Curcumin Prevents Aggregation in α-synuclein by Increasing the Reconfiguration Rate

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*Running Title: Intramolecular diffusion in αS with curcumin

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Background: α-synuclein is an aggregation-prone protein which reconfigures more slowly under aggregating conditions.

Results: Curcumin binds to monomeric α-synuclein, prevents aggregation and increases the reconfiguration rate, particularly at high temperatures.

Conclusion: Curcumin rescues the protein from aggregation by making the protein more diffusive.

Significance: The search for aggregation inhibitors should account for changes in chain dynamics by the small molecule.

SUMMARY

α-synuclein is a protein that is intrinsically disordered in vitro and prone to aggregation particularly at high temperatures. In this work we examine the ability of curcumin, a compound found in turmeric, to prevent aggregation of the protein. We find strong binding of curcumin to α-synuclein in the hydrophobic non-amyloid-β component (NAC) region and a complete inhibition of oligomers or fibrils. We also find that the reconfiguration rate within the unfolded protein is significantly increased at high temperatures. We conclude that α-synuclein is prone to aggregation because its reconfiguration rate is slow enough to expose hydrophobic residues on the same timescale that bimolecular association occurs. Curcumin rescues the protein from aggregation by increasing the reconfiguration rate into a faster regime.

INTRODUCTION

α-synuclein aggregation is involved in, and likely the cause of, Parkinson’s disease (1). Although α-synuclein is commonly thought of as intrinsically disordered, a recent report demonstrated that in human cells it exists in a helical tetramer that does not easily aggregate (2). This suggests the physiological pathway for aggregation is first unfolding of the tetramer to kinetically trapped monomers and then re-association to a disordered aggregate and eventually fibrillar Lewy bodies. Therefore preventing re-association of the monomers is a useful therapeutic strategy. Many researchers in the past several years have investigated the interaction of potential aggregation inhibitors with oligomers of various sizes and fibrils but there have been few observations of inhibitors with monomers, primarily because spectroscopic detection is difficult (3-7).

We have recently investigated the chain dynamics of disordered, monomeric α-synuclein under a variety of aggregation conditions and found that the internal reconfiguration rate (or the rate of intramolecular diffusion) is fast under conditions in which aggregation is inhibited and slows when aggregation is more likely (8). We interpret these observations with a model in which the first step of aggregation is kinetically controlled by the reconfiguration rate of the disordered monomer. When intramolecular
Diffusion is fast compared to bimolecular association, aggregation is unlikely because exposed hydrophobes quickly reconfigure, but if intramolecular diffusion slows to the same rate as bimolecular association, aggregation becomes more likely. A logical extension of this model is that aggregation inhibitors prevent bimolecular association by raising the reconfiguration, or the rate of intramolecular diffusion, of the disordered protein.

Intramolecular diffusion is the random motion of one part of the protein chain relative to another. To measure intramolecular diffusion we use the Trp-Cys contact quenching method by which Tryptophan is excited to a long-lived triplet state which is quenched on contact with cysteine within the same protein chain. Measurement of this rate of quenching at various temperatures and viscosities allows the extraction of the rate of diffusion between these two points in the chain.

In this work we investigate the effect of the small molecule curcumin on the intramolecular diffusion of α-synuclein. Curcumin, a compound found in the spice turmeric, has been shown to have many medicinal properties and inhibits aggregation of Alzheimer’s peptide Aβ (9). In α-synuclein, curcumin has been shown to inhibit fibril formation and increase solubility, but the physical basis of the aggregation inhibition is not known (10). We find curcumin strongly binds to the monomer and completely inhibits aggregation, and with curcumin intramolecular diffusion of α-synuclein is increased more than 10-fold at 40°C compared to the protein alone.

**EXPERIMENTAL PROCEDURES**

**α-synuclein mutation, expression and purification**

The α-synuclein plasmid was a kind gift from Gary Pielak at the University of North Carolina, Chapel Hill. 39W/69C and 69C/94W mutants of α-synuclein were created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The mutations were confirmed by DNA sequencing. The wild-type and mutant proteins were expressed in E. coli BL21 (DE3) cells transformed with the T7-7 plasmid and purified as described previously (11). The mutants purity was checked by SDS-PAGE to be >95%. The protein concentration was determined from the absorbance at 280 nm using extinction coefficient of 11460 M⁻¹ cm⁻¹. The stock solutions of ~200 µM were stored at -80 °C in 25 mM sodium phosphate buffer (pH 7.4) with 1 mM tris(2-carboxyethyl)phosphine (TCEP). An aliquot was thawed and filtered shortly before each experiment.

