Pacific Symposium on Biocomputing 6:67-78 (2001)

AN NMR-BASED QUENCHED HYDROGEN EXCHANGE INVESTIGATION OF MODEL AMYLOID FIBRILS FORMED BY COLD SHOCK PROTEIN A

ANDREI T. ALEXANDRESCU

Department of Molecular and Cell Biology, University of Connecticut,
75 N. Eagleville Rd. U-3125, Storrs, CT 06269-3125, USA

Acid-denatured cold shock protein A (CspA) self–assembles into polymers with properties typical of amyloid fibrils. In the present work, a quenched hydrogen exchange experiment was used to probe the interactions of CspA fibrils with solvent. Exchange was initiated by incubating suspensions of fibrils in D_2O, and quenched by flash freezing. Following lyophilization, exchange-quenched samples were dissolved in 90% DMSO/10% D_2O, giving DMSO-denatured monomers. Intrinsic exchange rates for denatured CspA in 90% DMSO/10% D_2O (pH* 4.5) were sufficiently slow (~1 x 10^{-3} min^{-1}) to enable quantification of NMR signal intensity decays due to H/D exchange in the fibrils. Hydrogen exchange rate constants for CspA fibrils were found to vary less than 3-fold from a mean value of 5 x 10^{-5} min^{-1}. The uniformity of rate constants suggests that exchange is in the EX1 limit, and that the mechanism of exchange involves a cooperative dissociation of CspA monomers from fibrils, concomitant with unfolding. Previous studies indicated that the highest protection factors in native CspA are ~10^3, and that protection factors for the acid-denatured monomer precursors of CspA fibrils are close to unity. Because exchange in is in the EX1 regime, it is only possible to place a lower limit of at least 10^5 on protection factors in CspA fibrils. The observation that all amide protons are protected from exchange indicates that the entire CspA polypeptide chain is structured in the fibrils.

1. Introduction

Deposits of protein amyloid fibrils are associated with a number of pathologies including systemic amyloidosis[1], inflammation-associated reactive amyloidosis[2], type II diabetes[3], as well as a large variety of neurological disorders.[4] The precise role of protein amyloid fibrils in these diseases remains unresolved.[4,5] Protein amyloid fibrils exhibit similar morphologies by electron microscopy, have a characteristic cross-β X-ray fiber diffraction pattern, and stain with the dye Congo Red.[6] Because amyloid fibrils are formed from a large variety of proteins and peptides with no sequence homology,[7-9] the extent to which conserved morphologies and staining properties reflect conserved structural properties is an open question. Ultimately, an improved understanding of amyloid diseases at the molecular level will require high-resolution methods to characterize fibrils and intermediates in fibril formation.

Hydrogen isotope exchange experiments can provide information on protein stability, dynamics, folding, and association.[10-15] Recently, hydrogen exchange

methods have been used to investigate folded and partially folded monomeric precursors of amyloid fibrils, and to correlate the stability of structure in these species with the propensity to form fibrils.[12,13] Information on the hydrogen exchange properties of polymeric assemblies formed during fibrilogenesis is currently lacking. In cases where direct measurements of hydrogen exchange are precluded, it is sometimes possible to transfer the hydrogen exchange information encoded in amide proton NMR signal decays, to a form of the protein that is tractable to NMR spectroscopy. Quenched hydrogen experiments have a number of applications. These include hydrogen exchange measurements in short-lived protein folding intermediates[14], partially folded equilibrium intermediates with NMR spectra subject to severe line-broadening[15], and protein[11] or protein-membrane complexes[16] that are too large for direct NMR detection. To be applicable to amyloid fibrils, a quenched hydrogen exchange experiment must satisfy at least two requirements. First, the amyloid fibrils need to be converted to a form of the protein amenable to NMR, such as unfolded monomers. Second, hydrogen exchange must be sufficiently slow under the conditions of NMR detection to prevent loss of amide protons trapped in the fibrils. Dimethyl sulfoxide (DMSO)[a] is a strong protein denaturant that has been reported to solubilize β-amyloid fibrils formed *in vitro*.[17] Indeed, DMSO has shown therapeutic potential for some types of amyloidoses.[17] A second important feature of this solvent is that base-catalyzed exchange is substantially reduced in DMSO/D_2O mixtures containing volume fractions greater than 90% DMSO.[18] As shown by Roder and co-workers[20], intrinsic exchange rates in unstructured model peptides are shifted to an apparent minimum near pH* 5 in 90% DMSO/10% D_2O, and are reduced ~100-fold compared to those in pure D_2O. Exchange is thus sufficiently slow to detect amide protons in unfolded polypeptides that would otherwise be completely exchanged during the course of NMR measurements in D_2O.

