Trimethylamine-*N*-oxide-induced folding of α-synuclein

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Abstract The effect of the natural osmolyte trimethylamine-*N*-oxide (TMAO) on the structural properties and fibril formation of the natively unfolded protein human α-synuclein was studied using several physico-chemical methods. TMAO induced folding of α-synuclein: at moderate concentrations, a partially folded intermediate with enhanced propensity for fibrillation accumulated; at higher concentrations, α-synuclein was tightly folded and underwent self-association to form oligomers. The latter conformation was significantly helical and probably represents the physiologically folded form of the protein. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: α-Synuclein; Fibril; Osmolyte; Natively unfolded; Trimethylamine-*N*-oxide; Oligomer

1. Introduction

α-Synuclein is a small (14 kDa), highly conserved presynaptic protein that is abundant in various regions of the brain [1–3]. Structurally, purified α-synuclein belongs to the family of natively unfolded proteins [4–6], which are characterized by a unique combination of low overall hydrophobicity and large net charge [7]. Deposition of aggregated forms of α-synuclein in neuronal or glial cytoplasm is a pathological hallmark of several neurodegenerative diseases, including Parkinson's disease, dementia with Lewy bodies, Lewy body variant of Alzheimer's disease, and multiple system atrophy [8,9]. In vitro, the protein forms fibrils with morphologies and staining characteristics similar to those extracted from disease-affected brain [10–15]. The kinetics of fibrillation are consistent with a nucleation-dependent mechanism [11,16], in which the critical early stage of the structural transformation involves a partially folded intermediate [5].

The function of α-synuclein is unknown. It is generally accepted that the biological functions of proteins are predetermined by their 3D structures. Hence, the existence of natively unfolded proteins raises the question of the structural basis of their function. It has been suggested that the large flexibility of natively unfolded proteins allows them to interact efficiently with several different targets [17,18]. Thus, natively unfolded proteins may be significantly folded in their normal cellular milieu due to binding to specific targets and ligands.

Recently it has been demonstrated that the naturally occurring osmolyte, trimethylamine-*N*-oxide (TMAO), can cause thermodynamically unstable proteins to fold, and regain high functional activity [19,20]. Osmolytes may fold unstructured proteins due to the osmophobic effect, a solvophobic thermodynamic force, arising from the unfavorable interaction between the osmolyte and the peptide backbone [21].

2. Materials and methods

2.1. Expression and purification of recombinant α-synuclein

The expression (in *Escherichia coli*) and purification of α-synuclein were as described previously [5].

2.2. Materials

Thioflavin T (TFT), TMAO and 1-anilinonaphthalene-8-sulfonic acid (ANS) were obtained from Sigma, St. Louis, MO, USA. All other chemicals were of analytical grade from Fisher Chemicals.

2.3. Circular dichroism measurements

CD spectra were obtained with an AVIV 60DS spectrophotometer (Lakewood, NJ, USA) using an α-synuclein concentration of 70 μM. Near-UV CD spectra were recorded in a 1.0 cm pathlength cell, far-UV CD with a 0.01 cm pathlength cell. CD spectra of the appropriate buffers were recorded and subtracted from the protein spectra.

2.4. Fluorescence measurements

Fluorescence measurements were made in semimicro quartz cuvettes (Hellma) with a 1 cm excitation light path using a FluoroMax-2 spectrofluorometer (Instruments S.A., Inc., USA). ANS emission spectra were recorded with excitation at 350 nm. ANS and protein concentrations were 15 and 5 μM, respectively. All data were processed using DataMax/GRAMS software. Time courses of changes in ANS fluorescence intensity were analyzed at 470 nm with excitation at 350 nm.

2.5. Acrylamide quenching

Acrylamide quenching studies of the intrinsic fluorescence of α-synuclein were performed by adding aliquots from a stock solution of the quencher into a cuvette containing the protein solution. Fluorescence intensities were corrected for dilution effects. Fluorescence quenching data were analyzed as described in [5], using the general form of the Stern–Volmer equation [22].

2.6. Small angle X-ray scattering (SAXS) experiments

SAXS measurements were made using Beam Line 4-2 at Stanford Synchrotron Radiation Laboratory [23,24]. Experimental conditions and protocol were as described in [5]. The radius of gyration (R_g) was calculated according to the Guinier approximation [25].