**Aggregation inhibition studies**

The effect of curcumin on the inhibition of α-synuclein aggregation was measured in two aggregation conditions. First, the fibril formation in the absence and presence of curcumin (curcumin/protein molar ratio =1.5) was initiated by stirring the protein, at a concentration of 48 µM, in 25 mM phosphate buffer, pH 7.4, 150 mM NaCl, 1 mM TCEP, 37°C (11). Second, soluble oligomers formation in the absence and presence of curcumin (curcumin/protein molar ratio =1.5) was started by incubating the 5 µM α-synuclein in 10% trifluorethanol (TFE) (v/v), 25 mM phosphate buffer, pH 7.4, 1 mM TCEP, 37°C (12).

At regular times, individual aliquots of 60 (10) µl of each sample pre-incubated without or with curcumin (curcumin:α-synuclein = 1.5:1) were mixed with 440 (490) µl 25 µM thioflavine T (ThT) solution and 25 mM phosphate buffer at pH 7.4 and the aggregation kinetics was followed by measurements of ThT fluorescence at 480 nm and far-UV circular dichroism (CD) at 217 nm, respectively. The ThT fluorescence was measured using a Jobin Yvon Spex Fluorolog-3 spectrofluorimeter equipped with a temperature-controlled cell holder. The excitation and emission wavelengths were 440 and 480 nm, respectively. A 10-mm path-length quartz cell and excitation and emission slit width of 5 nm were used. Far UV CD data were obtained with an Applied Photophysics Chirascan spectropolarimeter equipped with a temperature-controlled cell holder.

**Conformational Studies**
**RESULTS**

**Curcumin binds to monomeric α-synuclein**

Binding of curcumin was measured using a variety of optical absorption and fluorescence methods (see supplementary data for details). As shown in Fig. 1, curcumin binds strongly to monomeric α-synuclein with dissociation constant $K_D \sim 10^{-5}$ M without making any significant alteration in the unfolded state of α-synuclein (Fig S1). Measuring binding at various temperatures we determined the enthalpy ($\Delta H = -7.9$ kcal/mol) and entropy ($\Delta S = -0.0081$ kcal/mol/K) of binding, indicating the binding is enthalpically driven and suggesting it is due to non hydrophobic interactions. These observations are similar to previous reports of binding of curcumin with proteins such as αs1-casein (15), β-lactoglobulin (16) and FtsZ (17).

The binding profiles for the two loops investigated here are the same, indicating the Trp and Cys mutations are not significantly affecting the curcumin binding. There is no evidence curcumin significantly alters the conformational ensemble, but the tryptophan emission at position 94 shows significant quenching and a slight blue-shift in the spectrum upon binding of curcumin (see Fig. S2). No such effect is observed for the tryptophan at position 39. This suggests an affinity for binding in the NAC region.

**Curcumin strongly inhibits oligomer and fibril formation.**

We investigated the effect of curcumin on the aggregation of 69C/94W mutant of α-synuclein in two solution conditions. In 25 mM phosphate buffer of pH 7.4, 150 mM NaCl, 1 mM TCEP, α-synuclein at high concentration (48 μM) is known to form fibrils in 6 days upon stirring at 37°C (11), while in 10% TFE (v/v) at pH 7.4, α-synuclein at low concentration (5 μM) aggregate into soluble oligomers in 70 minutes at 25°C (12). Figure 2a and 2c show kinetics of 69C/94W mutant fibrillation and oligomerization, respectively in the absence and presence of curcumin monitored with ThT fluorescence and far-UV CD at 217 nm. For fibrillation, in the absence of curcumin (filled...
symbols), sigmoidal kinetics curves were observed with both the probes indicating a nucleation-polymerization reaction. Oligomer formation occurred without a lag phase, shows weak ThT binding, and saturated in 1 hour. However, at the highest curcumin concentration tested (curcumin/protein molar ratio 1.5), no increase in ThT fluorescence or CD values were observed in either case, indicating that both fibrillation and oligomerization are completely prevented.