The present work examines the applicability of quenched hydrogen exchange measurements to amyloid fibrils formed by CspA. Although CspA has no role in disease it serves as an effective model system for investigations of amyloid fibrils.[19,20] The native structure of CspA has been studied by both x-ray diffraction and NMR.[21,22] There is extensive information on the stability and folding kinetics of the protein.[23,24] Hydrogen exchange rates have been characterized for both native CspA, and for the acid-denatured monomer precursors of CspA fibrils.[20-25] In contrast to polypeptides that form fibrils spontaneously when dissolved in water[3], native CspA is a monomer at neutral pH,[21-25] and fibril formation can be triggered by a switch to pH 2.[20] These factors combined make CspA an ideal prototype for the quenched hydrogen exchange experiments described herein.

---

[a] Abbreviations: CspA, cold shock protein A; DCA, dichloroacetic acid; DCl, deuterium chloride; DMSO, dimethyl sulfoxide; D_2O, deuterium oxide; HSQC, heteronuclear single-quantum correlation; NaOD, sodium deuteroxide; pH*, apparent pH in mixtures of DMSO/H_2O or DMSO/D_2O, P_f, protection factor - the ratio of intrinsic to observed amide proton exchange rates ($P_f = k_{int}/k_{obs}$).

2. Materials and Methods

D_2O (isotopic purity > 99.98%) was from Glaser AG (Basel, Switzerland). Dichloroacetic acid (DCA), deuterium chloride (DCl), and d6-DMSO [$(CD_3)_2SO$] were from Aldrich. ^{15}N- and $^{15}N/^{13}C$-labled CspA were expressed in *E. coli* BL21(DE3) cells harboring the pET11-*cspA* vector[23], and purified as previously described.[19]

Initial experiments were used to establish that CspA fibrils are soluble in mixed DMSO/H_2O solvents. At volume fractions up to 50% DMSO, small particles of the translucent gel formed during fibrilogenesis remained undissolved. By contrast, fibrils dissolved in solvent mixtures containing volume fractions greater than 75% DMSO gave clear homogeneous solutions at pH* 7.0. ^{1}H-^{15}N HSQC spectra of CspA fibrils dissolved in >75% (v/v) DMSO showed chemical shifts and long T2 values characteristic of a denatured monomeric protein, and were closely similar to those of 'virgin' samples of the native protein dissolved directly in >75% DMSO.

A second series of experiments were used to establish that lyophilized CspA fibrils are soluble in 90% DMSO/10% H_2O. Solution pH was found to be a critical determinant of solubility. Lyophilized fibrils were soluble as long as the apparent solution pH was neutral or acidic (pH* between 7 and 3). In some cases, solutions of lyophilized fibrils dissolved directly in 90% DMSO/10% H_2O reached pH* values as high as 9, concomitant with precipitation of the protein. In addition to protein solubility, drifts in solution pH could affect intrinsic amide proton exchange rates. Consequently dichloroacetic acid (DCA) was incorporated as a pH buffer, as suggested by Roder and co-workers.[18]

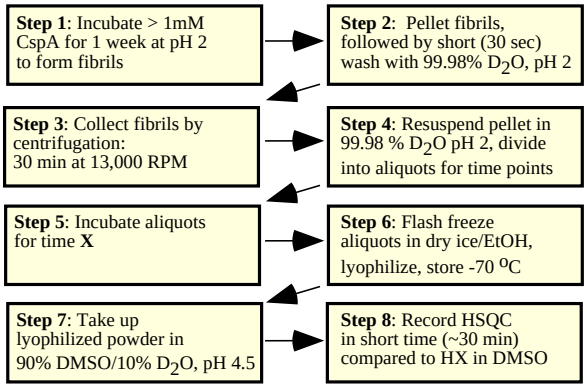

Figure 1. Scheme summarizing the quenched hydrogen exchange experiment used to characterize CspA fibrils.

The experimental protocol used to characterize hydrogen exchange in CspA fibrils is summarized in Fig. 1. A 3.1 mM solution of CspA in 180 µl of H_2O, was acidified from pH 5.8 to pH 2.0 with 1.7 µl of 4 N HCl. The solution was left undisturbed in a sealed Eppendorf tube at room temperature (22 °C) for 1 week.

After 1 week of incubation at pH 2.0, the fibrils were re-suspended in 1.4 ml of 99.98% D_2O (preadjusted to pH 2.0 with DCl), vigorously mixed for 30 sec with a *Vortex-Genie*, and collected by centrifugation for 30 min at 13,000 rpm using a desktop microcentrifuge. Analysis of the supernatant and pellet fractions by SDS-PAGE, showed that >90% of the protein was in the pellet. Following the D_2O wash, the pelleted fibrils were re-suspended a second time in 1.4 ml of 99.98% D_2O, vortexed for 30 sec, and divided into six aliquots of 250 µl for the hydrogen exchange experiment. The two serial re-suspensions of the initial 180 µl fibril sample into 1.4 ml of 99.98% D_2O, place an upper limit of 1.5% on the residual fraction of H_2O in the D_2O aliquots used for the hydrogen exchange experiments. The actual figure is probably less than 1% H_2O, since the volume of the fibril pellet after the first re-suspension in D_2O was ~100 µl.

