to substrate. For both BCA II and \( \alpha \)-amylase, a nearly 1.65-fold reduction in aggregation was achieved in the presence of 0.1 and 0.28 \( \mu \)M of NPfA, respectively (Fig. 3A,B). Both above and below this molar concentration of NPfA, increased aggregation was observed.

Determination of surface hydrophobicity

A significant increase in fluorescence intensity was observed when ANS was added to NPfA indicating the presence of many exposed hydrophobic surfaces on NPfA. However, the refolded mixture of NPfA + CPfA showed relatively lower fluorescence suggesting that some of the exposed hydrophobic surfaces of NPfA became masked by CPfA. In the case of a heated BCA II + NPfA mixture, ANS fluorescence was higher than their individual intensities. This indicates that, even after NPfA binding, sufficient hydrophobic patches remain exposed on the surfaces of the mixture contributed by both BCA II and NPfA (Fig. 4A).

Analysis of substrate–NPfA complex formation

Heat treatment of BCA II and \( \alpha \)-amylase resulted in clouding of solutions, indicating aggregation. In the

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**Fig. 2.** Thermal aggregation and its prevention. Light scattering profile during thermal aggregation of (A) BCA II (3 \( \mu \)M) heated at 65 °C in the absence (trace 1) and in the presence of 0.3, 0.6, 0.99 and 3 \( \mu \)M of NPfA (traces 2–5), respectively; (B) \( \alpha \)-amylase (72 \( \mu \)M) heated at 90 °C in the absence (trace 1) and in the presence of 14.4, 24 and 72 \( \mu \)M of NPfA (traces 2–4), respectively; (C) MSG (2 \( \mu \)M) heated at 50 °C in the absence (trace 1) and in the presence of 8 and 16 \( \mu \)M of NPfA (traces 2 and 3), respectively.
presence of NPfA, however, heating caused no change and the solutions remained transparent. Each sample after centrifugation was passed through a gel filtration column and the major peaks were analyzed on SDS/PAGE. Heat-treated transparent solutions of substrate + NPfA showed bands of both the proteins (Fig. 4B). This indicates strong interaction between substrate and NPfA which did not dissociate even after gel filtration.

Co-immunoprecipitation to show the interaction between NPfA and BCA II

Anti-His IgG directed against NPfA resulted in co-immunoprecipitation of BCA II in the pellet fraction of heat-treated BCA II + NPfA mixture. This fraction when analysed on SDS/PAGE revealed the presence of both the proteins. However, BCA II alone in both its native and heat-aggregated form did not immunoprecipitate with anti-His IgG (Fig. 4C).
Inhibition of polyQ-mediated amyloid formation by NPfA

PolyQ started forming aggregates after 21 h of incubation at 37 °C. By 62 h, only 30% of the initial amount of peptide remained in soluble monomeric form. However, a substantial proportion of the sample (~88%) remained in the soluble form when incubated in the presence of NPfA for the same period of time (Fig. 5A). This indicates that polyQ remains protected from undergoing aggregation in the presence of NPfA.

Dissociation of preformed amyloid-β fibrils

Without NPfA, preformed amyloid-β fibrils showed marginal changes in thioflavin T (ThT) fluorescence throughout the incubation period. In the presence of NPfA, the ThT fluorescence reduced dramatically (~3-fold) within 3 h of incubation. ThT fluorescence further decreased with increasing incubation time and after 9 h it was almost negligible indicating disappearance of the ThT-binding population. A control experiment in the presence of BSA resulted in marginal change in ThT fluorescence indicating that dissociation of preformed amyloid-β fibrils is specifically mediated by NPfA (Fig. 5B).

Transmission electron microscopy of amyloidic samples

PolyQ and amyloid-β samples incubated alone (as mentioned above) for the designated period showed fibrillar aggregates. When incubated in the presence of NPfA and observed after 3 days, the aggregates dissociated and appeared as small fragments (Fig. 5C,D).

