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Heme binding site in apomyoglobin may be effectively targeted with small molecules to control aggregation

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ABSTRACT

A number of ligands with affinities for the heme binding site of apomyoglobin were tested to control amorphous and fibrillar aggregation in the protein. Several techniques, including fluorescence, dynamic light scattering, transmission electron microscopy, dot blot analysis combined with viability studies were employed for structural characterization and cytotoxicity assessment of the intermediate and final protein structures formed during the aggregation process. Of the small molecules investigated, chrysin and Nile red with high structural similarities to heme were chosen for further studies. Only fibril formation was found to be prevented by Nile red, while chrysin, with a greater structural flexibility, was able to prevent both types of aggregate formation. The two ligands were found to influence aggregation at different stages of intermediate structure formation, an ability determined by their degrees of similarities with heme. Based on structural characterization and toxicity studies, it is concluded that ligands similar in structure to heme may be effective in influencing various stages of aggregate formation and toxicity potencies of the protein structures. Since metalloproteins constitute more than thirty percent of all known proteins, it is concluded that the present strategy may be of general significance.

1. Introduction

Myoglobin is a globular, all-alpha metalloprotein, whose "apo" form (i.e. devoid of heme) is well-characterized relative to its folding dynamics (Jamin, 2005; Fink et al., 1998). Native, molten globule and unfolded states have been obtained for the apo form of horse myoglobin at pH 6.5, pH 4.2 and pH 2, respectively (Fink et al., 1998; Tcherkasskaya and Ptitsyn, 1999). Amyloid formation of myoglobin has been reported for the apo structure at alkaline pH, while the heme-containing form (holomyoglobin) could not be driven toward this state (Fandrich et al., 2003). On the other hand, a mutant form (W7FW14F) of sperm whale apomyoglobin showed fibrillation at physiologic pH and room temperature (Sirangelo et al., 2004, 2009; Infusini et al., 2012). In the present study, different features of aggregation in wild-type horse apomyoglobin have been investigated, including determination of the roles of pH and electrostatic repulsion on aggregation pathways, which may result in amorphous aggregation or fibril formation. Small molecules were tested as inhibitors of both events and toxicities of intermediate structures were determined. It is suggested that stability of apomyoglobin could affect formation of "on pathway" or "off pathway" intermediates.

One common event that is thought to be of importance in aggregate formation of apo-metalloproteins is destabilization of the native protein structure as a result of metal removal. For example, amyloid fibrils of bovine α-lactalbumin form either at low pH or by disulfide reduction. These conditions cause release of Ca^{2+}, leading to reduction of thermal stability and formation of partially folded conformations (Goers et al., 2002; Veprintsev et al., 1997; Ebrahim-Habibi et al., 2010a). Similarly, loss of metal ions, disulfide reduction, and pathologic mutations may drive superoxide dismutase toward amyloid fibrillation (Oztug Durer et al., 2009; Fee and Phillips, 1975; Mei et al., 1992). Fibril formation is also observed in apo-carbonic anhydrase, which may take up a pre-molten globular structure after removal of its zinc ion (Es-haghi et al., 2012). More specifically, conditions leading to loss of heme group of bovine cytochrome c (i.e. mutations in the heme binding site, or incubation

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at 75 °C and under mild alkaline conditions) results in formation of unfolded structures and consequently, amyloid fibrils (Pertinhez et al., 2001; de Groot and Ventura, 2005). The structure stabilizing role of heme has been demonstrated for the heme-binding section of myoglobin (microglobin) (Ji et al., 2008), and further suggested to prevent apomyoglobin amyloid formation (Iannuzzi et al., 2007).

2. Materials and methods

Horse myoglobin, Thioflavin T, Congo red, borate, 2-butanone, dialysis tubing, MTT, Nile red, and chrysin were purchased from Sigma (St Louis, MO, USA). ANS was obtained from Fluka. Glycine, DMSO and all salts and organic solvents were obtained from Merck (Darmstadt, Germany). RPMI medium were purchased from Gibco. FBS, horse serum, streptomycin and penicillin were obtained from Biosera (England). Ultra centrifuge filters were purchased from Millipore and A11 antibody was obtained from Chemicon.

2.1. Preparation of apomyoglobin (Teale, 1959)

Apomyoglobin was prepared by phase extraction process. Holomyoglobin was dissolved in 0.01 M HCl and mixed with 2-butanone on ice. Upon phase separation, the organic layer was removed and the procedure was repeated until clear solution appeared. Then, the sample was dialysed overnight in distilled water to remove extra butanone. Ultra centrifugation was employed to concentrate the apoprotein solutions.

