α-Synuclein Structures Probed by 5-Fluorotryptophan Fluorescence and ^{19}F NMR Spectroscopy

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α-Synuclein, the main protein component of fibrillar deposits found in Parkinson's disease, is intrinsically disordered in vitro. Site-specific information on the protein conformation has been obtained by biosynthetic incorporation of an unnatural amino acid, 5-fluorotryptophan (5FW), into the recombinant protein. Using fluorescence and ^{19}F NMR spectroscopy, we have characterized three proteins with 5FW at positions 4, 39, and 94. Steady-state emission spectra (maxima at 353 nm; quantum yields ≈ 0.2) indicate that all three indole side chains are exposed to the aqueous medium. Virtually identical single-exponential excited-state decays ($\tau \approx 3.4$ ns) were observed in all three cases. Single ^{19}F NMR resonances were measured for W4, W39, and W94 at -49.0 ± 0.1 ppm. Our analysis of the spectroscopic data suggests that the protein conformations are very similar in the regions near the three sites.

Introduction

More than 15 000 proteins in the Swiss Protein Database are predicted to contain disordered regions of 40 or more residues. Moreover, at least 100 proteins currently are characterized as natively unfolded.[1−6] These unstructured polypeptides pose tremendous challenges to the traditional structure−function approach of biochemical science. α-Synuclein (α-syn), the main protein component of amyloid deposits found in Parkinson's disease (PD),[7,8] appears to be unstructured in vitro.[9] It is well established that α-syn becomes highly helical in the presence of acidic micelles or phospholipid vesicles,[10−15] suggesting that protein−membrane interactions may be important for function.[16,17] Furthermore, the fibrils implicated in PD are mostly β-sheets.[18] Interestingly, several different types of protofibrillar structures also have been characterized, including spheres and annuli, by atomic force microscopy.[19,20] The finding that α-syn undergoes large environmentally induced conformational changes is of great interest;[21] one or more of these species could disrupt cellular function, leading to disease. The development of spectroscopic probes of α-syn conformation is central to investigations of fibril formation.

Fluorescence energy transfer (FET) kinetics can be used to obtain distributions of donor−acceptor distances ($P(r)$) in biopolymers.[22−26] Our FET studies of α-syn have revealed that the protein adopts at least three conformations with significant populations of extended polypeptides, deviating from a random coil description at physiological pH.[27] In addition, perturbations of solution conditions produced only subtle redistributions of the structures within the protein ensemble. We recognize that these nanosecond time scale snapshots report only on equilibrium structures and that the time scales of large amplitude motions in the polypeptide chain could play a central role in the aggregation process implicated in pathogenesis. To characterize the conformational dynamics of the protein, we measured the rates of reaction between the triplet excited state of tryptophan, ^{3}W* and 3-nitro-tyrosine, Y(NO_2) in six different W−Y(NO_2) α-synucleins, probing loop sizes between 15 and 132 residues with contact times of 140 ns and 1.2 μs for 15 and 132 residue loops.[28] Our results from FET and electron transfer data establish that α-syn structures are highly dynamic, interchanging between collapsed and extended populations on the microsecond time scale.

We have extended our work on α-syn structures to include experiments employing an unnatural amino acid, 5-fluorotryptophan (5FW), as a dual fluorescence/^{19}F NMR spectroscopic probe. Unlike tryptophan whose fluorescent excited state decays via multiple pathways,[29−31] 5FW in proteins exhibits monoexponential luminescence decay kinetics.[32] A 5FW probe has been incorporated biosynthetically at each of three different aromatic sites (F4, Y39, F94) in α-syn, using glyphosate to inhibit cellular production of aromatic amino acids.[33] We report here an analysis of both fluorescence and ^{19}F NMR data acquired for the proteins.

Materials and Methods

N-(Phosphonomethyl)glycine (glyphosate) and 5-fluoro-D,L-tryphtophan were obtained from Sigma-Aldrich and used without further purification.

