**Supplementary Information** 

## Kinetics of Fast Tetramerization of the Huntingtin Exon 1 Protein Probed by Concentration-Dependent On-Resonance $R_{1\rho}$ Measurements

Alberto Ceccon, Vitali Tugarinov\* and G. Marius Clore\*

Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0520

\*Address correspondence to these authors:

G.M.C., mariusc@mail.nih.gov; V.T., vitali.tugarinov@nih.gov

## **Materials and Methods**

**NMR sample preparation**. U-[<sup>15</sup>N/<sup>13</sup>C]-labeled huntingtin exon-1 protein (htt<sup>ex1</sup>) was expressed and purified as described previously.<sup>1</sup> Following purification, the lyophilized htt<sup>ex1</sup> fractions were dissolved in a 1:1 trifluroacetic acid (TFA):hexafluoroisopropanol (HFIP) solvent mixture to ensure complete removal of pre-existing aggregates that can seed the aggregation of monomeric polypeptides.<sup>2</sup> The solvent mixture was removed under a stream of N<sub>2</sub> gas and the resulting peptide film further lyophilized for 16 h to ensure complete removal of both solvents. Prior to final lyophilization, the peptide film was dissolved in 0.1 mM TFA. All NMR samples of htt<sup>ex1</sup> were prepared by dissolving an aliquot of the lyophilized peptide in a 13.8 mM monobasic sodium phosphate buffer, pH 4.6, containing 50 mM NaCl in 10% D<sub>2</sub>O/90% H<sub>2</sub>O (v/v). The initial use of low pH serves to improve peptide solubility.<sup>2</sup> The pH of the buffer was subsequently adjusted to 6.5 by adding dibasic sodium phosphate for a final sodium phosphate buffer, pH 6.5, 50 mM NaCl, 10% D<sub>2</sub>O/90% H<sub>2</sub>O (v/v)) to the appropriate final concentrations. Since htt<sup>ex1</sup> does not contain any tryptophan, its concentration was determined from the absorbance at 205 nm<sup>3</sup> as described previously.<sup>1</sup>

**NMR spectroscopy.** All NMR experiments were recorded at 5 °C using an 800 MHz Bruker Avance-III spectrometer, equipped with a TCI triple resonance *z*-axis gradient cryogenic probe. All NMR data were processed using the NMRDraw-NMRPipe software package.<sup>4</sup> For exchange-induced chemical shift measurements, the time domain in the indirect dimension (<sup>15</sup>N or <sup>13</sup>C) was extended two-fold through the application of sparse multidimensional iterative lineshape-enhanced (SMILE) reconstruction.<sup>5</sup>

Measurement of <sup>13</sup>C $\alpha$  and <sup>15</sup>N exchange-induced chemical shifts ( $\delta_{ex}$ ). Changes in <sup>13</sup>C $\alpha$  and <sup>15</sup>N chemical shifts as a function of htt<sup>ex1</sup> concentration were obtained from 2D constant-time (CT) <sup>1</sup>H-<sup>13</sup>C and 2D <sup>1</sup>H-<sup>15</sup>N heteronuclear single quantum coherence (HSQC) spectra as described previously.<sup>1</sup> Briefly, serial dilutions of <sup>15</sup>N/<sup>13</sup>C-labeled htt<sup>ex1</sup> from 1.4 mM to 50  $\mu$ M were obtained by addition of the NMR buffer (see above), with the protein concentration verified at each dilution step. The <sup>15</sup>N/<sup>13</sup>C $\alpha$ - $\delta_{ex}$  values at peptide concentration *i* were calculated as,  $\delta_{ex}(i) = \delta_{obs}(i) - \delta_{ref}$ , where  $\delta_{obs}$  is the observed chemical shift and  $\delta_{ref}$  the chemical shift at a concentration of 50  $\mu$ M. Measurement time for each <sup>1</sup>H-<sup>15</sup>N HSQC and <sup>1</sup>H-<sup>13</sup>C HSQC spectrum was ~2 and ~3 hrs, respectively.

<sup>15</sup>N- $R_{1\rho}$  measurements. <sup>15</sup>N- $R_{1\rho}$  and <sup>15</sup>N- $R_1$  data were collected using the pulse schemes and procedures described previously.<sup>6</sup> At each protein concentration, on-resonance <sup>15</sup>N- $R_{1\rho}$  values were obtained at three spin-lock (SL) radiofrequency (RF) field strengths: 750, 1,500 and 3,000 Hz using two-time-point measurements. The measurement time was ~6 hours per RF field strength. <sup>15</sup>N- $R_{2,eff}$  values were calculated from the relationship,  $R_{2,eff} = (R_{1\rho} - R_1 \cos^2 \theta)/\sin^2 \theta$ , where  $\theta$  is the angle subtended by the direction of the effective spin-lock field with respect to the *z*-axis of the laboratory frame (external magnetic field B<sub>0</sub>).

