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Length of polyglutamine tract affects secondary and tertiary structures of huntingtin protein

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ABSTRACT

The role of polyglutamine (polyQ) tract on protein stability and disease pathology remains ambiguous. We monitored the unfolding/refolding patterns of huntingtin proteins with varying polyQ lengths. In the presence of urea, minor differences in unfolding and refolding efficiencies were observed. However, in the presence of guanidinium hydrochloride, the protein with a longer polyQ stretch was able to regain its secondary but not tertiary structure on step-wise removal of denaturant. Thus, in case of Huntington's disease, the higher aggregation propensity of the mutant protein is likely to be due to the lower stability of the protein due to elongated polyQ tract.

1. Introduction

Polyglutamine (polyQ) diseases belong to the class of protein misfolding diseases in which the homopolymeric stretch of glutamine residues in a protein undergoes elongation, resulting in neurodegenerative conditions like spinocerebellar ataxias, Huntington's disease, etc. In these pathological conditions, an endogenous protein like ataxin or huntingtin undergoes mutation leading to an increased length of the polyQ stretch. This confers the protein with deleterious functions. Proteins with extended polyQ stretches form intracytoplasmic or intranuclear aggregates. Whether the formation of aggregates is a defence mechanism of the cell or if it is responsible for cellular toxicity, has been a matter of debate [1–4].

The role of the polyQ tract in deciding the stability of such a protein is crucial. Chow et al. had compared the stability of ataxins containing normal (15Q)- and elongated (78Q)-length polyQ stretches [5]. They reported that the presence of the longer tract led to instability and promoted the aggregation of the ataxin variant with the longer polyQ (78Q) stretch. The less stable ataxin-78Q unfolded readily to form an aggregation-prone intermediate, which then formed the characteristic fibrillar aggregates [5]. Destabilization of the native state in the presence of an elongated polyQ stretch in ataxin has also been reported by others [6]. A later, more comprehensive study by the earlier group led to the proposal of an alternate pathway of ataxin folding [7]. According to this model, the elongated polyQ stretch in ataxin has no effect on its stability. Instead, the longer stretch lowers the energy barrier for the partial unfolding of the protein, leading to faster fibrillation. In case of normal ataxin (Q15), this energy barrier is very high and transition from native to partially unfolded intermediate states is thermodynamically unfavourable and does not occur [7]. The proteins involved in polyQ diseases have no structural or conformational similarity with each other except for the polyQ stretch. Also, as the above analysis shows, the role of polyQ stretch in deciding the (in)stability of even a single protein, has remained ambiguous.

In case of huntingtin (htt), the aggregation propensity of the protein has been shown to be strongly dependent on the length of the polyQ region [8,9]. However, no study has been carried out correlating the length of the polyQ tract with the folding/unfolding behaviour of the protein. The large size of huntingtin (~350 kDa) makes it a difficult-to-express and -characterize protein. Most of the studies have therefore been carried out with the N-terminal fragment where the polyQ stretch is located, conjugated to different tags for ease of expression, purification and visualization [8–12]. Fusion with glutathione-S-transferase (GST) has been used successfully in studies with huntingtin [8,10–12] and ataxin [13,14]. Masino et al. have shown that the GST tag is useful in deciphering the structural information about the polyQ tract using NMR and far-UV CD spectroscopies [11]. Measurement of correlation times from ^{1}H–^{15}N relaxation and chemical shifts of Q resonances have confirmed that normal and elongated polyQ stretches remain in a random coil conformation in the fusion proteins [11], indicating that the presence of GST has no effect on the folding behaviour

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of the polyQ stretch in either protein. Hence, we have used GST tagged HD20Q (N-terminal fragment of huntingtin containing 20 glutamine residues) and GST tagged HD51Q (N-terminal fragment of huntingtin containing 51 glutamine residues) for carrying out the comparative stability studies of these two proteins.

2. Methods

2.1. Expression and purification of GST-HD20Q and GST-HD51Q

Plasmid vectors for the expression of GST-tagged normal (pGEX-5X1-HDexon1-CAG20) and mutant (pGEX-5X1-HDexon1-CAG51) fragments of huntingtin protein were received as gifts from Prof. E. Wanker (Max-Delbrück-Centrum für Molekulare Medizin (MDC), Berlin, Germany). Details of the plasmids have been described earlier [8,10]. *Escherichia coli* BL21 (DE3) cells were transformed separately with pGEX-5X1-HDex1CAG20 (coding for GST-HD20Q) and pGEX-5X1-HDex1CAG51 (coding for GST-HD51Q) plasmids by the $CaCl_2$ method [15]. Expression and purification of the proteins were carried out as described earlier [8]. The concentration of protein in different fractions was determined by the dye binding method [16] using bovine serum albumin as the standard protein. Samples were centrifuged at 1,00,000 × *g* for 45 min at 4 °C to remove any preformed aggregates before carrying out the studies.