Discussion

The studies on stability and interactions between the individual NPfA and CPfA resulted in a novel finding. NPfA not only acts as specific internal chaperone by mediating folding of its own C-domain (CPfA), but also functions as a non-specific molecular chaperone by preventing aggregation of unrelated proteins. In multi-domain proteins, isolated domains often display decreased stability compared with the parent protein and each domain gets stabilized preferentially by inter-domain interactions [34]. For example, in human γ-D-crystallin, folding of the isolated unstable N-terminal domain was found to be nucleated by the relatively stable C-terminal domain [35]. The appearance of CPfA in the supernatant fraction upon refolding in the presence of NPfA indicates that the latter might be

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**Fig. 5.** Effect of NPfA on amyloids. (A) Plot of residual percent monomer of polyQ (35 μM) after incubation in the absence (●) or in the presence (●) of an equimolar concentration of NPfA. (B) Time-dependent ThT fluorescence of amyloid-β protein (44 μM) incubated in the absence (●) and in the presence of 4.6 μM NPfA (▲) or in the presence of 4.5 μM BSA (▼). ThT binding by NPfA alone (●) is shown as control. Transmission electron microscope images after 3 days incubation of (C) polyQ amyloid fibrils in the absence (left) or in the presence (right) of NPfA and (D) amyloid-β fibrils in the absence (left) and in the presence (right) of NPfA.
working as nucleating partner, assisting the CPfA to fold into soluble form (Fig. 1A).

While studying the molecular organization of isolated domains, we found that NPfA formed large oligomeric complexes of polydispersed nature (Fig. 1B). Owing to the polydispersion, the number of monomers in the oligomeric assembly could not be determined. An ~5-fold enhancement in the fluorescence of ANS pointed towards the presence of significant surface hydrophobicity of the folded oligomeric NPfA (Fig. 4A). All these characteristics are typical of small molecular chaperones, which generally form large polydispersed oligomeric complexes surrounding the partially denatured substrate proteins through hydrophobic interactions, keeping the latter in a folding-competent state [36]. Small molecular chaperones are also characterized by the presence of a typical ~90 amino acid α-crystallin domain. A BLAST search revealed that NPfA does not contain any such stretch. Proteins lacking an α-crystallin domain have been reported to show chaperoning activity [37, 38]. The sequence of NPfA was individually aligned with sHSPs from Methanocaldococcus janaschii, Hsp16.9 from wheat, Pyrococcus furiosus sHSP, zebrafish α-A crystallin, human α-B crystallin and other non-HSP chaperones such as human clusterin and human β-tubulin. None of these alignments resulted in any significant sequence similarity with NPfA except β-tubulin, where eight amino acids at its C-terminus were found to be identical with the N-terminus of NPfA (within a stretch of 25 amino acids). Barring lack of sequence similarity, all other properties of NPfA matched well with those of small molecular chaperones. This led us to hypothesize that NPfA also functions as a non-specific molecular chaperone.

To test our hypothesis, we carried out in vitro assays where reduction in aggregation of substrate proteins by NPfA was used as a measure of the extent of its chaperoning efficiency. We studied the aggregation of a variety of proteins that exist as monomers, with size ranging from a small, single-domain, globular protein like BCA II (29 kDa) to a relatively large protein α-amylase (55.5 kDa) and the much larger multidomain protein MSG (82 kDa). In each case, reduction of light scattering in the presence of NPfA clearly indicated its chaperoning effect (Figs 2 and 3). The chaperoning efficiency varied depending on the size and nature of the target protein. To obtain similar reduction in aggregation, the requirement of NPfA increased with increase in the size of the substrate protein. For the small protein BCA II, the presence of an equimolar concentration of NPfA completely rescued the former from aggregating, whereas higher concentrations of NPfA were required for α-amylase and MSG. Even at such high concentrations of NPfA, complete prevention of aggregation could not be achieved (Fig. 2A–C). This could be because of the dependence of the chaperoning activity of small molecular chaperone/sHSPs on the mass ratios of participating proteins [23,39]. An alternative explanation could be based on the size of the substrate proteins. With increase in the size of the substrate, the collisional frequency with chaperones vis-à-vis the kinetics of interaction decreases, resulting in reduced chaperoning activity. In a substrate populated environment, the kinetics of interaction amongst partially denatured substrates predominates, causing more aggregation.