2.7. Fibril formation assay

Assay solutions contained 15 μM TFT and protein at a concentration of 70 μM (1.0 mg/ml) in 20 mM Tris–HCl, 0.1 M NaCl, pH 7.5, and the desired TMAO concentration. A volume of 100 μl of the mixture was pipetted into a well of a 96-well plate (white plastic, clear bottom) and a 1/8″-diameter Teflon sphere (McMaster-Carr, Los Angeles, CA, USA) was added. Each sample was run in triplicate or quadruplicate. The plate was loaded into a fluorescence plate reader

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(Fluoroskan Ascent) and incubated at 37°C with shaking at 300–600 rpm with a shaking diameter of 1 mm. The fluorescence was measured at 30 min intervals with excitation at 450 nm and emission at 485 nm, and curve-fit as described in [26].

3. Results

3.1. TMAO induces biphasic secondary structure formation transitions in α-synuclein

In order to investigate the effect of TMAO on the conformation of human α-synuclein, several structural approaches were used. At pH 7.5 and at 20°C, the far-UV CD spectrum of recombinant human α-synuclein in the absence of TMAO shows a spectrum typical of an unfolded protein. As the concentration of TMAO is increased, changes are observed corresponding to formation of secondary structure, Fig. 1A. At high TMAO concentrations (>3 M), α-synuclein showed a far-UV CD spectrum typical of a well-folded protein.

Fig. 1B represents the TMAO-dependencies of $[\theta]_{222}$ and $[\theta]_{200}$. The α-synuclein folding is described by two successive sigmoidal curves separated by a pronounced plateau, with ~30% secondary structure being formed in the first step. Interestingly, the shape and intensity of the far-UV CD spectra measured in the vicinity of 2 M TMAO are close to those reported for the previously observed partially folded intermediate [5,27]. Thus, TMAO initially induces formation of the intermediate ($C_m = 1.4 \pm 0.1$ M), which subsequently transforms into a species with well-organized secondary structure ($C_m = 2.3 \pm 0.1$ M).

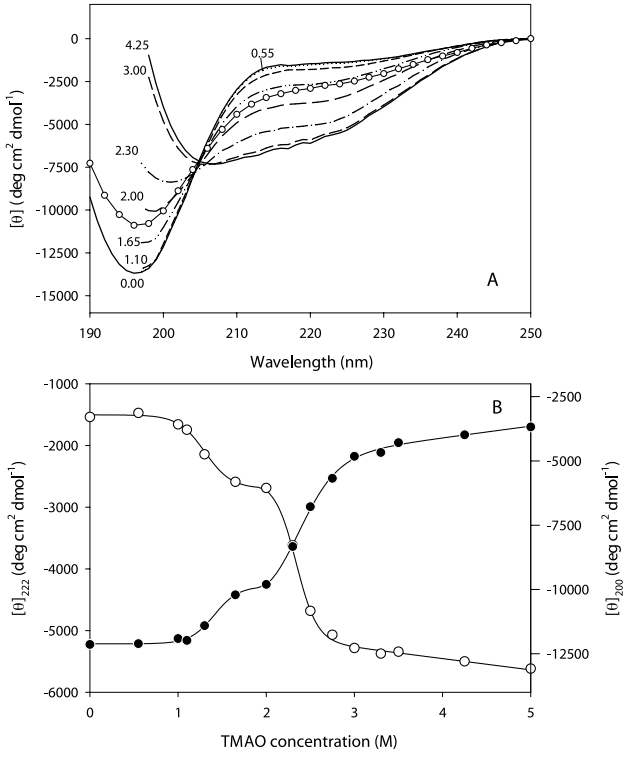

Fig. 1. Effect of TMAO on the structural properties of human α-synuclein. A: Far-UV CD spectra as a function of TMAO concentration. The spectrum of the partially folded intermediate induced in α-synuclein at pH 3.0 is shown for comparison (circles). B: TMAO-dependent conformational changes: $[\theta]_{222}$ (open circles) and $[\theta]_{200}$ (black circles). Measurements were carried out at 20°C. Protein concentration was 70 μM.