The six aliquots of CspA fibrils suspended in 99.98% D_2O (pH 2.0), were incubated undisturbed at room temperature for periods ranging from 30 minutes to 9.5 days. After each incubation period, the aliquot was immediately flash-frozen in a dry ice/ethanol bath, lyophilized, and stored at –70 °C for subsequent use.

For analysis by ^{1}H-^{15}N HSQC NMR spectroscopy, lyophilized CspA fibrils were dissolved in 220 µl of a "read-out buffer" consisting of 90% DMSO/10% D_2O, 20 mM DCA, pH* 4.5. The read-out buffer contained D_2O rather than H_2O to prevent back-exchange of protons into the protein. Assuming an insignificant loss of protein during the quenching protocol, each of the six NMR samples had a protein concentration of 0.4 mM in 220 µl of the 90% DMSO/10% D_2O read-out buffer. A value of pH* 4.5 was chosen for the NMR experiments, close to the ~ pH* 5 minimum for intrinsic exchange rates in 90% DMSO/10% D_2O.[18] It was found that the pH* of the read-out buffer consistently increased by ~0.6 pH units on addition of the lyophilized fibrils. The pH* of the read-out buffer was thus pre-adjusted to a value of 3.9 with DCl and NaOD, prior to the addition of lyophilized protein. The pH* value of each of the six dissolved fibril samples was checked after acquisition of ^{1}H-^{15}N HSQC spectra, and was invariant within the range between pH* 4.4 and 4.5. Based on model peptide studies, intrinsic exchange rates in 90% DMSO/10% D_2O at pH* 4.5 and a temperature of 10 °C, are predicted to be ~0.0003 min^{-1}, or 100-fold smaller than the corresponding intrinsic exchange rates in D_2O at pH 4.5.[18] Intrinsic exchange rates were measured for an ^{15}N-labled CspA sample under the conditions of this study (90% DMSO/10% D_2O, 20 mM DCA, pH* 4.4, 10 °C). The intrinsic exchange rates for backbone amide protons span a range from 0.03 to 0.0002 min^{-1}, with a median value of 0.003 min^{-1} (not shown). The indole nitrogen proton of Trp11 gives the fastest measurable rate (0.04 min^{-1}), while exchange rates of side-chain amide protons from 2 Gln and 3 Asn residues in CspA are too fast to measure.

NMR data were recorded on Bruker and Varian spectrometers operating at 600 MHz. All NMR samples contained solution volumes of ~220 µl, transferred into 5 mm susceptibility-matched Shigemi tubes (Allison Park, PA). In order to obtain residue-specific information on hydrogen exchange in CspA fibrils, ^{1}H and ^{15}N NMR resonances of denatured CspA in 90% DMSO were assigned using a 3D-HNCACB experiment.[26] The 3D-HNCACB experiment was recorded at 25 °C on a virgin 0.6 mM $^{13}C/^{15}N$-labeled CspA sample dissolved in 90% d6-DMSO/10%

H_2O, 20 mM DCA, pH* 4.5. This buffer corresponds to the protonated analogue of the DMSO/D_2O read-out buffer used to monitor hydrogen exchange in CspA fibrils. Since DMSO is an aprotic solvent, amide protons from the DMSO-denatured protein can only exchange with the H_2O component of the solution. Consequently, exchange labile sites are fully protonated in DMSO/H_2O. The 3D-HNCACB experiment was acquired with 1024 x 88 x 32 complex points (spectral widths of 6000 x 8400 x 1380 Hz) for the ^{1}H x ^{13}C x ^{15}N dimensions. d6-DMSO was used for the deuterium lock.

To analyze the decay of amide proton signal intensities due to hydrogen exchange from fibrils incubated in D_2O at pH 2.0, ^{1}H-^{15}N HSQC spectra were recorded on six lyophilized exchange-quenched samples dissolved in a pH* 4.5 90% d6-DMSO/10% D_2O, 20 mM DCA read-out buffer. A temperature of 10 °C was selected in order to minimize amide proton exchange with the D_2O component of the 90% DMSO/10% D_2O buffer. The HSQC spectra were recorded with 1024 x 128 complex points (spectral widths of 7508 x 1338 Hz) for the ^{1}H x ^{15}N dimensions. Placing the deuterium lock on D_2O was found to result in an artifactual attenuation of signals in the downfield region of the spectrum that could be misinterpreted as hydrogen exchange. Consequently, the d6-DMSO signal was used for the deuterium lock. The ^{1}H carrier frequency was placed at 4.7 ppm. The ^{1}H-^{15}N HSQC spectra employed pulse field gradients for coherence selection, and suppression of signals from the solvent.[26] Acquisition of each of the six ^{1}H-^{15}N HSQC spectra was completed in times randomly distributed between 26 and 31 min from the time of dissolving the exchange quenched samples in the 90% DMSO/10% D_2O read-out buffer. In order to interfere with measurements of amide proton intensities, exchange in the 90% DMSO/10% D_2O read-out buffer would have to be significant on time scales comparable to the <5 min differences in the recording of the HSQC spectra. The fastest exchanging amide proton in CspA (Trp11 indole) has an exchange half-life of 25 min in the read-out buffer under these conditions. The majority of backbone amide protons (94%) have exchange half-lives longer than 1.5 hr, so that exchange is negligible compared to the 5 min differences in the acquisition of the HSQC spectra.