The NPfA also facilitated reduction in refolding-mediated aggregation, although at slightly lower efficiency, but significant enough to be noticed (Fig. 3). Recently it has been reported that the chaperoning efficiency of α-crystallin is determined by the differential interaction with aggregation-prone intermediates of different structural stabilities [40]. In our case, the extent of reduction in aggregation was more pronounced in the case of thermal aggregation than refolding-mediated aggregation. This could be because of differential binding affinities of NPfA towards unfolding and refolding intermediates. Such a phenomenon has been observed in the case of α-crystallin [41]. NPfA being thermally stable remains folded at the experimental temperature (Fig. 1D) and binds to thermally denatured aggregation-prone substrates. During the refolding reaction, however, there is a narrow concentration range within which NPfA acts as chaperone (Fig. 3). This is because, at lower NPfA concentrations, there may not be enough NPfA to compete for the available surfaces on partially folded substrate proteins. On the other hand, at higher concentrations, NPfA prefers self-recognition and undergoes homo-oligomerization over hetero-association with substrate proteins.

Efficient prevention of protein aggregation has been achieved by the inclusion of additives like sucrose, glycerol and PEG [42]. These findings were considered to be more of bulk solvent effect rather than direct protein-additive interaction. However, in our case, the co-appearance of both substrate and NPfA bands in the same lane on SDS/PAGE of heat-treated, gel filtration chromatography purified mixtures suggested direct physical interaction between the proteins. This was observed regardless of the type of substrate protein (Fig. 4B). The direct interaction was also evident from studies where heated BCA II co-immunoprecipitated with NPfA-directed antibody (Fig. 4C). The NPfA + CPfA refolded mixture displayed lower ANS fluorescence compared with NPfA alone (Fig. 4A). This
suggests that the exposed hydrophobic surface on NPfA gets involved in interaction with CPfA during refolding. This also reflects the general property of small molecular chaperones that are known to bind substrates through hydrophobic interactions [26,27]. In the case of BCA II aggregation, the presence of NPfA did not lower the ANS fluorescence (Fig. 4A). This may be because of the presence of residual hydrophobic surfaces on both the interacting proteins which bind to ANS.

The predominance of β-sheet structure in folded NPfA (Fig. 1D) fitted well with structural information available for other sHSPs [9,33]. Moreover, some sHSPs are shown to be associated with protein misfolding diseases, such as Huntington’s and Alzheimer’s disease [43,44]. The proteins involved in these diseases, namely polyQ and amyloid-β respectively, form fibrillar amyloidic aggregates. Cross β-sheet structures are the hallmark of such aggregates which propagate by engaging more homologous proteins in the β conformation. Our data showed that NPfA prevents the formation of amyloidic aggregates of polyQ and dissociates the preformed amyloid-β proteins (Fig. 5). In the case of polyQ, this effect is presumably because NPfA mimics the homologous complementary oligomerizing surfaces by extending its own β-sheet scaffold, which engages in heterologous association, thereby preventing amyloid propagation. A possible mechanism by which amyloid-β fibrils are disaggregated by NPfA may be the halting of elongation followed by dissociation, as has been reported in other cases [45,46]. Alternatively, the intermolecular associative forces between NPfA and amyloid-β are possibly stronger than the homomolecular aggregative forces amongst amyloid-β molecules. Irrespective of whether NfA binds to the fibrils at the ends or sidewise, the dominant interaction of NPfA with amyloid-β releases the latter from their fibrillar structure. Binding of NPfA at the ends will gradually decrease fiber length whereas lateral binding will fragment the preformed fibril at different positions. The latter appears to prevail in our case as we could see smaller oligomers in the incubated samples (Fig. 5D, right-hand panel). The exact mechanism of amyloid dissociation is under investigation.