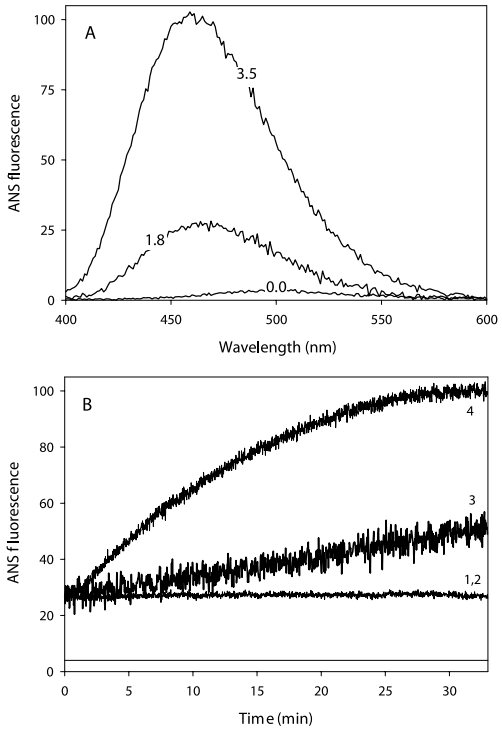

Fig. 2. Effect of TMAO on ANS binding to α-synuclein. A: ANS spectra as a function of TMAO concentration. B: Kinetics of ANS spectral changes induced by TMAO (1.8 M (curves 1 and 2) and 3.5 M (curves 3 and 4). Measurements were carried out for different protein concentrations: 1 μM (curves 1 and 3) and 5 μM (curves 2 and 4). The ANS fluorescence intensity in the absence of TMAO is shown as straight line.

3.2. TMAO-induced changes in ANS binding

Changes in ANS fluorescence are characteristic of the interaction of this dye with solvent-exposed hydrophobic surfaces of partially folded proteins [28]. Fig. 2A shows that the addition of TMAO to α-synuclein in the presence of ANS leads to dramatic changes in the ANS fluorescence spectrum; a significant increase in the fluorescence intensity and a pronounced (~30 nm) blue shift in λ_{max}. Interestingly, the ANS spectrum for α-synuclein in the presence of 1.8 M TMAO is close to that reported for partially folded α-synuclein [5,27]. However, a further increase in TMAO concentration is accompanied by an additional increase in the ANS fluorescence intensity (Fig. 2A).

In order to investigate the underlying molecular mechanism of TMAO-induced folding of α-synuclein, we looked at the time course of the increase in ANS fluorescence intensity in solutions containing 1 and 5 μM of the protein (Fig. 2B). Interestingly, for both protein concentrations in the presence of 1.8 M TMAO, the conformational changes occurred rapidly, within the dead time of the manual mixing experiment (~5 s). This illustrates that under the conditions studied, the osmolyte-induced partial folding of α-synuclein is an intramolecular process and is not accompanied by association of the partially folded molecules.

However, in the solutions with higher TMAO concentration (3.5 M), the kinetics are biphasic and different for different protein concentrations. Fig. 2B illustrates that a ~25% increase in the ANS fluorescence intensity occurs within the dead time of the experiments, independent of the α-synuclein concentration. Subsequent slower changes are protein concen-

tration-dependent, with rate constants of $3.7 \pm 0.2 \times 10^{-4}$ s^{-1} and $1.1 \pm 0.1 \times 10^{-3}$ s^{-1} for 1 and 5 μM of the protein, respectively, indicating that the slower kinetic phase reflects association of α-synuclein. Thus, in the presence of 3.5 M TMAO, α-synuclein folds rapidly into the partially folded conformation, which then associates in a protein concentration-dependent manner.

3.3. TMAO-induced changes in the environment of tyrosine residues

Further evidence for TMAO-induced structural changes in α-synuclein are shown in Fig. 3. Information on the relative solvent exposure of tyrosine residues (there are no tryptophans in α-synuclein) can be obtained from analysis of the effect of quencher molecules such as acrylamide [22]. Fig. 3A shows the Stern–Volmer plots for α-synuclein as a function of TMAO. The upward curvature indicates the presence of both dynamic and static quenching [22]. In the absence of osmolyte $K_{SV} = 15.9 \pm 0.2$ M^{-1}, which is somewhat smaller than the value for free L-tyrosine ($K_{SV} = 21.1 \pm 0.2$ M^{-1}) [32]. In the presence of 1.8 M TMAO, K_{SV} decreases to 13.1 ± 0.1 M^{-1}, whereas in 3.5 M TMAO, K_{SV} decreases to 8.5 ± 0.2 M^{-1}. This means that the tyrosine residues of natively unfolded α-synuclein are partially protected from the quencher molecules and the degree of protection increases as the TMAO concentration increases. The value of K_{SV} of α-synuclein in the presence of 3.5 M TMAO is representative of typical folded globular proteins.