Hydrogen exchange rates were obtained from decays of ^{1}H-^{15}N HSQC crosspeak intensities as a function of exchange time, defined as the period from the suspension of CspA fibrils in D_2O, to the quenching of hydrogen exchange by flash-freezing the samples in dry ice/ethanol. The intensity of each ^{1}H-^{15}N HSQC crosspeak was determined from the 2D data matrix point with the largest value, using the program Felix 98. Crosspeak maxima were determined separately for each spectrum to compensate for possible small variations in chemical shifts. Rate constants for exchange were obtained from non-linear least-squares fits of cross peak intensity decays as a function of exchange-time, to the single exponential function:

$$I = I_O \exp(-k_{ex}\, t) \qquad (1)$$

The initial intensity (I_O), and the exchange rate constant (k_{ex}), were free variables in the fits. Uncertainties in k_{ex} values were taken from the standard errors of the fits.

3. Results

Assignments for CspA in 90% DMSO/10% H_2O were obtained from a 3D HNCACB spectrum[26] recorded at a temperature of 25 °C. The HNCACB experiment has proven extremely useful for assigning NMR spectra of denatured proteins, including the acid- and urea-denatured forms of CspA.[19] In the case of DMSO-denatured CspA, assignments could be established for 64 of the 67 backbone ^{1}H-^{15}N correlations (96%) expected from the amino acid sequence of the protein (Fig. 2).

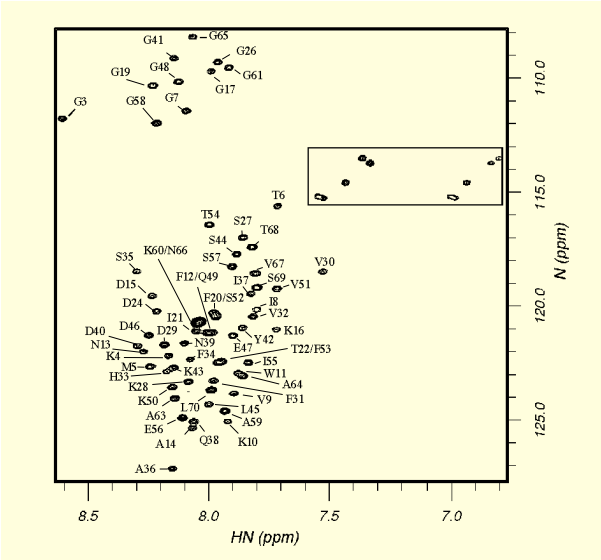

Figure 2. ^{1}H-^{15}N HSQC spectrum of ^{13}C/^{15}N-labeled CspA freshly dissolved in 90% d6-DMSO/10% H_2O, 20 mM DCA, pH* 4.5, 25 °C. The protein is fully protonated in the DMSO/H_2O buffer and all amide protons expected from the sequence of CspA are observed. NMR assignments are based on a 3D-HNCACB experiment recorded on the same sample. The rectangle highlights side-chain correlations from the three Asn and two Gln residues in CspA.

To minimize contributions from intrinsic amide proton exchange, a lower temperature of 10 °C was used to record ^{1}H-^{15}N HSQC spectra of the six exchanged-quenched fibril samples dissolved in 90% DMSO/10% D_2O (Fig. 3a,b). NMR assignments were readily transferred from 25 °C to 10 °C, however, a larger proportion of signals were partially or completely overlapped at the lower temperature. Of 64 assigned ^{1}H-^{15}N correlations, 16 were insufficiently resolved to

enable accurate measurements of signal intensities. In total, hydrogen exchange rates were obtained for 48 of the 70 residues (69 %) in the protein (Fig. 4b).