Fig 2b and 2d show the far-UV CD spectra of the reactants and products formed in the absence and presence of curcumin under two aggregation conditions. In the fibrillation condition monomeric \( \alpha \)-synuclein is characterized by a deep negative minimum at 198 nm. It has been previously shown that at very low concentration (~0.5 \( \mu \)M) \( \alpha \)-synuclein exists as monomer between 0-60 % TFE (12), and we see a similar partially folded spectrum with and without curcumin in 10% TFE (v/v). In the absence of curcumin, monomeric reactants incubated in either condition show a transition from monomeric to a \( \beta \)-sheet structured aggregate with a negative minimum at 217 nm (Fig 2b and 2d). In the presence of curcumin, the spectra of the products in both aggregation conditions are almost unchanged after incubation. Taken together, these results suggest that curcumin at a molar ratio of 1.5:1 completely prevents the fibrillation and oligomer formation of \( \alpha \)-synuclein.

Curcumin significantly affects \( \alpha \)-synuclein intramolecular diffusion

We investigated the effect of curcumin on intramolecular contact rates in two \( \alpha \)-synuclein mutants, 39W/69C and 69C/94W by the Trp-Cys contact quenching method. Tryptophan triplet kinetics observed for these mutants exhibit a rapid decay on the microsecond timescale due to quenching of the tryptophan triplet by contact with cysteine and a second decay on the millisecond timescale due to other photophysical processes (18). In the presence of curcumin, similar triplet decay kinetics was observed for these mutants. The observed rate of triplet decay consists of two processes, intramolecular diffusion and irreversible quenching of the triplet by cysteine on close contact. In equilibrium intramolecular diffusion brings the Trp and Cys within the same polypeptide together with a diffusion limited forward rate \( k_{D+} \), where it may be quenched with rate \( q \) or diffuse away with rate \( k_{D-} \). The observed rate is given by (19)

\[
k_{obs} = \frac{k_{D+}q}{k_{D-} + q}
\]

If \( q \ll k_{D-} \), then \( k_{obs} \sim k_{D+} \), and the observed rate is diffusion-limited. However cysteine is not a diffusion-limited quencher of free tryptophan in water so \( q \ll k_{D-} \), and Eq. 1 can be rewritten as

\[
\frac{1}{k_{obs}} = \frac{1}{k_{D+}} + \frac{1}{qK} \sum \frac{1}{k_{D-}(T,\eta)} + \frac{1}{k_{x}(T)}
\]

We assume the reaction-limited rate \( k_R \) depends only on temperature (T) but \( k_{D+} \) depends on both temperature and viscosity of the solvent (\( \eta \)). Therefore, by making measurements at different viscosities for a constant temperature we can extract both \( k_R \) and \( k_{D+} \), by fitting a plot of \( 1/k_{obs} \) vs. \( \eta \) at a given temperature to a line in which the intercept is \( 1/k_R \) and the slope is \( 1/\eta k_{D+} \).

This measurement typically assumes that the cysteine is the only significant quencher in the sample (20), but it is likely that curcumin is also an efficient quencher. Fig. 3 shows measured \( k_{obs} \) of \( \alpha \)-synuclein 69C/94W and N-acetyl-L-tryptophanamide (NATA) in various concentrations of curcumin. The rates for NATA increase significantly with curcumin, indicating curcumin is a very efficient quencher, but the rates of 94W actually decrease slightly. This suggests that the curcumin is quite tightly associated with the protein and not free to quench Trp through bimolecular diffusion. Furthermore, although the curcumin is probably bound fairly close to 94W based on the fluorescence measurements (see Fig. S2), it is apparently not accessible to efficiently quench the triplet state, suggesting it is buried in a hydrophobic pocket within the chain.