Preparation and Purification of 5-Fluorotryptophan-Containing α-Synucleins. The wild-type human α-syn expression plasmid (pRK172) was provided by M. Goedert (Medical Council Research Laboratory of Molecular Biology, Cambridge, U. K.).[34] Single Trp residues were introduced at three different aromatic-residue positions (F4, Y39, and F94) by site-directed mutagenesis. These proteins also had all three native Tyr residues in the C-terminus mutated to Phe residues (Y125F/Y133F/Y136F). All site-directed mutagenesis reactions were performed using a QuickChange kit (Stratagene). All mutations were confirmed by DNA sequencing (Caltech DNA Sequencing Core Facility). Circular dichroism (CD) spectra demonstrated that the mutations had no apparent effects on the protein structure in solution (disordered) or in the presence of micelles (α-helical) (data not shown).

For incorporation of 5-fluorotryptophan (5FW) into α-syn, *Escherichia coli* cells (BL-21(DE3)pLysS, Invitrogen) were

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grown in a modified new minimal medium (NMM).[35] Trace metals (Cu^{2+} and Mn^{2+}) and biotin were not used, but uracil (10 mg/L) was supplemented. A mixture of all amino acids except tryptophan and proline was added to a final concentration of 40 mg/L. All bacterial growths were under the selective pressure of 34 mg/L chloramphenicol and 100 mg/L ampicillin. A starter culture (25 mL) was inoculated with a freshly transformed single colony of bacterial cells, grown in Luria broth (LB) media overnight at 30 °C, and used further to inoculate 1 L of LB medium containing 20 mM glucose. As soon as the culture reached an $OD_{600\ nm} \approx 0.6-0.8$, the cells were harvested and washed once with M9 medium. Resuspended cells then were transferred to 2 L of NMM medium containing glyphosate (1 g/L) and 5-fluoro-D,L-tryptophan (40 mg/L). After 30 min, protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (ITPG) for 5−6 h at 30 °C.

Recombinant α-syn was purified according to published procedures.[27] Protein concentrations were determined using a molar extinction coefficient estimated on the basis of amino acid content: $\epsilon_{280} = 6980\ M^{-1}\ cm^{-1}$ (5FW4 and 5FW94) and $\epsilon_{280} = 5700\ M^{-1}\ cm^{-1}$ (5FW39).[36] The purity of all protein samples was assessed by SDS-PAGE on a Pharmacia Phast system using silver-staining methods. The protein molecular weights were confirmed by electrospray ionization mass spectrometry (ESI-MS) (Caltech Protein/Peptide Microanalytical Laboratory). Absorption and luminescence spectra were measured on a Hewlett-Packard 8452 diode array spectrophotometer and a Spex Fluorolog2 spectrofluorimeter, respectively. All purified proteins were concentrated using Centriprep YM-3 (MWCO 3kD) (Millipore) and stored at −80 °C.

Time-Resolved Fluorescence Measurements. Fluorescence decay kinetics measurements were carried out as previously described.[27] Protein samples (3−5 μM in 20 mM NaPi, pH 7.4) were deoxygenated by repeated evacuation/Ar fill cycles on a Schlenk line. Buffer solutions were filtered through 0.22 μm to remove any particulate matter. A polarized laser pulse (35° from vertical) from the third harmonic (292 nm) of a regeneratively amplified femtosecond Ti:sapphire laser (Spectra-Physics) was used as an excitation source, and a picosecond streak camera (Hamamatsu C5680) was used in the photon-counting mode for detection. 5FW emission was selected by an interference filter ($\lambda = 355 \pm 5$ nm). All experiments were conducted at 25 °C using a temperature-controlled cuvette holder. All protein samples were filtered through Microcon YM-100 (MWCO 100kD) (Millipore) spin filter units to remove oligomeric material prior to experiments.

Estimation of 5-Fluorotryptophan Incorporation. Model absorption spectra were constructed from individual amino acids, Phe, Tyr, *N*-acetyl-tryptophanamide (NATA), and 5FW in 20 mM NaPi buffer, pH 7.4. Two different total concentrations were used, 20 and 50 μM. Fluorescence lifetimes of 5FW with additions of NATA (10% and 20%) also were measured to determine the effect of the tryptophan chromophore.