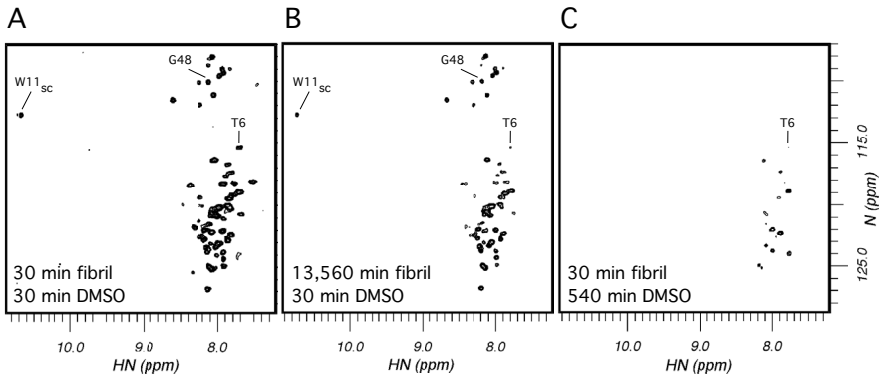

Figure 3. Representative time points from the experiment used to monitor hydrogen exchange in CspA fibrils. (**A**) The shortest exchange time point obtained for a 30-min incubation of CspA fibrils in D_2O. (**B**) The longest exchange time point for a 9.5-day incubation of CspA fibrils in D_2O. (**C**) Control experiment recorded on the sample in (A) after incubation for 9 hr at 22 ^oC in the 90% DMSO/10% D_2O read-out buffer, illustrating the slow intrinsic amide proton exchange rates of denatured CspA in 90% DMSO/10% D_2O. The unique indole ^1H-^{15}N correlation of Trp11 ('$W11_{sc}$') is aliased in the ^{15}N-dimension of the HSQC spectrum (^1H – 10.68 ppm, ^{15}N – 134.7 ppm).

Figure 3A shows the ^1H-^{15}N HSQC spectrum for the fibril sample incubated for the shortest time in D_2O (30 min). The side-chain amide protons from 3 Asn and 2 Gln residues are fully exchanged in the 90% DMSO/10% D_2O read-out buffer, during the course of the HSQC experiment (compare Figs. 2 and 3), so the extent of protection of these sites in the fibrils could not be determined. By contrast, all of the backbone amide protons observed for fully protonated CspA in 90% DMSO/10% H_2O (Fig. 2), are also observed in the spectrum of the exchange-quenched sample in 90% DMSO/10% D_2O (Fig. 3a). Figure 3B shows the ^1H-^{15}N HSQC spectrum for the fibril sample incubated for the longest time in D_2O (9.5 days). The spectra in Fig. 3 were recorded with the same acquisition parameters, and are plotted at the same contour levels for direct comparison. At lower contour levels, the spectrum in Fig. 3B shows all of the correlations observed in Fig. 3A. Over the 9 day D_2O-exchange period separating the spectra in Fig. 3A and 3B, backbone amide proton crosspeak intensities decrease to between 66% and 27% of their initial values, with a mean decrease of 50%. Representative data for the decays of amide proton signal intensities as a function of fibril incubation time in D_2O are shown in Fig. 4A. The indole nitrogen proton of the unique tryptophan at position 11 ($W11_{sc}$ in Fig. 3a,b) exchanges with a rate comparable to those of the backbone amide protons.

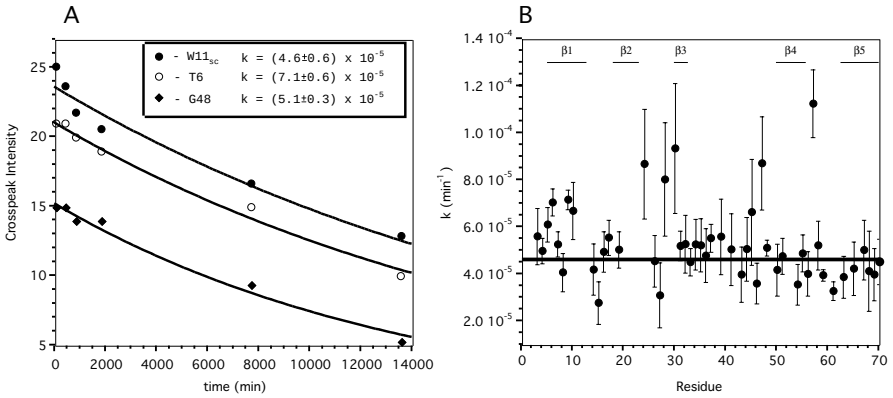

Figure 4. Amide proton exchange kinetics. (**A**) Crosspeak intensities as a function of fibril incubation time in D_2O for the representative amide protons indicated in Fig. 3: ● - Trp11 side-chain, ❍ - Thr6 backbone, ◆ - Gly48 backbone. Curves represent nonlinear least-squares fits of the decay data to a single exponential function (Eq. 1). (**B**) Amide proton exchange rates as a function of position in the amino acid sequence of CspA. Note that all backbone amide protons are protected in the CspA fibrils. The gaps in the graph represent two proline residues in CspA, three unassigned amide protons, and sixteen amide protons for which NMR spectral overlap precluded an accurate estimation of exchange rates. The horizontal line indicates the mean of the hydrogen exchange rate constants.