Figure 4a and 4b show plots of exponential decay times (1/k_{obs}) vs. viscosity for various temperatures at pH 7.4 in the absence of curcumin and with a curcumin/\( \alpha \)-synuclein molar ratio of 1.5:1 respectively. Without curcumin, the intercept decreases and the slope increases dramatically.
with temperature. At the highest temperatures, the intercept is consistent with zero, implying that \( k_{\text{obs}} \) is diffusion-limited. In contrast, in the presence of curcumin, the change in slope is much more gradual and at no temperature is the observed rate diffusion-limited. This trend is more similar to trends observed in other unfolded peptides and proteins (13,21).

The reaction-limited (\( k_R \)) and diffusion-limited (\( k_{D+} \) at the viscosity of water at each temperature) rates are plotted in Fig 5 for 69C/94W and Fig S3 for 39W/69C for various concentrations of curcumin. The protein concentration was held fixed at 10 \( \mu M \) and the highest concentration of curcumin was 15 \( \mu M \), beyond which the curcumin absorbed too much light at 442 nm to make the measurement of the Trp triplet absorption accurately. In order to interpret these rates we use a theory by Szabo, Schulten and Schulten (SSS) which models intramolecular diffusion as diffusion on a 1-dimensional potential of mean force determined by the probability of intrachain distances (\( P(r) \)) (22).

The measured reaction-limited and diffusion-limited rates are given by (23)

\[
k_R = \int_{d_u}^{\infty} q(r)P(r)dr
\]

(3)

\[
\frac{1}{k_{D+}} = \frac{1}{k_{D+}^D} \int_{d_u}^{\infty} dr \left( \int_{d_u}^{\infty} (q(x) - k_R)P(x)dx \right)^2
\]

(4)

where \( r \) is the distance between the tryptophan and cysteine, \( D \) is the effective intramolecular diffusion constant and \( q(r) \) is the distance dependent quenching rate. The distance-dependent quenching rate for the Trp-Cys system drops off very rapidly beyond 4.0 Å, so the reaction-limited rate is mostly determined by the probability of the shortest distances (24). Very generally, \( k_R \) and \( k_{D+} \) are both inversely proportional to the average volume of the chain and \( k_{D+} \) is directly proportional to \( D \). Therefore in the absence of curcumin the large increase in \( k_R \) represents a significant compaction in the size of the protein and the moderate decrease in \( k_{D+} \) represents a significant slowing in diffusion as temperature is increased. The reconfiguration rate can be defined as the rate to diffuse one part of the chain across the diameter of the protein \( k_r = 4D/(2R_G)^2 \).

To determine the diffusion constant we assume the probability distribution is given by a Gaussian chain

\[
P(r) = \frac{4\pi r^2}{N} \left( \frac{3}{2\pi r^2} \right)^{3/2} \exp \left( -\frac{3r^2}{2(\langle r^2 \rangle)} \right)
\]

(5)

where \( \langle r^2 \rangle \), the average Trp-Cys distance, is an adjustable parameter, and \( N \) is a normalization constant such that \( \int P(r) = 1 \). For each measured \( k_R \), \( \langle r^2 \rangle \) was found such that it matched the measured rate using Eq. 3. These distances are plotted in Fig 5c. Then the correct \( P(r) \) was used in Eq. 4 with the measured \( k_{D+} \) to determine \( D \), plotted in Fig 5d. Without any curcumin, \( D \) decreases by \( \sim \)50-fold from 0 to 40 C. The addition of curcumin has a small effect on the size of the chain and the diffusion constant at low temperature but the effect increases dramatically at high temperature. Since the binding curves suggested that curcumin was preferentially binding near 94W we repeated these measurements with mutant 39W/69C (see Fig. S3) and found qualitatively similar results, suggesting curcumin affects the global dynamics of the protein.

**DISCUSSION**

We have previously shown that \( \alpha \)-synuclein, uniquely among disordered sequences, compacts and diffuses more slowly as temperature is increased (14). Examining other conditions in which aggregation is enhanced (low pH or the familial mutation A30P) we find good correlation between the rate of intramolecular diffusion and the rate of aggregation. When diffusion is fast (\( D \sim 10^6 \text{ cm}^2\text{s}^{-1} \)), such as is observed for most intrinsically disordered sequences, the protein reconfigures too fast to make stable bimolecular interactions with another protein chain, but when reconfiguration rate is about the same as the
bimolecular encounter rate, stable interactions are more likely and aggregation can proceed. This accounts for the dramatic increase in aggregation rate of \( \alpha \)-synuclein at 40 C compared to 0 C.