^{19}F NMR. ^{19}F NMR spectra were obtained at 470.3 MHz with a Varian INOVA500 spectrometer. Sample volumes of 400−550 μL were used. All protein samples were exchanged into 20 mM sodium phosphate buffer, pH 7.4, using a PD-10 gel filtration column, concentrated to 50−70 μM using Microcon YM-3 (MWCO 3kD) (Millipore), and filtered through Microcon YM-100 (MWCO 100kD) (Millipore) spin filter units to remove oligomeric material prior to experiments. NMR samples were prepared with 10% D_2O (v/v) as the lock solvent. ^{19}F resonances were referenced to trifluoroacetic acid as an external standard (0 ppm). No pH correction was made for D_2O content; no proton decoupling was used. Spectra were collected in 1500 transient blocks over 2−11 h at 15 °C with the following parameters: relaxation delay of 1.0 s, pulse width of 7.6 μs, and sweep width of 20 kHz. For data processing, MestReC software was used with a 10 Hz line broadening exponential function.

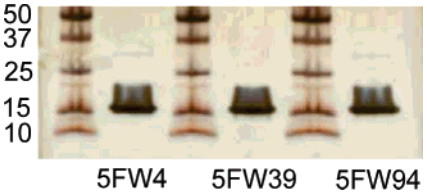

Figure 1. Representative SDS-PAGE analysis of purified 5FW-containing α-synucleins.

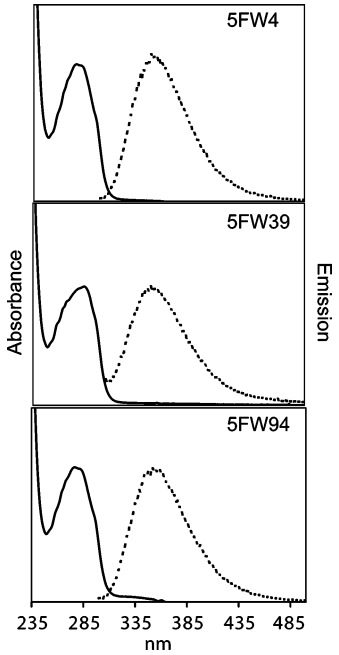

Figure 2. Absorption and emission spectra of 5FW-containing α-synucleins (20 mM sodium phosphate buffer, pH 7.4, 25 °C).

Results and Discussion

Tryptophan has been widely exploited as a fluorescent probe of protein microenvironment.[37−39] The emission properties of the indole side chain, which are extremely sensitive to solvent polarity, allowed us to investigate site-specific conformational changes in three Trp-labeled (4, 39, 94) α-synucleins (e.g., binding to SDS micelles yields a pronounced blue shift in the N-terminus (position 4) compared to positions 39 and 94). Since the Trp fluorescence decays were not well modeled by single-exponential functions ($\tau_1 \approx 4$ ns (35−50%); $\tau_2 \approx 1.6$ ns (35−50%); $\tau_3 \approx 0.5$ ns (<10%)[27]), we have turned to a better fluorophore, not only to simplify data analysis for the monomeric protein, but also to characterize the aggregation state of α-syn at various solution concentrations and during the aggregation process through reliable lifetime measurements. Recently, Broos and co-workers reported that seven out of eight 5FW-modified mannitol permease sites exhibit monoexponential fluorescence decay kinetics,[32] suggesting that 5FW is a promising candidate for α-syn fluorescence experiments. In addition, with ^{19}F incorporation, we can explore protein local conformations by NMR spectroscopy.