This is a first report where the specific domain of a hyperthermophilic protein has been shown to be endowed with chaperoning activity, while the full-length PfA failed to show this effect. This could be because NPfA is a non-natural product and is an isolated folded polypeptide with its own structural and functional properties very different from its wild-type parent. This finding is of immense physiological relevance as NPfA may be used in heterologous protein expression systems as co-expression partner in improving the solubility of proteins, thereby reducing losses due to inclusion body formation. A thorough investigation of the chaperoning activity of NPfA in a heterologous co-expression system is required. The effect of NPfA on disintegrating amyloid-β proteins is significant as it may be used for therapeutic intervention of protein-aggregation-related diseases.

Materials and methods

Cloning and expression of PfA domains

For cloning the N- and C-terminal domains separately, a previously developed PfA clone was used as template [1]. Primer pairs 5′-CT GCT AGC GTG AAA ATT CTT CTA ATT CTT ATT ATT CTT CTA GTT ATC AAA CTA GAG ATC TTC TC-3′ (280): 14 440, 12 950 and 27 390M and 5′-AG GGA TCC TTA GTT AAC CAC CAC GAG ATC TTC TC-3′ were used for PCR amplification of the DNA sequences corresponding to NPfA while primer pairs 5′-TA GCT AGC GTC CTA GTT ATC ATC AAA CTA ATC TCC C3′ and 5′-GGC GGG ATC CTA ATC TCT AAG CTC TTC C3′ were used for CPfA. The PCR amplified products corresponding to each domain were ligated separately in pET28a vector (Novagen, MA, USA) using the NheI and BamHI (New England Biolab, Hitchin, UK) sites, followed by transformation into E. coli DH5α and subsequently into expression host E. coli Rosetta (DE3). Cultures grown in LB medium (HiMedia, Mumbai, India) containing 50 μg·mL-1 kanamycin and 17 μg·mL-1 chloramphenicol (Sigma, St. Louis, MO, USA), were induced with 1 mM isopropyl-β-d-thiogalactopyranoside (Sigma) (at an A600 of 0.6) and harvested 14 h post induction by centrifugation, followed by sonication. Expression was analyzed by 12% SDS/PAGE.

Purification of domains

For both NPfA and CPfA, the standard Ni-nitrilotriacetic acid (NTA) affinity based purification procedure was followed. Briefly, supernatant of cell lysate obtained after sonication in lysis buffer (10 mM Tris, 100 mM sodium dihydrogen phosphate, 8 M urea, pH 8.0) at 4 °C was loaded on a pre-equilibrated Ni-NTA agarose column (Qiagen, Venlo, Netherlands). After washing, proteins were eluted using lysis buffer, pH 4.5. Fractions containing each domain were pooled and subjected to dialysis against refolding buffer (25 mM Tris, 50 mM NaCl, pH 8.0; for CPfA pH 9.0). Following centrifugation, the pellet and supernatant fractions were checked by 12% SDS/PAGE.

Co-refolding of both the domains from their denatured state was done by dialyzing a mixture of NPfA and CPfA in an equimolar ratio. Protein concentration was determined by UV absorbance at 280 nm using molar extinction coefficients (ε280): 14 440, 12 950 and 27 390 m-1·cm-1 for NPfA, CPfA and an equimolar mixture of NPfA + CPfA, respectively.
Determination of molecular mass

Molecular mass and oligomeric nature were determined by MALDI-TOF mass spectrometry (AB SCIEX TOF-TOF 5800, MA, USA) and by dynamic light scattering with a Zetasizer NanoZS instrument (Malvern, Worcestershire, UK), respectively.