Amide proton protection in CspA fibrils is remarkably uniform (Fig. 3A,B). A concern in light of this observation is that the amide proton signals detected in ^{1}H-^{15}N HSQC spectra might be due to a residual background of fully protonated CspA molecules. The CspA fibrils used for the quenched hydrogen exchange experiment were formed in an initial volume of 180 µl H_2O, and diluted twice in 1.4 ml of 99.98% D_2O. Based on this protocol it is possible to place an upper limit of at most 1.5% H_2O in the exchange-quenched samples used to read-out the hydrogen exchange kinetics of CspA fibrils. Estimating that the protein concentration of the exchange-quenched samples is at most 0.4 mM CspA in 90% DMSO/10% D_2O, places an upper limit on the concentration of protonated CspA molecules of 0.006 mM. This concentration of protein is too dilute to detect within the sensitivity of the ^{1}H-^{15}N HSQC spectra. The exponential decays of amide proton intensities as a function of fibril incubation time in D_2O (Fig. 4a), and the complete exchange of Asn and Gln side-chain protons in the 90% DMSO/10% D_2O read out buffer, are inconsistent with signals originating from a residual background of protonated CspA molecules. A more direct experimental control is provided by the data in Fig. 3c. The ^{1}H-^{15}N HSQC spectrum in Fig. 3c was recorded for the same exchange-quenched CspA sample as in Fig. 3a, after 9 hr of incubation at 22 °C in the 90% DMSO/10% D_2O buffer. The latter spectrum evinces a substantial loss of amide proton signal intensities, as intrinsic exchange in 90% DMSO/10% D_2O starts to make a significant contribution (signal intensities range from 70% to less

than 3% of the initial values in Fig. 3A). If signals originated from a background of protonated CspA molecules, intensities would remain constant as a function of time in 90% DMSO/10% D_2O.

Figure 4B summarizes hydrogen exchange rate constants for CspA fibrils calculated from Equation 1. The rate constants span only a four-fold difference in magnitude, from a maximum of 1.1×10^{-4} to a minimum of 2.8×10^{-5} min^{-1}. Of 48 residues for which exchange rates could be accurately determined, 29 (60%) have rate constants within experimental uncertainty of the mean of 5.3×10^{-5} min^{-1}.

Discussion

Exchange rate constants in proteins can vary as much as 10^8 between protons in regions of stable hydrogen-bonded secondary structure and protons in surface exposed loops.[27] In marked contrast, exchange rate constants obtained for CspA fibrils vary by less than a factor of 4 (Fig. 4B). The uniformity of exchange rate constants can be appreciated directly from the observations that all backbone amide protons are observed after 9 days in D_2O, and that decreases in HSQC signal intensities as a function of exchange time are similar for most sites in the protein (Fig. 3A,B).

NMR hydrogen exchange data are usually interpreted in terms of the model

$$\text{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{open} \xrightarrow{k_{int}} \text{exchanged} \qquad (2)$$

where "closed" and "open" refer to individual amide protons in exchange-resistant and exchange-susceptible conformations, respectively.[10,27,29] The observed hydrogen exchange rate is

$$k_{obs} = \frac{k_{op} k_{int}}{(k_{cl} + k_{int})} \qquad (3)$$

where k_{cl} is the closing rate and k_{op} is the opening rate. The intrinsic rate (k_{int}) depends on factors such as pH, temperature, and the location of an amide proton in the protein sequence.[29] There are two limiting cases of this model.[10,27,29] In the EX1 limit $k_{cl} << k_{int}$, and observed rates are determined by those for the opening reactions ($k_{obs} = k_{op}$). In the EX2 limit $k_{int} << k_{cl}$, and observed rates are proportional to the equilibrium constants $K_{HX} = (k_{op}/k_{cl})$ relating the concentrations of *open* and *closed* conformations [$k_{obs} = (k_{op}/k_{cl})k_{int}$]. The uniform exchange rate constants observed for CspA fibrils are most consistent with an EX1 mechanism.[10] Intrinsic rate constants (k_{int} values) calculated[29] for CspA at

pH 2.0 and 22 °C, vary by more than two orders of magnitude from 6.0 to 0.01 min^{-1}. The k_{int} value for the indole nitrogen proton of Trp11 can be estimated[29] to be on the order of 500 min^{-1}. An EX2 mechanism would require that the ratio k_{op}/k_{cl} coincidentally varied from residue to residue as the inverse of k_{int}, so as to give a product [$k_{obs} = (k_{op}/k_{cl})k_{int}$] that is uniform for all amide protons (Fig. 4B). A much more tenable possibility is that exchange is in the EX1 limit ($k_{obs} = k_{op}$), and thus independent of k_{int}.[b]