We elected to introduce 5FW into α-syn using glyphosate, an inhibitor of the 5-enolpyruvylshikimic acid-3-phosphate synthase reaction of aromatic amino acid biosynthesis.[33] Al-

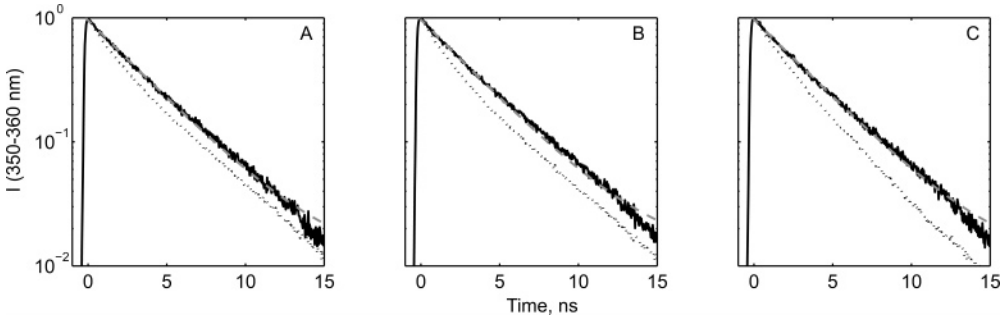

Figure 3. Decay of 5FW4 (A), 5FW39 (B), and 5FW94 (C) fluorescence in 20 mM sodium phosphate buffer, pH 7.4. Dashed gray lines are single-exponential functions used to fit the 5FW kinetics. Previously measured multiexponential Trp kinetics[27] are shown as dotted lines.

though 5FW is toxic to cells, sufficient growth could be obtained using previously published protocols.[33,36] Three 5FW α-syn variants were prepared and characterized: 5FW4/Y125F/Y133F/Y136F, 5FW39/Y125F/Y133F/Y136F, and 5FW94/Y125F/Y133F/Y136F. Sample homogeneity was assessed to be >95% pure by SDS-PAGE analysis (Figure 1). Electrospray mass spectrometry analyses confirm that the proteins are fluorinated (5FW4, 14 469 (calcd, 14 470); 5FW39, 14 451 (calcd, 14 454); 5FW94, 14 469 (calcd, 14 470)), and the presence of unlabeled protein is negligible. The substitution of a fluorine atom on the tryptophan ring produces a red shift in the absorption spectrum; this shift facilitates identification and quantification of the chromophore when incorporated into a protein. Accordingly, α-synucleins obtained from growths supplemented with glyphosate and 5FW exhibit characteristic absorption bands (Figure 2); they can be modeled adequately using spectra constructed from the individual aromatic amino acids, Phe, Tyr, and 5FW (data not shown). In combination with mass spectral and absorption data, we estimate that the incorporation yield is ∼90−95%.

All three 5FW probes (two incorporated at residues near the N-terminus (4 and 39) and one at the C-terminus, 94) exhibit fluorescence maxima at 353 nm (Figure 2), indicating that the indole side chains are largely water-exposed. (The corresponding 5-fluoro-D,L-tryptophan peak in water is at 355 nm.) The quantum yields were determined to be ∼0.2 for all three variants using 5-fluoro-D,L-tryptophan as a standard (0.15).[40] This increase in quantum yield also is manifested in a longer lifetime (3.4 ns) compared to 2.76 ns for the free amino acid (Figure 3). The 5FW fluorescence decay in the protein can be modeled by a single-exponential function (≥90% of the amplitude). As anticipated, comparisons to previous Trp measurements reveal that the fluorine substitution simplifies the decay kinetics. Under identical solution conditions (20 mM sodium phosphate buffer, pH 7.4), the Trp proteins require at least three exponential functions to model the excited-state decay (Figure 3). The complex excited-state decay pattern observed in Trp mutants of α-syn complicates the extraction of distance information from FET kinetics. The typical treatment of this problem involves the assumption that the nonexponential Trp* decay pattern arises from *local* conformational heterogeneity that has no effect on the longer-range distribution of donor−acceptor distances in the polypeptide ensemble.[41,42] Our observation of exponential 5FW* decay in α-syn provides independent experimental validation for this assumption and supports our prior analysis of Trp to 3-nitro-tyrosine energy transfer data. In this treatment, we modeled Trp* decay with the function $I(t) = I_o(t) \int P(r) \exp(k_{et}(r)t)\, dr$, where $I_o(t)$ is the Trp fluorescence decay in the absence of energy transfer, $P(r)$ is the probability of observing a conformation with donor−acceptor distance r, and $k_{et}(r)$ is the rate of energy transfer at distance r. If nonexponential Trp* decay arises from local conformation heterogeneity, then a single distance distribution function, $P(r)$, will describe the energy transfer kinetics of all conformers. In comparison to other 5FW-containing proteins,[32,43] the lifetimes obtained for α-syn are slightly shorter. (For example, the 5FW decays in 4−5 ns in mannitol permease.[32]) Our data reveal no differences in the local environments of the three sites, even though they are in different regions of the polypeptide.

