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Amyloid fibril formation by a helical cytochrome

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Abstract The substitution of alanines for the two cysteines which form thioether linkages to the haem group in cytochrome c_{552} from *Hydogenobacter thermophilus* destabilises the native protein fold. The *holo* form of this variant slowly converts into a partially folded *apo* state that over prolonged periods of time aggregates into fibrillar structures. Characterisation of these structures by electron microscopy and thioflavin-T binding assays shows that they are amyloid fibrils. The data demonstrate that when the native state of this cytochrome is destabilised by loss of haem, even this highly α-helical protein can form β-sheet structures of the type most commonly associated with protein deposition diseases.

Key words: Cytochrome c_{552}; Amyloid fibril; Circular dichroism; Electron microscopy

1. Introduction

The *c*-type cytochromes are electron-transfer proteins that contain a haem prosthetic group which is covalently attached through thioether linkages to two cysteine residues that are in a CXXCH motif in the protein [1]. We have been studying a variant of cytochrome c_{552} from *Hydogenobacter thermophilus*. The wild-type form of this protein adopts the helical class 1 cytochrome fold containing four α-helices but no β-sheet structure [2]. In the variant we have been characterising the two cysteine residues involved in covalent linkages to the haem (Cys 11 and Cys 14) that are replaced with alanine residues. This modification generates a *b*-type cytochrome with a non-covalently bound haem [3], a change that significantly reduces the stability of the protein. The folded structure of the *holo* state of wild-type cytochrome c_{552} is stable to temperatures in excess of 100°C reflecting the fact that *H. thermophilus* is a thermophilic bacterium which grows optimally at 70°C [3]. In contrast, the *b*-type variant has a significantly reduced stability with a melting temperature (T_m) of 58°C for the oxidised form [3]. Here we report the biophysical characterisation of structural changes to the protein which are consequent on this reduced stability and occur in samples of the *b*-type variant of cytochrome c_{552} over a period of time. The results give insight into the range of conformations which are potentially accessible to protein molecules and identify a possible explanation for the covalent nature of the haem group ligation in *c*-type cytochromes.

2. Materials and methods

2.1. Circular dichroism (CD) measurement

Spectra were recorded on a Jasco J-720 spectropolarimeter at room temperature using a cell with a 0.1 mm optical pathlength. The samples contained 0.25 mM C11A/C14A cytochrome c_{552} in 20 mM phosphate buffer at pH 7.3. The reported spectra are the average of two scans; solvent spectra were subtracted from the measured spectra of the protein samples.

2.2. Electron microscopy

Aliquots of a 0.37 mM C11A/C14A cytochrome c_{552} sample were applied to formvar-coated grids and negatively stained with a solution of 2% (w/v) uranyl acetate in water. The samples were then washed, air-dried and examined in a JEOL JEM1010 transmission electron microscope operating at an accelerating voltage of 80 kV.

2.3. Thioflavin-T binding assay

Samples contained a 10 μl aliquot of C11A/C14A cytochrome c_{552} fibrils stained with 0.5% thioflavin-T on paraformaldehyde-fixed sections. Each sample was air-dried, mounted with PVA and then viewed under UV light using a fluorescence microscope.

3. Results and discussion

The *holo* form of C11A/C14A cytochrome c_{552} retains a large amount of the helical secondary structure present in the wild-type protein [3]. This is illustrated by the CD spectrum shown in Fig. 1 (solid line) which has minima at 207 and 222 nm. Formation of the *apo* protein by complete removal of the haem from C11A/C14A cytochrome c_{552} results in a substantial change to the structure of the protein. The far UV CD spectrum (Fig. 1, dotted line) displays a significant decrease in the signal strength of the 222 nm minimum, accompanied by a small shift of the band at 207 nm towards lower wavelengths. A large increase in random coil structure (characterised by a single minimum below 200 nm) can account for the shift of the 207 nm band towards lower values. The loss of intensity of the 222 nm band indicates that this increase is accompanied by a considerable reduction in α-helical structure. The helical content (calculated using the method of Chen et al. [4]) decreases from 43% in the *holo* state to approximately 14% in the *apo* form. Similar behaviour has been observed for other

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Abbreviations: CD, circular dichroism