2.2. Turbidity measurements

Different buffers (phosphate, borate, sodium acetate), pH (4.2, 6.5, and 9), protein concentrations (0.2–0.4 mg/ml) and temperatures (60–70 °C) were used when screening for the best conditions.

Finally, solutions containing 0.3 mg/ml apomyoglobin in sodium acetate (10 mM), sodium phosphate (10 mM) and pH 6.5 and different ligands were prepared and incubated at 65 °C. To monitor protein aggregation, turbidity measurements were made at 350 nm on a Shimadzu UV–visible spectrophotometer (Kyoto, Japan) (Rezaei-Ghaleh et al., 2007a). Temperatures were controlled to within ±0.1 °C.

2.3. Thioflavin T (ThT) binding assay

All fluorescence experiments were carried out on a CaryEclipse VARIAN fluorescence spectrophotometer. To investigate fibrillation, apomyoglobin samples were added to ThT solution in a molar ratio of 1:2.5, then mixed thoroughly and incubated for 5 min. Fluorescence excitation and emission were set at 440 and 482 nm and slit widths were set at 5 nm and 10 nm, respectively.

2.4. 8-Anilino-1-naphthalene sulfonate (ANS) fluorescence assays

The excitation wavelength was 350 nm and emission spectra were recorded between 400 and 600 nm. Excitation and emission slit widths were set at 5 nm. The final concentrations of ANS and protein were 88 μM and 0.03 mg/ml respectively.

2.5. Nile red fluorescence assay

Apomyoglobin (85 μM) was added to Nile red (0.3 μM) at pH 7 and the increase and shift occurring in the fluorescence emission was monitored. The excitation wavelength was 580 nm and emission spectra were recorded between 600 and 700 nm. Excitation and emission slit widths were both set at 5 nm.

2.6. Congo red absorbance assays

Ten microliters of well-mixed incubation sample were added to 190 μl of the Congo red solution and incubated for 30 min. Absorbance spectra were recorded (400–600 nm) using a Shimadzu UV–visible spectrophotometer (Kyoto, Japan).

2.7. Circular dichroism (CD) measurement

CD spectra in the far-UV region (190–260 nm) were obtained on an AVIV 215 spectropolarimeter (Aviv Associates, Lakewood, NJ, USA), using a 1 mm path cell at room temperature. The protein concentration was 0.4 mg/ml.

2.8. Transmission electron microscopy (TEM)

Copper 400 mesh grid was covered with carbon-coated formvar film followed by adsorption of 10 μl of apomyoglobin samples. After 2 min, excess fluid was removed with a paper filter, and 1% uranyl acetate added. After another minute, excess dye was removed. Finally, the grids were monitored with a CEM 902A Zeiss microscope (Oberkochen, Germany) and Philips (Japan).

2.9. Dynamic light scattering

Dynamic light scattering (DLS) studies were performed on a zeta potential and particle size analyzer (Brookhaven Instrument, Holtsville, NY 11742-1896, USA).

The size distribution/abundance of particles was studied in the absence and presence of desired ligands. The final protein concentration used was 58 μM. A laser of 657 nm with a fixed detector angle of 90° was used. DLS studies were carried out at least in triplicates.

2.10. Dot blot analysis

Dot immunoblot analysis was performed to investigate reactivity of anti-oligomeric A11 antibody against apomyoglobin oligomers. Aliquots of protein samples were spotted onto nitrocellulose membranes and dried. Membranes were blocked with 10% dry milk and incubated with A11 antibody (1:1000 dilution) overnight, then incubated with goat-anti rabbit HRP as the secondary antibody (1:2000 dilution) for 1 h. Dot visualization was carried out in a dark room.

2.11. Docking studies

Docking was performed with Autodock vina (Trott and Olson, 2010). The 1DWR.pdb file (protein devoid of heme group) was used as receptor. Grid box of $48 \times 48 \times 44$ points was used with a spacing of 1.0 Å, and the grid box center was placed on $x = 13.583$, $y = 21.646$, and $z = 8.308$. The box encompassed the whole protein molecule. Ligands were prepared using Molecular Operating Environment (MOE), 2010.10 (Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7). Gasteiger charges were assigned to protein and ligand molecules. Exhaustiveness was set on 20 and 100 poses were generated for the ligands. Preparation of the image representing the best pose of each ligand was performed with MOE 2010.10.

2.12. MTT assay

Cell viability assay was assessed using rat pheochromocytoma (PC12) cell line. After 24 h of incubation with samples pretreated under amyloidogenic conditions in the absence and presence of ligands, cells were washed with PBS. 100 μl of MTT stock solution