2.2. Chaotrope-induced unfolding and refolding of HD20Q and HD51Q

GST-tagged fusion proteins (1 μM each) were denatured in the presence of different concentrations of urea (0–8 M) and guanidinium HCl (0–6 M) for 6 h at 25 °C. For refolding experiments, GST-tagged fusion proteins (8 μM each) were denatured in the presence of 8 M urea and 6 M guanidinium HCl for 6 h at 25 °C. The protein samples were then diluted step-wise with phosphate buffer (137 mM NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4), pH 7.4. Changes in secondary and tertiary structures were monitored using different spectroscopic techniques. Spectra of appropriate controls, with the same concentration of protein as at each stage of dilution but without any chaotrope, were also recorded.

2.3. Far-UV CD spectroscopy

Far-UV CD spectra of the samples were recorded in the wavelength range of 200–250 nm. Control spectra of buffer containing appropriate concentration of denaturant were subtracted in each case. Bandwidth was kept at 1 nm. Protein concentrations were kept equal within a set of measurements. Pathlength of the cuvette was 0.1 cm.

2.4. Intrinsic fluorescence spectroscopy

Intrinsic fluorescence spectra of the samples were recorded in the wavelength range of 300–400 nm after excitation at 290 nm. Control spectra of buffer containing appropriate concentration of denaturant were subtracted in each case. Protein concentration was the same in all cases. Excitation and emission slitwidths were 5 nm each. The scanning speed was 8 nm/s. Protein concentrations were kept equal within a set of measurements.

3. Results and discussion

HD20Q and HD51Q were purified by affinity chromatography. The pattern of purification seen for both proteins (Fig. S1A–C) was the same as has been reported in the literature [8,10]. Significant differences between the secondary structures of the two proteins, as determined by far UV CD spectroscopy, were observed (Fig. S1D). The α-helix and β-sheet contents of the elongated length htt fragment (HD51Q) were found to be 26% and 16%, respectively, as compared to the corresponding values of 14% and 31% for HD20Q. The fractions of random coil in the structure were 54% and 58% for HD20Q and HD51Q, respectively. The large value of randomness in both structures matches well with what has been reported earlier [11]. Significant differences in secondary structures have also been reported in case of ataxin variants with 27Q and 78Q tracts [6]. The increasing value of α-helicity with increasing polyQ stretch is consistent with earlier observations with thioredoxin–polyQ systems [17]. In this hypothesis, transition from α-helical to β-sheet conformer in the elongated polyQ-tract containing protein preceded the formation of amyloid fibrils. X-ray crystallographic data with MBP–polyQ fusion proteins have shown that the N-terminal stretch of the protein is a helix-rich region while the order is lost in the polyQ tract [18]. The far UV-CD spectrum of GST alone was typical of that expected of α/β-proteins with predominantly α-helical content (Fig. S1D), and matches with what is reported in the literature [11]. The elongated length htt formed fibrillar structure on prolonged incubation (Fig. S1E). GST-tagged proteins have been used in this case since the fusion tag has been reported to keep HD51Q in solution for a longer time [8]. With longer lengths of polyQ stretches, the htt fragments aggregate almost immediately on purification, even in the presence of GST tag [8]. The length of the polyQ stretch has an effect on the aggregation propensity of the protein [8,9], most likely resulting from a conformational change in the protein due to the variable polyQ tract. The presence of the polyQ tract, whether 20Q or 51Q, does not alter the structure of GST since the binding of GST-tagged proteins to the glutathione-agarose matrix remained unaffected. Proteins with fusion tags have often been used in stability studies where the presence of the tag has no effect on the properties of the protein under study or vice versa [3,6].

3.1. Equilibrium denaturation studies

In order to determine if varying stretches of polyQ length resulted in differences in the stability of proteins, we carried out equilibrium denaturation studies of the two proteins. Following incubation with urea and GdHCl, decrease in θ_{222} values was observed in case of both HD20Q and HD51Q (Fig. 1a). θ_{222} is a measure of the α-helicity of a protein. The concentration of the denaturant at which 50% of the protein is denatured (C_m) was calculated to be 4.2 M for HD20Q and 5.2 M for HD51Q. Thus, a marginally higher concentration of urea is required to destabilize mutant htt as compared to the normal protein, introducing a higher degree of rigidity in the protein due to the elongated polyQ stretch. Far UV–CD spectra of guanidinium HCl-denatured samples were recorded at different concentrations of the denaturant. A decrease in the θ_{222} values with increase in the concentration of guanidinium HCl was observed with both HD20Q and HD51Q (Fig. 1b). With increase in the concentration of guanidinium HCl, the structure became more disordered, as reflected by decreasing values of θ_{222}. However, in the presence of the stronger denaturant, viz. guanidinium HCl [19], there was no significant difference between the denaturation patterns of the two proteins. The slightly higher C_m value of HD51Q observed in the presence of urea thus represents only marginally higher stability of the huntingtin fragment with elongated polyQ stretch.

For monitoring changes in the tertiary structures of the two protein fragments following denaturation by chaotropes, protein samples were incubated for 6 h with different concentrations of urea and analysed by intrinsic fluorescence spectroscopy. Intrinsic fluorescence intensity of urea-denatured HD20Q decreased with