SHORT COMMUNICATION

The role of tryptophan in protein fibrillogenesis: relevance of Trp7 and Trp14 to the amyloidogenic properties of myoglobin

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In order to understand the role of tryptophan in the mechanisms of fibrils formation, the ability of a series of analogs of the residue 7–18 span of myoglobin to form amyloid-like fibrils was investigated. Alternatively one or both tryptophans were substituted with alanine and leucine, to determine the contribution of hydrophobicity and aromaticity. The scale of aggregation propensity of the peptides determined indicates that tryptophan is crucial for the amyloidogenic process. Since the rare tryptophan residue is generally engaged in structural roles in proteins, or when exposed serves as binding sites, we surmise that its exposure in the amyloidogenic fragments allows for intermolecular clustering with residues from other molecules leading to the formation of amyloid aggregates.

Keywords: fibrils/peptide synthesis/protein fragments/tryptophan

Amyloid fibril formation is a common feature of a variety of unrelated diseases. A general view is that fibrils can be formed by a large variety of proteins and peptides under suitable experimental conditions; the ability to form amyloid fibrils appears to be a general property of the polypeptide chain (Fändrich et al., 2001; Dobson, 2003). Despite numerous studies, the role of specific amino acid residues and sequences of residues that trigger fibrils formation are still not well defined. Short peptides have been frequently used to identify specific polypeptide sequences, which are responsible for protein aggregation and amyloid formation. Several factors that positively affect the aggregation propensity of a protein have been defined, such as β-sheet propensity, hydrophobicity and net charge (Chiti et al., 2003). Among these, it has been shown that hydrophobicity plays a key role. Indeed, statistical and experimental studies on protein sequences have revealed that stretches of hydrophobic residues rarely occur, suggesting that they have been negatively selected during protein evolution (Schwartz and King, 2006; Monsellier et al., 2007). Interactions between aromatic groups seem to be critical, in particular those involving phenylalanine (Phe) residues, able to establish π–π stacking in amyloid fibrils (Azriel and Gazit, 2001) and these are frequently observed in amyloid-forming proteins (Gazit, 2002). On the other hand, the role of tryptophan (Trp) has not been extensively tackled. Trp has the largest surface area and it is a highly preferred component of residue clusters in protein structure (Heringa and Argos, 1991). Although relatively rare, Trp often has a unique role in folded protein structures and appears at the active binding sites of many proteins (Samanta et al., 2000). In native proteins, Trp is mostly involved in long-range interactions, highlighting the importance of this residue in the formation of a tertiary structure (Eidenschink et al., 2009).

In the present work, to understand the poorly studied role of Trp residues in the amyloidogenic properties of a protein, we have analyzed several peptides, derived from the residue 7–18 span of the 153-residue chain of myoglobin (Fig. 1). We had previously found that the N-terminal residue 1–29 fragment of myoglobin, obtained by proteolysis, is very prone to aggregate and that the modulation of its net charge by changing the pH of the solution can be crucial for fibrils formation (Picotti et al., 2007). Moreover, the residue 7–18 span was identified as the aggregation-promoting sequence. This region contains the two Trp residues (positions 7 and 14) of the protein, which are highly conserved in mammalian myoglobins. The smaller peptides studied herein were obtained by solid-phase synthesis, purified by reverse phase-high performance liquid chromatography and characterized for their ability to form fibrils. The Trp residues were substituted with alanine (Ala), which typically does not alter main-chain conformations and does not impose electrostatic or steric effects (Cunningham and Wells, 1989), leading to peptides denoted WA, AW and AA (Fig. 1). To establish whether the aromatic character of the region is essential for triggering the aggregation or if hydrophobicity alone is sufficient to favor self-assembly, a peptide (LL) containing two leucines (Leu) instead of Trp was also prepared (Fig. 1). Moreover, two serine (Ser) residues were added at the N- and C-terminal of the peptides to increase their solubility. All the peptides have the same length and a net charge value of 0 under neutral pH conditions and +1 at pH 2.0.

The secondary structure of the peptides was evaluated by circular dichroism (CD) (Fig. 1A). At pH 2.0, as well as at
At pH 7.5 (not shown), the peptides are unfolded, as indicated by the pronounced minimum at \( \approx 200 \text{ nm} \) evident in the CD spectra (Fig. 1A). In order to induce the formation of fibrils, peptides solutions (1 mg/ml in 10 mM HCl, pH 2.0) were incubated for 24 h at room temperature and analyzed by CD, Fourier transform infrared (FTIR) spectroscopy (Fig. 1B and C) and transmission electron microscopy (TEM) (Fig. 2A). The shape of the CD spectrum of wild-type peptide WW is modified upon incubation and shows an intense positive band at 193 nm and negative intensity (at 217 nm) as expected for a \( \beta \) structure, but the minimum of the negative band (at 227 nm) is anomalous. This signal could be ascribed to the superimposition of contributions of Trp residues that present an absorption band near to 224 nm (Manning and Woody, 1989), to the perturbation of the subunit packing during aggregation (Arnold et al., 1992), or to the low-energy component of a Trp/Trp exciton coupl (Andersen et al., 2006). The Trp-containing peptides WA and AW also show an increase in \( \beta \)-sheet structure, the minimum at 217 nm and the maximum at \( \approx 200 \text{ nm} \) in the CD spectra (Fig. 1B, long-dashed and dotted-dashed lines), with the 227 nm band entirely absent. Peptides LL and AA are still unfolded upon 1-h incubation since their CD spectra are not changed (Fig. 1B, dotted and short dashed lines).