The EX1 limit is typically observed only under conditions that promote high values of k_{int}, such as high pH and temperature.[10,27,29] This is because the closing (e.g. folding) rates of proteins often have values higher than 10^4 min^{-1}. Consequently, the EX1 limiting condition ($k_{cl} << k_{int}$) is rarely satisfied at neutral or acidic pH. In the present case, conversion of exchange-susceptible denatured monomers to exchange–resistant fibrils involves structure formation coupled to intermolecular assembly. Although the kinetics for these processes are unknown, NMR measurements on a 0.72 mM solution of acid denatured CspA indicate that during the exponential phase of fibril growth monomers are lost at a rate of 10^{-3} min^{-1}.[20] It is entirely probable that under the conditions of the hydrogen exchange measurements, the re-association of unfolded CspA molecules into fibrils proceeds with rates considerably smaller than intrinsic proton exchange rates at pH 2.0 (e.g. $k_{cl} << 0.1$ min^{-1}).

An EX1 mechanism alone cannot account for the observation that exchange rates in CspA fibrils vary by less than a factor of 4. In typical proteins, EX1 exchange is observed only for the subset of slowest exchanging protons. Exchange rates within this subset, however, can vary by more than 5 orders of magnitude.[28,29] The uniform exchange rate constants for CspA fibrils strongly suggest that exchange occurs through a highly cooperative process, such as depolymerization of CspA molecules from fibrils into exchange-susceptible unfolded monomers. If dissociation involved only CspA monomers on the surface of a fibril, this should be reflected in an asymmetric pattern of protection. Amide protons exposed to solvent in the fibril state, should exchange faster than protons buried in protein association interfaces and thus give larger apparent exchange rates. That exchange rates are uniform suggests that dissociation of exchange-susceptible CspA molecules from the fibrils is cooperative, such that on average the distinction between monomers on the surface and in the interior of the fibrils is lost.

The time course of CspA fibril formation at pH 2.0 is described by a lag phase followed by an exponential increase in polymerization.[20] ^{1}H-^{15}N HSQC spectra recorded during the course of fibril formation show a single set of signals corresponding to soluble acid-denatured monomers. As the protein becomes incorporated into aggregates that are too large for NMR detection, ^{1}H-^{15}N HSQC correlations decay to a baseline value reflecting the solubility limit of the acid-denatured monomers.[20] Hydrogen exchange rates were measured for a 0.4 mM

---

[b] EX1 and EX2 mechanisms can usually be distinguished from the pH dependence of k_{obs}.[10,27-29] Such an approach, however, would require verification that the structure and stability of CspA fibrils is invariant to pH.

solution of CspA in D_2O, immediately after acidification to pH 2.0.[20] The hydrogen exchange experiment was completed in ~1 hr, a timescale well within the ~11 hr lag phase for fibril formation of the 0.4 mM CspA sample. Exchange rates measured for 22 of the 67 backbone amide protons in CspA were on the order of 0.1 min^{-1}. These fast exchange rates preclude a contribution from denatured CspA monomers to the protection observed in CspA fibrils at pH 2. Protection factors ($P_f = k_{int}/k_{obs}$) for acid-denatured CspA monomers were close to 1. The P_f values of unity indicate that the stability of structure in acid-denatured CspA monomers is insufficient to provide significant protection of amide protons from solvent exchange, and supports a mechanism in which hydrogen exchange from fibrils occurs through dissociation into monomers that are unfolded at pH 2.

Data on hydrogen exchange rates of native CspA were obtained at pH 5.4.[25] In native CspA, the most strongly protected amide protons come form strands β1-β4, and show protection factors in the range between 10^2 and 10^3. With the exception of Leu70, hydrogen exchange rates for amide protons in loops and in strand β5 are too fast to measure in water.[25]

Hydrogen exchange in CspA fibrils is in the EX1 regime, so that $k_{obs} = k_{op}$. Nevertheless, it is possible to put an upper limit on the value of the equilibrium constant relating the concentrations of exchange-susceptible (*open*) and exchange-resistant (*closed*) conformations, $K_{HX} = (k_{op}/k_{cl})$. From the definition of EX1 exchange, $k_{cl} << k_{int}$. It follows that $K_{HX} << k_{op}/k_{int}$. The protection factor ($P_f = 1/K_{HX}$), similarly corresponds to the lower limit $P_f >> k_{int}/k_{obs}$. Intrinsic amide proton exchange rates calculated for the CspA sequence at pH 2.0 and 22 °C, place a lower limit on P_f values in CspA fibrils of ~10^5. The P_f values in CspA fibrils are thus at least 100-fold greater than the largest in native CspA. The intrinsic exchange rate for the indole nitrogen proton of Trp11 can be estimated to be ~500 min^{-1}.[29] This rate is 100-fold larger than that of any backbone amide protons, and suggests a P_f value that in excess of ~10^7.