In this work we have examined the effect of curcumin binding on intramolecular diffusion of \( \alpha \)-synuclein. There is little difference in \( D \) at T=0 C but the difference widens with increasing temperature. At T=40 C and equal molar ratio of curcumin to protein, \( D \) is 15 times higher than with no curcumin. This difference widens to 30 times at 1.5:1 curcumin:protein, the highest ratio measurable in our instrument which suggests that multiple curcumin bound to a single protein further increases \( D \).

The Trp fluorescence data suggests one preferred binding site for curcumin is near position 94. Molecular mechanics simulations of Alzheimer’s peptides have shown that curcumin preferentially associates with alanine and other aliphatic residues (25). Between residues 60 and 100 there are 15 aliphatic residues (alanine and valine, plus L100), and in particular there are three alamines in a row at positions 89-91. We propose this as a possible binding site. Having made one or more bonds between the side chains and the curcumin, the aromatic rings of the molecule are then available to interact with any of the nearby hydrophobic residues, creating a hydrophobic cluster of residues close in sequence.

Thus it appears that one or more curcumin bound to \( \alpha \)-synuclein rescues the protein from the slow diffusion regime that promotes aggregation. Since the reaction-limited rates are correlated with temperature and the diffusion-limited rates are inversely correlated, by extension the chain volume and diffusion coefficient are inversely correlated. We conclude curcumin disrupts long-range interactions within the chain, allowing it to more quickly reconfigure. Fig. 6 shows a schematic of this behavior. Typically \( \alpha \)-synuclein is a fairly compact disordered protein with many long-range interactions within the chain (gray circles). This makes reconfiguration fairly slow (top row) and allows exposed hydrophobes to associate with other chains making oligomers, which eventually rearrange into larger fibrillar species. With the addition of curcumin (middle row), the chains become less compact and intramolecular interactions are more short range allowing faster reconfiguration. Faster reconfiguration allows the chains to escape from bimolecular association (bottom row) and prevents further aggregation steps.

Future work should investigate whether this property is common in aggregation inhibitors. For example, as a control experiment we have measured intramolecular diffusion of the protein in the presence of N-acetyl-Leucine, a hydrophobic amino acid and find the diffusion coefficient is unchanged (see figure S4), suggesting that curcumin’s ability to affect reconfiguration is somewhat unique.

This assay yields unique information about the mechanism of aggregation inhibition at the first step of process. More common assays, such as ThT fluorescence, are not sensitive to monomer/monomer interactions which are the preferred step for an inhibitor to act on. One potential danger with inhibiting a later step of aggregation pathway is that accumulation of a toxic intermediate could make toxicity worse (26). Therefore this measurement should become a common assay in the development of new Parkinson’s drug candidates that prevent aggregation at the first step.

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The abbreviations used are: αS, α-synuclein; Cur, Curcumin; NAC, non-amyloid-β component; TCEP, tris(2-carboxyethyl)phosphine; ThT, thioflavine T; TFE, trifluorethanol; NATA, N-acetyl-L-tryptophanamide

References

Figure Legends

Figure 1. Curcumin-α-synuclein binding as measured by (a) curcumin difference absorption at 404 nm, (b) curcumin fluorescence at 512 nm after exciting the protein at 430 nm and (c) and (d) tryptophan fluorescence at 355nm after exciting the samples at 295 nm. In panel a and b curcumin concentration is 10 μM and in panels c and d α-synuclein concentration is 10 μM. Lines are fits to Eq. S2 for (a) and (c) n=1 (black lines) and n=2 (grey lines) and for (b) as marked. All lines in (d) are for n=1.