FTIR measurements after 24 h of aggregation were performed to analyze the type and contribute of secondary structure of the peptides and, specifically, to evaluate the increase in cross \( \beta \)-sheet structure upon fibrils formation. The FTIR spectrum of peptide WW shows a maximum centered at 1620 cm\(^{-1}\), corresponding to cross-\( \beta \) structure (Fabian et al., 1993; Zandomeneghi et al., 2004) (Fig. 1C, continuous line). The peptides WA and AW show more complex FTIR spectra, indicating the presence of different types of structures (long-dashed and dotted-dashed lines, respectively). The spectrum of WA peptide presents a main band centered at 1622 cm\(^{-1}\), corresponding to cross-\( \beta \) structure, and a less intense one at 1660 cm\(^{-1}\), indicative of turns and/or coil structures. The FTIR spectrum of the peptide AW shows the cross-\( \beta \) sheet band at 1618 cm\(^{-1}\), coupled to a band at 1684 cm\(^{-1}\), which is characteristic of antiparallel aggregated \( \beta \)-sheet. The spectra of peptides AA and LL show a main peak at 1640–1644 cm\(^{-1}\), corresponding to mostly random...
structure. In the case of LL there is a low-intensity band at 1618 cm\(^{-1}\), indicative of the formation of a smaller amount of cross-\(\beta\) structure, if compared to that of peptides WW, WA and AW.

The morphology of the aggregates formed by the distinct peptides was analyzed by TEM. Filamentous structures with amyloid-like appearance were observed for all peptides (Fig. 2A). They are long, unbranched and laterally aligned, with diameters spanning from 7 to 10 nm. In the case of peptides AA and LL, the TEM pictures show that the frequency of appearance of the fibrils is significantly lower when compared with the wild-type species. The peptide AW shows an intermediate behavior, since the fibrils are fewer than those formed by the peptide WW but more than those formed by peptides LL and AA. The quantity of the fibrils formed by each peptide was estimated by measuring the concentration of the peptides in the supernatants obtained after ultra-centrifugation of the mixture after 24 h of incubation. Ultracentrifugation was performed at 4°C for 1.5 h at 90 000 r.p.m. (380 000 g). The white bars refer to the percentage of cross-\(\beta\) structure indicated by the FTIR spectrum (see Fig. 1C) formed by each peptide as deduced by curve fitting and calculated by summing the percent contribution of the bands at 1620 and 1685 cm\(^{-1}\).

The main finding of this work is that Trp residues play a fundamental role in promoting the aggregation of the myoglobin peptides. It is likely that Trp plays a key role in triggering the aggregation process, due to its inherent tendency to establish interactions. In the natively folded myoglobin, Trp7 and 14 are located in the A helix of the protein, their side chains are oriented on the same side and are involved in long-range contacts with neighboring residues (Tcherkasskaya et al., 2000). In the isolated peptides described here, in the absence of other interactions, these residues turn out to be exposed and, at the same time, are likely to look for a stabilizing contact in intermolecular

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**Fig. 2.** Morphology of the aggregates formed by the peptides incubated in 10 mM HCl pH 2.0 for 24 h visualized by TEM (A). A drop of the peptide solution (0.2 mg/ml) was placed on a Butvar-coated copper grid (400-square mesh) (TAAB-Laboratories Equipment Ltd, Berks, UK) and dried. The samples were negatively stained with a drop of 1% (w/v) uranyl acetate solution and observed with a Tecnai G2 12 Twin transmission electron microscope (FEI Company, Hillsboro, OR, USA), operating at an excitation voltage of 100 kV. Scale bars correspond to 100 nm. (B) The percentage of peptide represented by the precipitated fibril pellet (black bars); estimated by measuring the concentration of the peptides in the supernatant after ultracentrifugation of the mixture after 24 h of incubation. Ultracentrifugation was performed at 4°C for 1.5 h at 90 000 r.p.m. (380 000 g). The white bars refer to the percentage of cross-\(\beta\) structure indicated by the FTIR spectrum (see Fig. 1C) formed by each peptide as deduced by curve fitting and calculated by summing the percent contribution of the bands at 1620 and 1685 cm\(^{-1}\).
interactions. In general, under conditions that favor protein folding, the hydrophobic amino acids, such as Trp, are sequestered in the interior of the protein or are located at interfaces between two protein domains. These types of residues are not in contact with the aqueous solvent, because their non-polar side chains are insoluble in water (Gerstman and Chapagain, 2005). In this way, the native fold plays a protective role hiding in the interior regions or specific residues prone to aggregation (Routledge et al., 2009). In an unfolded protein chain or in a peptide, this protective effect runs out and the exposure of Trp residues can lead to aberrant and non-native interactions, such as amyloid aggregation.