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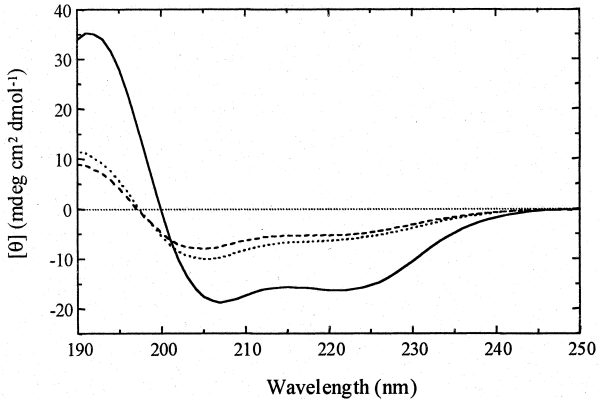

Fig. 1. CD spectra of C11A/C14A cytochrome c_{552} in the *holo* state recorded for a freshly prepared sample (solid line) and a sample after a period of 22 days in solution (dashed line). The dotted line shows the CD spectrum of the protein in the *apo* state after complete removal of the haem group. C11A/C14A cytochrome c_{552} was prepared as reported in [3].

cytochromes following removal of the haem group [5–9]. Furthermore there is evidence that horse *apo* cytochrome *c*, in the presence of high concentrations of salts at both low and neutral pH, forms a non-native collapsed or molten globule state with a content of helical secondary structure significantly attenuated from that of the *holo* protein [7,8].

During the course of characterising further the structural properties of *holo* C11A/C14A cytochrome c_{552} we identified some unexpected changes to our samples over long periods of time. The dashed line in Fig. 1 shows the CD spectrum of a sample of the *holo* protein that was left at room temperature for 22 days. The positions of the minima in the spectrum and their intensities are almost identical to those observed for the *apo* state of C11A/C14A cytochrome c_{552} (dotted line). Over time, therefore, in this variant where the prosthetic group is not covalently attached to the protein, the haem group dissociates from the interior of the protein and is precipitated. Thus the *holo* protein gradually converts to the partially folded *apo* state.

In addition, over a longer time period some precipitation of the protein as well as the haem in the samples was observed. Further characterisation revealed that fibrillar structures were forming in the solution of these protein samples. Fig. 2A shows an electron micrograph of a sample of 0.37 mM C11A/C14A cytochrome c_{552} solution at pH 7.3 after 8 weeks of incubation at room temperature. The solution contains structures which are long, smooth and unbranched with a diameter of ca. 6–13 nm typical of amyloid fibrils [10]. The binding of thioflavin-T to the fibrils, one diagnostic of amyloid structures, was probed by using fluorescence microscopy. The areas rich in fibrous material were found to be stained with the dye and to give a bright yellow fluorescence against a dark green background, (Fig. 2B) the observation of bright patches confirming the amyloid character of the aggregated material [11].

Overall, therefore, in cytochrome c_{552} mutation of the two cysteine residues, which bind to the haem group in the native protein, to alanines allows the haem group to dissociate from the protein, resulting in a loss of the cooperative native structure. Such a loss will lead to at least a partial unfolding of the protein, giving rise to an increased exposure of the polypeptide backbone and an enhanced propensity to aggregate [10]. For C11A/C14A cytochrome c_{552} this aggregation was found to lead to the formation of amyloid fibrils of the type commonly associated with pathological conditions [12–14]. This behaviour is of particular interest as cytochrome c_{552} has a helical native structure while such fibrils are predominantly β-sheet [15]. Furthermore, secondary structure predictions for the cytochrome c_{552} sequence show that the protein has a very low β-propensity (with the profile network method [16] 47.5% α-helix and 3.8% β-sheet is predicted). In this case at least, therefore, the secondary structure propensity of the protein sequence is not a key factor in the ability of the protein to form amyloid fibrils.