Fig. 3B represents the near-UV CD spectra of α-synuclein measured at different TMAO concentrations. In the absence of osmolyte or in the presence of 1.8 M TMAO, there is an essentially flat spectrum. This indicates that natively unfolded or partially folded α-synuclein has no rigid tertiary structure. However, in the presence of 3.5 M TMAO, a very prominent band in the vicinity of 275 nm appears. Thus, self-association of the partially folded intermediate is accompanied by the appearance of significant tertiary structure.

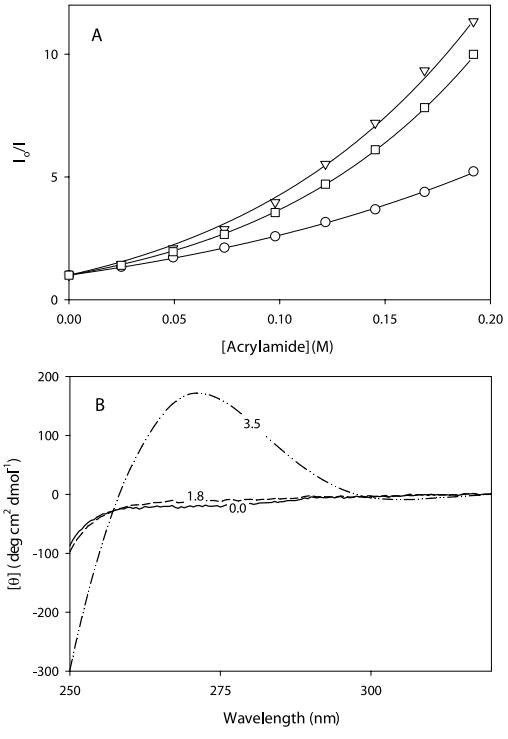

Fig. 3. A: Stern–Volmer plots for α-synuclein in the absence (▽) or presence of 1.8 M (□) or 3.5 M TMAO (○). Fluorescence quenching by acrylamide. B: Near-UV CD spectra for α-synuclein in the presence of 0, 1.8 or 3.5 M TMAO. Measurements were carried out at 20°C.

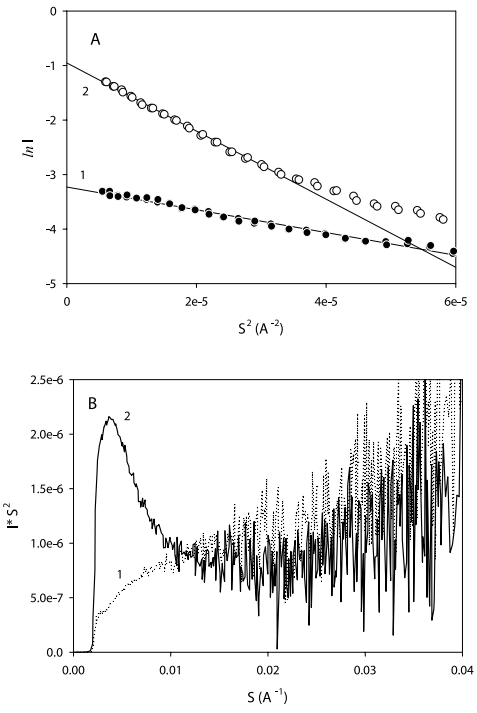

Fig. 4. Guinier (A) and Kratky plot (B) representation of the results of SAXS analysis of α-synuclein: 1: without TMAO; 2: 3.5 M TMAO. Measurements were carried out at 23°C. Protein concentration was 210 μM.

3.4. SAXS shows oligomers at high TMAO

SAXS provides information about the packing density, shape and dimensions of biopolymers in solution [25]. Guinier analysis of the scattering data shows that the addition 3.5 M TMAO leads to a significant increase in R_g for α-synuclein, from 41.1 ± 1.2 to 64.9 ± 0.3 Å (Fig. 4A). We attribute the large increase in R_g induced by 3.5 M TMAO to self-association. Analysis of the SAXS forward-scattering intensity values, $I(0)$, show that in the presence of 3.5 M TMAO, the $I(0)$ value for α-synuclein is ~10-fold larger than that determined for the protein in the absence of osmolyte (0.3855 ± 0.0035 vs. 0.03985 ± 0.0012, respectively). Thus, α-synuclein is monomeric in the absence of TMAO, but associates to form oligomers (probably decamers) at high concentrations of TMAO.