Interestingly, the position of Trp along the sequence of the peptides seems to affect the extent of aggregation (WA > AW). Both peptides WA and AW contain five hydrophobic residues. However, in WA, these are distributed along the entire sequence, whereas in AW there are four consecutive non-hydrophobic residues clustered in the N-terminal portion of the peptide. Patterns of alternating hydrophilic and hydrophobic residues, which favor β-sheet formation, have been shown to be less frequent in natural proteins and this has suggested that evolutionary selection has reduced the probability of occurrence of this sequence (Broome and Hecht, 2000). This hypothesis is supported by the observation that peptides corresponding to the N-terminal region of myoglobin with the same composition but with scrambled sequences present different behaviors in terms of kinetic of aggregation (Monsellier et al., 2007).

In an unfolded polypeptide chain the properties inherent in its sequence are determinant factors in driving the aggregation process (Jahn and Radford, 2008). The amino acid substitutions in regions of the sequence crucial for the aggregation of a protein can modulate its aggregation potential (Otzen et al., 2000; Calamai et al., 2003; Chiti and Dobson, 2006). The importance of aromatic moieties in amyloidogenesis has been demonstrated for many peptides (Westmark, 1992; Kayed et al., 1999; Azriel and Gazit, 2001; Gazit, 2002; Naito et al., 2004; Makin et al., 2005) and the occurrence of aromatic residues is very high in peptides or fragments of proteins involved in fibrillogenesis. Actually, the most frequently involved residue is Phe, because of the restricted geometry of interaction between planar aromatic systems (Gazit, 2002). Aggregation studies on short sequences containing aromatic residues revealed that Phe residues could act as structural elements that direct the self-assembly, via π-stacking (Gazit, 2002; Makin et al., 2005). However, other authors reported that aromatic residues affect aggregation only because of their hydrophobicity and propensity to form a β-sheet structure (Tracz et al., 2004; Bemporad et al., 2006). Despite its hydrophobicity, Trp possesses a moderate β-sheet propensity when compared with the other two aromatic residues Phe and Tyr (Street and Mayo, 1999) and generally has a distinct structural or functional role in folded proteins. For example, in mammalian myoglobins, Trp7 and 14 are highly conserved. They are involved in van der Waals contacts with neighboring residues within a surrounding sphere of 5 Å (Tcherkasskaya et al., 2000). The substitution of these residues results in a dramatic effect on the structural properties of myoglobin. The apomyoglobin mutant W7FW14F, in which the two Trp are substituted with Phe, forms amyloid like fibrils at physiological pH (Sirangelo et al., 2002). The difficulty of this apomyoglobin mutant to reach its native folding is due to the fact that the Phe residues are not able to maintain the same long-range interactions as Trp does. Dyson et al. (2006) have shown that Trp14 and helices A, G and H are involved in the folding initiation of apoMb. A double mutant was prepared, in which Trp14 was replaced with Gly and Gly73 in the E helix, which is supposed to interact with Trp14, was replaced with Trp. During the folding process of this protein, the intermediate contains helices E, G and H, instead of A, G, H, as in the folding of apoMb, and the A helix folds later. Therefore, it is clear that the recognition of specific key residues and the hydrophobic interaction largely contribute to the stability of the initial folding unit that drives the folding of the remaining part of the polypeptide chain. Another example is the case of hen egg white lysozyme, where extensive clusters of hydrophobic structure exist within the protein even under strongly denaturing conditions. These clusters are disrupted by a single mutation (W62G) located at the interface between the two major structural domains in the native state, demonstrating that the formation of the native-like structure from the denatured protein is assured by W62 through nonnative and long-range interactions (Klein-Seetharaman et al., 2002).

In summary, the results presented in this work show that tryptophan plays an important role in favoring the aggregation of apomyoglobin peptides, not only because of its hydrophobic and aromatic character, but mainly for its tendency to establish interactions with other residues of the polypeptide chain. Hence, in the protein model herewith studied the abundance of Trp residues, together with the clustering of hydrophobic residues (Schwartz and King, 2006; Monsellier et al., 2007), are key determinants for misfolding and protein aggregation. Indeed, this observation may be in keeping with the fact that Trp is the least frequent amino acid in the human proteome (Cagney et al., 2003).

References

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Amyloid fibrils from myoglobin fragments