Thermal stability of NPfA

To check the stability of NPfA against temperature, far-UV CD spectra in the range 200–250 nm were recorded both after heating (up to 90 °C) and after cooling (to 25 °C) of the protein sample (0.1 mg·mL⁻¹) on a Peltier-attached JASCO J-815 spectropolarimeter (Tokyo, Japan). Measurement was done in a 0.2-cm path-length quartz cell at a scan speed of 50 nm·min⁻¹. Three spectra were recorded and averaged and finally plotted after subtracting the buffer baseline.

Determination of surface hydrophobicity

The extent of surface hydrophobicity of the proteins as well as the aggregation mixture was monitored by ANS (Sigma) fluorescence. Samples of NPfA (0.22 mg·mL⁻¹), NPfA + CPfA refolded mixture (0.4 mg·mL⁻¹), BCA II (0.3 mg·mL⁻¹) and NPfA + BCA II (0.22 mg·mL⁻¹ + 0.3 mg·mL⁻¹) were made. Each sample was made in respective buffer to which ANS was added from a stock solution (200 μM) to obtain a final concentration of 60 μM. These samples were incubated for 5 min at 25 °C. Fluorescence scans in the range 400–600 nm were recorded for samples taken in a 1-cm path-length cuvette in a Cary eclipse spectrofluorimeter (Agilent, CA, USA) after excitation at 370 nm. Excitation and emission slits were set at 5 nm each.

Effect of NPfA on thermal and refolding-mediated aggregation of substrate proteins

To assess the chaperoning activity, thermal and refolding-mediated aggregation of substrate proteins, BCA II, α-amylase and MSG were measured by monitoring Rayleigh light scattering in the absence and in the presence of increasing concentrations of NPfA. For thermal aggregation, a cuvette containing the sample (3 mL) was inserted into the preheated sample chamber of a spectrofluorimeter fitted with a Peltier-controlled stirred cell and scattering was monitored with time. The excitation and emission monochromators were set at 400 nm. For BCA II, protein at 0.1 mg·mL⁻¹ (3 μM) was heated at 65 °C in 25 mM potassium phosphate buffer, pH 7.6, containing 1 mM dithiothreitol and 1 mM EDTA. For α-amylase, 4 mg·mL⁻¹ (72 μM) protein was heated at 90 °C in 25 mM sodium phosphate buffer, pH 7.2, containing 50 mM NaCl and 1 mM dithiothreitol. For MSG, 0.16 mg·mL⁻¹ (2 μM) protein was heated at 50 °C in 25 mM Tris/HCl buffer, pH 8.0, containing 50 mM NaCl. After heating, the samples were cooled and centrifuged and the supernatants were passed through a 50-fold dilution of the overnight incubated samples in buffer without GdnCl, dithiothreitol or EDTA. To establish interaction between BCA II and NPfA during thermal aggregation, the mixture (1 : 1 molar ratio) was heated, cooled and incubated with anti-His mouse monoclonal antibody (27E8; Fermentas) in 25 mM potassium phosphate buffer, pH 7.6, with 1 mM EDTA, 1 mM dithiothreitol and 50 mM NaCl. A suitable control experiment for each protein was set separately. Following overnight incubation at 4 °C on a rotatory spin mixer, the protein–antibody complex was immobilized on protein A/G plus agarose resins (Santa Cruz, TX, USA) by continuous head to head mixing for 2–3 h. Samples were centrifuged at 1 700 g for 10 min to separate the supernatant and pellet fractions. The immunoprecipitated pellet was washed several times with buffer containing 0.1% Triton X100 (Sigma) and 1 mM protease cocktail (Roche, Basel, Switzerland) to remove non-specifically attached proteins. Precipitated fractions were analyzed by 12% SDS/PAGE.