Figure 2. Effect of curcumin on fibrillation ((a) and (b)) and soluble oligomer formation ((c) and (d)) of α-synuclein measured using ThT fluorescence and ellipticity at 217 nm as marked. (a) Kinetics of fibrillation in 25 mM phosphate buffer of pH 7.4, 150 mM NaCl, 1 mM TCEP, at the protein concentrations of 48 µM, upon stirring at 37 C (b) Far-UV CD spectra of native α-synuclein and products of fibrillation obtained in condition “a” in the absence and presence of curcumin (curcumin/α-synuclein molar ratio = 1.5) (c) Kinetics of oligomerization at the protein concentrations of 5 µM, in 10 % TFE (v/v), 25 mM phosphate buffer of pH 7.4, 1 mM TCEP at 25C measured using ThT fluorescence and ellipticity at 217 nm. (d) Far-UV CD spectra of reactants obtained at the protein concentration of 0.4µM in 10% TFE (v/v) and products formed at the protein concentrations of 5 µM in 10% TFE (v/v) of oligomerization process in the absence and presence of curcumin (curcumin/α-synuclein molar ratio = 1.5). Since at very low concentration (<0.5 µM) α-synuclein exists as monomer in the presence of TFE (12), the CD spectrum at 0.4 µM monomeric protein in 10 % TFE was measured to obtain the reactant of oligomerization.

Figure 3. Effect of curcumin on observed rates of tryptophan triplet decay in N-acetyl-L-tryptophanamide (NATA) and α-synuclein as marked.

Figure 4. Observed lifetimes of the tryptophan triplet state of 69C/94W mutant (a) in the absence of curcumin (b) in the presence of curcumin/α-synuclein molar ratio of 1.5:1 at various temperatures and viscosities. The lines are independent fits to viscosity at each temperature.

Figure 5. Reaction-limited (a) and diffusion-limited (b) rates for 69C/94W mutant in the absence and presence of various concentrations of curcumin. The diffusion-limited rates are calculated for the viscosity of buffer at each temperature. (c) Average Trp-Cys distance calculated using Eqs. 3 and 5. (d) Diffusion coefficients calculated from measured $k_{D,1}$ and Gaussian probability distributions using Eq. 4.

Figure 6. Schematic of the action of curcumin on α-synuclein on bimolecular association and subsequent aggregation steps.
FIGURE 1

(a) Graph showing the relationship between absorbance (OD) and [α-synuclein] (µM).
(b) Graph showing the relationship between curcumin fluorescence and [α-synuclein] (µM).
(c) Graph showing the relationship between tryptophan fluorescence and [curcumin] (µM).
(d) Graph showing the relationship between relative tryptophan fluorescence and [curcumin] (µM) at different temperatures (5, 15, 20, 25 °C).
FIGURE 2

(a) pH 7.4, 48 µM αS
- ThT, Cur/αS = 0
- ThT, Cur/αS = 1.5
- CD, Cur/αS = 0
- CD, Cur/αS = 1.5

(b) pH 7.4, 48 µM αS
- Cur/αS = 0 (5 min)
- Cur/αS = 1.5 (5 min)
- Cur/αS = 0, 143 hrs
- Cur/αS = 1.5, 143 hrs

(c) 10% TFE (v/v), 5 µM αS
- ThT, Cur/αS = 0
- ThT, Cur/αS = 1.5
- CD, Cur/αS = 0
- CD, Cur/αS = 1.5

(d) 10% TFE (v/v)
- Cur/αS = 0, 0.4 µM αS (5 min)
- Cur/αS = 1.5, 0.4 µM αS (5 min)
- Cur/αS = 0, 5 µM αS (70 min)
- Cur/αS = 1.5, 5 µM αS (70 min)
FIGURE 3

![Graph showing the relationship between [curcumin] (μM) and \( k_{ob}(1/s) \). The graph compares NATA and α-Synuclein.]
FIGURE 5

(a) Plot of $k_R$ (s$^{-1}$) vs. $T$ (C) for different ratios: 0:1, 0.5:1, 1:1, 1.5:1.

(b) Plot of $k_D$ (s$^{-1}$) vs. $T$ (C) for different ratios: 0:1, 0.5:1, 1:1, 1.5:1.

(c) Plot of $\langle \rho^2 \rangle^{1/2}$ (Angstrom) vs. $T$ (C) for different ratios: 0:1, 0.5:1, 1:1, 1.5:1.

(d) Plot of $D \times 10^6$ (cm$^2$ s$^{-1}$) vs. $T$ (C) for different ratios: 0:1, 0.5:1, 1:1, 1.5:1.
Without curcumin, slow diffusion

With curcumin, fast diffusion