Based on time-dependent changes in ^{15}N T2 relaxation parameters, it was suggested that soluble aggregates formed during the exponential phase of fibril growth were predominantly stabilized through mispairing of the N-terminal half of CspA, corresponding to strands β1-β3 in the native structure.[20] The C-terminal half of the protein was suggested to be flexible in these aggregates.[20] The present observation that all amide protons are protected from solvent exchange indicates that in mature fibrils the entire polypeptide chain is involved in stable structure.

Acknowledgement

I thank Klara Rathgeb-Szabo for preparing CspA samples, Dr. Wolfgang Jahnke for recording the 3D HNCACB spectrum used to assign DMSO-denatured CspA, and Drs. J.L. Markley, D. Shortle, and P.L Yeagle for helpful discussions. This work was supported by grants from the Swiss NSF and by start-up funds from the University of Connecticut.

1. M.B. Pepys et al., *Nature* **362**, 553 (1993).
2. S. Urieli-Shoval, R.P. Linke, and Y. Matzner, *Curr. Opin. Hematol.* **7**, 64 (2000).
3. G.J.S. Cooper et al., *Proc. Natl. Acad. Sci. USA* **84**, 8628 (1987).
4. E.H. Koo, P.T. Lansbury, and J.W. Kelly, *Proc. Natl. Acad. Sci. USA* **96**, 9989 (1999).
5. J. Janson, R.H. Ashley, D. Harrison, S. McIntyre, and P.C. Butler, *Diabetes* **48**, 491 (1999).
6. M. Sunde and C. Blake, *Adv. Prot. Chem.* **50**, 123 (1997).
7. J.C. Rochet and P.T. Lansbury, *Curr. Opin. Struct. Biol.* **10**, 60 (2000).
8. N.D. Lazo and D.T. Downing, *Biochem. Biophys. Res. Commun*. **253**, 675 (1997).
9. K. Janek, et al., *Biochemistry* **38**, 8246 (1999).
10. Y. Bai, T.R. Sosnick, L. Mayne, and S.W. Englander *Science* **269**, 192 (1995).
11. Y.Paterson, W.S. Englander, and H. Roder, *Science* **249**, 755 (1990).
12. E.J. Nettleton, and C.V. Robinson *Methods Enzymol.* **309**, 633 (1999).
13. L.L. Hosszu, et al., *Nature: Struct. Biol.* **6**, 740 (1999).
14. J.B. Udgaonkar, and R.L. Baldwin, *Nature* **335**, 694 (1988).
15. A.K. Chamberlain, and S. Marqusee *Biochemistry* **37**, 1736 (1998).
16. L. Czerski, O. Vinogradova, and C.R. Sanders *J. Magn. Reson.* **142**, 111 (2000).
17. W. Regelson, and S.W. Harkins *Ann. N. Y. Acad. Sci.* **826**, 348 (1997).
18. Y-Z. Zhang, Y. Paterson, and H. Roder, *Protein Sci.* **4**, 808 (1995).
19. A.T. Alexandrescu, and K. Rathgeb-Szabo *J. Biomol. NMR* **11**, 461 (1998).
20. A.T. Alexandrescu, and K. Rathgeb-Szabo *J. Mol. Biol.* **291**, 1191 (1999).
21. H. Schindelin, W. Jiang, M. Inouye, and U. Heinemann, *Proc. Natl. Acad. Sci. USA* **91**, 5119 (1994).
22. W. Feng, R. Tejero, D.E. Zimmerman, M. Inouye, and G.T. Montelione, *Biochemistry* **37**, 10881 (1998).
23. S. Chatterjee, W. Jiang, S.D. Emerson, and M. Inouye, *J. Biochem.* **114**, 663 (1993).
24. K.L. Reid, H.M. Rodriguez, B.J. Hillier and L.M. Gregoret *Protein Sci.* **7**, 470 (1998).
25. V.A. Jaravine, K. Rathgeb-Szabo and A.T. Alexandrescu, *Protein Science* **9**, 290 (2000).
26. L.E., Kay, *Prog. Biophys. Molec. Biol.* **63**, 277 (1995).
27. T.G. Pedersen, N.K. Thomsen, K.V. Andersen, J.C. Madsen, and F.M. Poulsen, *J. Mol. Biol.* **230**, 651 (1993).
28. C.B. Arrington, and A.D. Robertson, *J. Mol. Biol.* **296**, 1307 (2000).
29. Y. Bai, J.S. Milne, L. Mayne, and S.W. Englander, *Proteins* **17**, 75 (1993).

Pacific Symposium on Biocomputing 6:67-78 (2001)