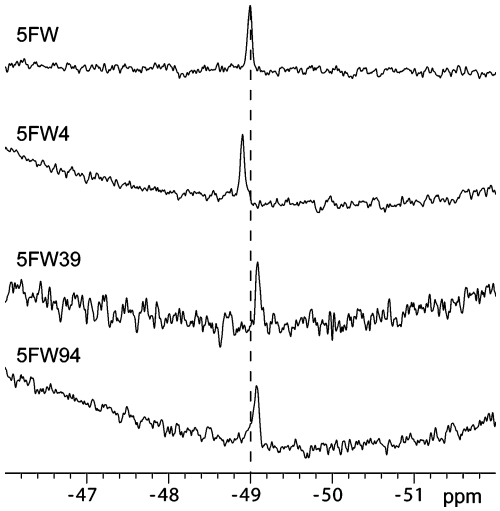

Figure 4. The 470 MHz ^{19}F NMR spectra of free 5FW and 5FW-containing α-synucleins. The spectra were collected at 15 °C to avoid protein aggregation. The top trace shows a peak at −49 ppm for free 5FW. Single resonances observed for all three variants (−48.9 ppm (5FW4), −49.1 ppm (5FW39), −49.1 ppm (5FW94)) are similar to that of the model complex.

We also have employed ^{19}F NMR to investigate α-syn conformations at positions 4, 39, and 94. ^{19}F NMR is particularly useful in studying larger proteins, because of the relatively large variations in fluorine chemical shifts (Phe, Tyr, and Trp); for example, shifts greater than 10 ppm have been observed in hen egg-white lysozyme for 4-fluorotryptophan and permit identification of individual resonances.[36,44−46] Proton-coupled ^{19}F NMR spectra of 5FW4, 5FW39, and 5W94 α-synucleins along with free 5FW are shown in Figure 4. Single resonances were observed in all three variants (−48.9 ppm (5FW4), −49.1 ppm (5FW39), and −49.1 ppm (5FW94)) near the chemical shift of the model complex, 5FW (−49.0 ppm) (Figure 4). The data confirm the absence of secondary and tertiary structure in labeled α-syn, as found with a chemically denatured 5FW-containing protein.[36] Although modest, the chemical shifts differ from one another, a finding that suggests that there are minor differences in the three protein microenvironments.

Conclusion

Although widely exploited as a fluorescent probe of protein environment and structure, Trp has complex photophysical properties. It is typical of Trp-containing peptides and proteins to exhibit nonexponential decay kinetics. This behavior attributable to multiple, slowly interconverting (>10 ns) side-chain conformations[30] as well as other decay pathways[31] complicates the extraction of distance information from FET kinetics. As an independent probe we have incorporated 5FW in engineered Trp sites in α-synuclein and demonstrated that the nonexponential Trp* decay pattern in the protein arises from *local* conformational heterogeneity. Importantly, these results validate our previous modeling methods for obtaining the distribution of donor−acceptor distances in the polypeptide ensemble.[27] As expected, 5FW is a superior fluorophore compared to Trp, and the presence of ^{19}F permits NMR investigations as well. Although both fluorescence and NMR experiments confirm that α-syn is a highly disordered polypeptide in solution, we anticipate that FET kinetics measurements using 5FW and 3-nitro-tyrosine as a donor−acceptor pair will be a powerful tool in characterizing intermediates during amyloid formation.

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