Co-immunoprecipitation of NPfA–BCA II conjugates

Purified NPfA contained six histidine residues at its N-terminus. To establish interaction between BCA II and NPfA during thermal aggregation, the mixture (1 : 1 molar ratio) was heated, cooled and incubated with anti-His mouse monoclonal antibody (27E8; Fermentas) in 25 mM potassium phosphate buffer, pH 7.6, with 1 mM EDTA, 1 mM dithiothreitol and 50 mM NaCl. A suitable control experiment for each protein was set separately. Following overnight incubation at 4 °C on a rotatory spin mixer, the protein–antibody complex was immobilized on protein A/G plus agarose resins (Santa Cruz, TX, USA) by continuous head to head mixing for 2–3 h. Samples were centrifuged at 1 700 g for 10 min to separate the supernatant and pellet fractions. The immunoprecipitated pellet was washed several times with buffer containing 0.1% Triton X100 (Sigma) and 1 mM protease cocktail (Roche, Basel, Switzerland) to remove non-specifically attached proteins. Precipitated fractions were analyzed by 12% SDS/PAGE.

Reverse-phase HPLC based sedimentation assay for monitoring polyQ-mediated aggregation

Purified and lyophilized polyQ peptide (PGQ9) dissolved in 1,1,1,3,3,3- hexafluoro-2-propanol and trifluoroacetic acid (Merck, Darmstadt, Germany) at a 1 : 1 ratio was incubated overnight at room temperature for disaggregation [47]. Solvent was evaporated to form a thin film by nitrogen flushing and was vacuum dried for 2 h in a desiccator.
The dried peptide was then dissolved in 800 μL water/trifluoroacetic acid solvent (pH 3.0) and ultracentrifuged (Thermo Fisher Scientific, MA, USA) for 4 h at 140 000 g to remove traces of micro aggregates. The concentration of soluble monomeric peptide in the supernatant was calculated from the peak area of elution obtained by analytical reverse-phase HPLC C18 column chromatography (Agilent 1260 Infinity). For the aggregation reaction 35 μM soluble peptide with or without NPfA (35 μM) was incubated at 37 °C in NaCl/Pi buffer, pH 7.4, with 100 mM NaCl. NPfA alone incubated identically served as control. Samples taken at different time intervals were ultracentrifuged at 50 000 g for 30 min at 37 °C. The supernatant was carefully withdrawn and the aggregation reaction was stopped by adding 20% formic acid before analysis by HPLC. Using Agilent in-built software, the peak area of polyQ was calculated by integration and finally by comparing with the peak area from the standard curve. The amounts of soluble polyQ left at different time points were obtained in micrograms.

**Effect of NPfA on amyloid-β fibrils**

Amyloid-β (1–42) peptide (rPeptide, USA) was solubilized by adding dimethylsulfoxide (Sigma) and sonicated extensively before setting aggregation reactions. For amyloid formation amyloid-β at 0.2 mg·mL⁻¹ (44 μM) in 50 mM sodium phosphate buffer, pH 7.4, with 100 mM NaCl was incubated at 37 °C with vigorous shaking for 24 h. For detecting amyloid formation, aliquots (10 μL) were taken at different time points and added to 50 mM glycine/NaOH buffer, pH 8.3, containing 25 μM ThT. The fluorescence intensity at 489 nm was recorded for the samples using a Perkin Elmer fluorimeter (USA) to monitor the aggregation kinetics. Saturation of ThT fluorescence confirmed the formation of amyloids. NPfA at 0.1 mg·mL⁻¹ (4.6 μM) was added to these preformed amyloids and ThT fluorescence was monitored with time. Identical reactions were set up in the absence of BSA (Sigma) at 0.3 mg·mL⁻¹ as control.

**Transmission electron microscopy of amyloïdic samples**

Following incubation, as mentioned in the previous section, samples of both amyloid-β and polyQ in the absence or presence of NPfA were adsorbed on copper grids, air dried and negatively stained with 1% uranyl acetate. Samples were observed with a transmission electron microscope (Philips CM12) operated at 100 kV.

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**References**


l-asparaginase domain with chaperoning activity

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s website:

Fig. S1. Molecular weight determination by mass spectrometry. Molecular weight of purified refolded NPfA showing a monomeric single peak of 22 kDa.