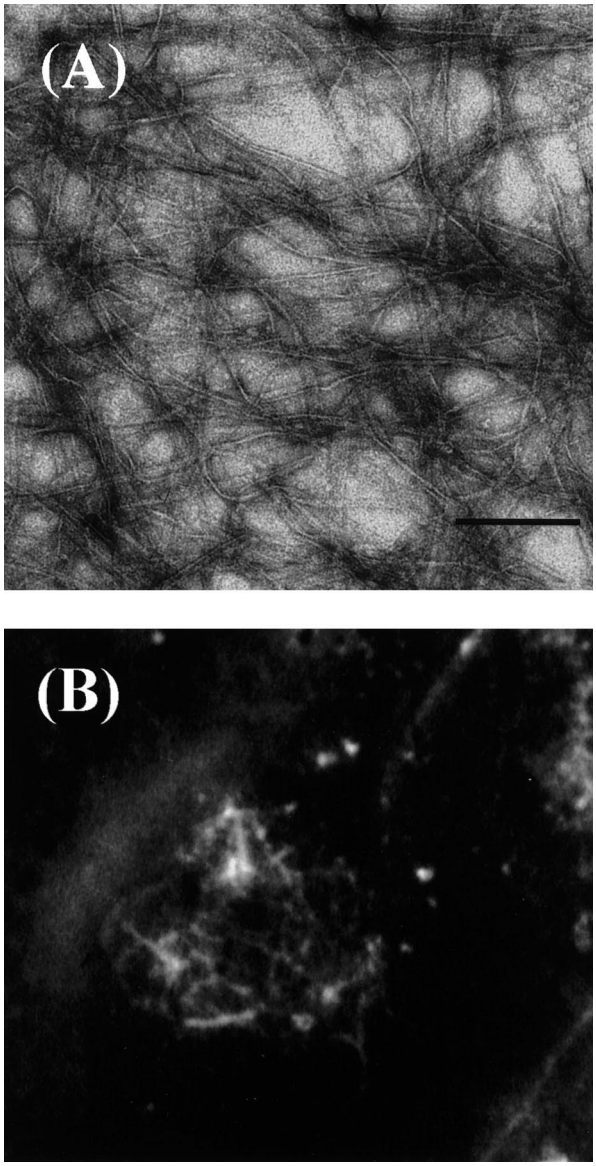

Fig. 2. Characteristics of the fibrils formed by C11A/C14A cytochrome c_{552}. (A) shows an electron micrograph of a negatively stained preparation of C11A/C14A cytochrome c_{552} at pH 7.3 after 8 weeks of incubation at room temperature. The diameter of the fibrils shown is ca. 6–13 nm (bar = 200 nm). (B) shows a thioflavin-T binding assay to the fibrils formed in C11A/C14A cytochrome c_{552} solution. The areas rich in the fibrous material are stained with the dye and give bright yellow fluorescence patches, confirming the amyloid character of the fibrillar material.

The present results add significantly to previous evidence that amyloid fibril formation does not require a significant population of a monomeric form of the protein with extensive β-sheet structure to be present in solution [17]. Here the fibrils appear to form from the partially folded *apo* state which has a CD spectrum characteristic of a mainly random coil conformation with approximately 14% α-helical secondary structure. A random coil, however, consists of rapidly interconverting conformations of widely differing character some of which will be substantially extended [18]. Interestingly, however, CD spectra of supernatant solutions in which the fibrils are identified have characteristics of β-sheet structure. Such spectra, however, have a very low intensity and probably reflect the presence of protein aggregates with a low molecular weight in the solution. Similar species have been inferred during aggregation of other systems [19].

The observations with C11A/C14A cytochrome c_{552} support the view that the ability to form amyloid fibrils is a generic property of polypeptide chains and not one restricted to the proteins involved in diseases such as Alzheimer's and the spongiform encephalopathies, nor to proteins with a high β-sheet propensity [12–14]. Indeed the data presented here, together with the observation that amyloid fibrils can also be formed by myoglobin [17], show that proteins that are substantially α-helical in their soluble forms can readily form the cross β-structure of amyloid fibrils. It is now well established that fibril formation results when the cooperative native fold is disrupted under conditions where non-covalent interactions such as hydrogen bonds remain favourable [10]. In the case of C11A/C14A cytochrome c_{552}, the *holo* protein has a closely similar structure to that of the wild-type protein but the mutations permit the gradual loss of the haem group. The resulting *apo* form of the protein is evidently sufficiently unstable that it can readily convert into amyloid fibrils. One might speculate, therefore, that one of the important roles of the covalent linkages of the protein to the haem group in wild-type cytochrome c_{552}, the purpose of which is still debated [20], could be to prevent a chain of events such as that reported here from occurring in vivo. Such a suggestion is consistent with the proposal that prevention of aggregation could be one of the key driving forces in the evolution of cooperative protein structures [13,21].

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