Analysis of the X-ray scattering in the form of a Kratky plot shows that in aqueous solution, as expected, α-synuclein does not have a well-developed globular structure (Fig. 4B; cf. [5,13]). However, the profile of the Kratky plot in the presence of 3.5 M TMAO shows a pronounced maximum at low angles, which indicates that the subunits in the oligomer are globular (i.e. tightly folded). Analogous behavior has been observed previously during the association of partially folded intermediates of staphylococcal nuclease [29,30].

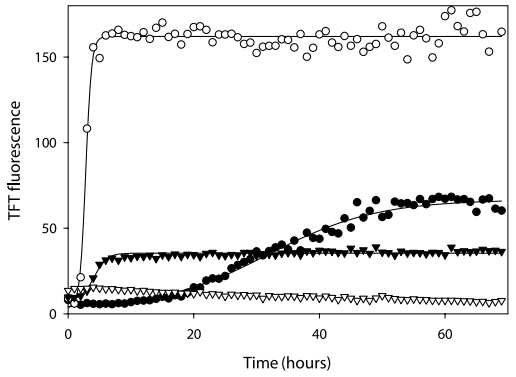

Fig. 5. Effect of TMAO on α-synuclein fibril formation, monitored by TFT fluorescence. The symbols represent TFT fluorescence intensities determined experimentally, and the lines are results of data fitting according to [31]. TMAO concentrations were 0 (●), 1 (○), 2 (▼) and 3 M (▽), the protein concentration was 70 μM. Conditions were pH 7.5, 37°C.

3.5. TMAO modulates fibrillation of α-synuclein

TFT is a fluorescent dye that interacts with amyloid fibrils leading to an increase in the fluorescence intensity in the vicinity of 480 nm [31,32]. Fig. 5 shows that TMAO affected the kinetics of α-synuclein fibrillation in a concentration-dependent fashion. The rate of fibril formation was substantially enhanced in the presence of 1 and 2 M TMAO, whereas it was essentially completely inhibited in solutions of 3 M TMAO. The acceleration of fibril formation in the presence of low concentrations of TMAO is due to the osmolyte-induced stabilization of the partially folded α-synuclein intermediate, which was previously shown to be a critical step in the early stage of fibrillogenesis [5,13,27]. The fact that in the presence of 3.5 M TMAO, α-synuclein self-associates to form well-folded oligomers with globular structure, yet does not fibrillate, suggests that these oligomers are not on the fibrillation pathway, and are quite stable. This also may explain the lower TFT signal observed for this protein in 2 M TMAO solutions (see Fig. 5).

4. Discussion

Our results demonstrate that TMAO causes natively unfolded α-synuclein to fold in a biphasic manner. Moderate TMAO concentrations (between 1 and 2 M) result in a partially folded intermediate that possesses structural properties previously described for this protein at high temperatures, low pH [5], and in the presence of certain metal cations [27] or pesticides [33]. The present results indicate that the osmophobic effect must be added to the previously described factors that induce the transformation of natively unfolded α-synuclein into the partially folded intermediate. Higher concentrations of TMAO lead to formation of an oligomer, probably a decamer, based on the SAXS data.

The partially folded conformation is not only stabilized under conditions of moderate osmolyte concentrations, but is also transiently populated in the kinetic pathway of oligomer formation at high TMAO concentration. Thus, depending on the conditions, the intermediate may self-associate leading to fibrils, amorphous aggregates or soluble globular oligomers. Furthermore, under all the situations in which the intermediate is populated, α-synuclein fibrillates significantly faster than in control experiments, consistent with the intermediate being a critical species in the fibrillation pathway. Interestingly, however, the addition of high osmolyte concentrations transforms the protein into oligomers in which the subunits are folded, and which do not fibrillate. It is not clear if it is the globular structure, or the strong intermolecular interactions between individual molecules, that prevents fibrillation in the oligomers.

The CD spectrum of α-synuclein in high TMAO concentration reveals significant helical structure. This conformation is thus likely to represent the conformation of α-synuclein when associated with its intracellular partners. The fact that this form of the protein does not fibrillate may also be significant with respect to the in vivo situation.

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