A 'two-time-point' measurement was employed for  $R_{1\rho}$ . When exchange is slow on the time-scale of spin relaxation, the component of the eigenvector in the solutions of the differential equations provided by Yuwen et al.,<sup>7</sup> that is responsible for the non-single-exponentiality of the magnetization decay (' $C_{\text{fast}}$ ' in Yuwen et al.,<sup>7</sup> or ' $C_{\text{fast}}^{\text{max}}$ ' in our later publication<sup>8</sup>), is inversely proportional to the square of the ratio of the exchange rate ( $k_{\text{ex}}$ ) and the spin relaxation rate of the (minor) state B,  $R_2^{\text{B}}$ ,

$$C_{\text{fast}}^{\text{max}} \approx \frac{p_{\text{B}}}{\left(1 + k_{ex} / R_2^{\text{B}}\right)^2},$$

where  $p_{\rm B}$  is the fractional population of the minor state B. In the case of htt<sup>ex1</sup> tetramerization,  $k_{\rm ex} \sim 20,000 \, {\rm s}^{-1}$ , while  $R_2^{\rm B}$  is on the order of 20 s<sup>-1</sup> for the htt<sup>ex1</sup> tetramer, leading to the squared ratio of the two approaching a value of  $\sim 10^6$ , and as a result,  $C_{\rm fast}^{\rm max}$  approaching  $\sim 10^{-6}$ . Clearly, such small deviations from single-exponential behavior are beyond the limits of experimental detection. No significant errors in  $R_{1\rho}$  'two-time-point' measurements can therefore be expected in the case of htt<sup>ex1</sup> tetramerization.

Combined analysis of concentration dependent <sup>15</sup>N/<sup>13</sup>C $\alpha$ - $\delta_{ex}$  and <sup>15</sup>N- $R_{2,eff}$  data. Following our previous study of htt<sup>ex1</sup> oligomerization,<sup>1</sup> the following assumptions were made with respect to the kinetic model in Figure 3A (main text): the values of chemical shift changes (<sup>15</sup>N- $\Delta\omega$  and <sup>13</sup>C $\alpha$ - $\Delta\omega$ ) of the dimer E<sub>2</sub> and the tetramer E<sub>4</sub>, were assumed to be the same; the intrinsic (exchange-free) transverse spin relaxation rates of the dimer and tetramer were assumed to be equal to  $2R_{2,E}$  and  $4R_{2,E}$ , respectively, where  $R_{2,E}$  is the relaxation rate of the monomer;  $R_1$  values of all species were assumed to be the same and equal to  $R_1$  of the monomer. The full set of concentration dependent <sup>15</sup>N/<sup>13</sup>C $\alpha$ - $\delta_{ex}$  and <sup>15</sup>N- $R_{2,eff}$  data obtained for full-length htt<sup>ex1</sup> are shown in Figures S1 and S2. All experimental data were fitted simultaneously by minimizing the following target function *F* consisting of the differences squared between the observed ('*obs*') and calculated ('*calc*') values of  $\delta_{ex}$  and  $R_{2,eff}$ 

$$F = \alpha_1 \sum_{i} \sum_{j} \sum_{k=1}^{3} \left( \frac{R_{2,\text{eff}}^{obs,i,j,k} - R_{2,\text{eff}}^{calc,i,j,k}}{\sigma_{R_{2,\text{eff}}}^{obs,i,j,k}} \right)^2 + \alpha_2 \sum_{i} \sum_{j} \sum_{l=1}^{2} \left( \frac{\delta_{\text{ex}}^{obs,i,j,l} - \delta_{\text{ex}}^{calc,i,j,l}}{\sigma_{\delta_{\text{ex}}}^{obs,i,j,k}} \right)^2$$
(S1)

where the indices *i*, *j*, *k*, and *l* correspond to the residue number, htt<sup>ex1</sup> concentration, spin-lock RF field strength (750; 1500; 3000 Hz), and type of nucleus (<sup>15</sup>N; <sup>13</sup>C $\alpha$ ), respectively. The empirically determined coefficients  $\alpha_1 = 1$  and  $\alpha_2 = 1$  were used for weighting different types of data. The set of global variable parameters in the minimization of the target functions comprised: { $K_1^{\text{diss}}$ ;  $K_2^{\text{diss}}$ ; *k*.1; *k*.2} (see Figure 3A and main text for definitions). The space of local (residue-specific) parameters included: { $\Delta \omega^N$ ;  $\Delta \omega^{CA}$ ;  $R_{2,E}$ ;  $\varphi^N$ ;  $\varphi^{CA}$ } where  $\Delta \omega^N$ ,  $\Delta \omega^{CA}$  are the differences between the chemical shifts of the dimer/tetramer and the monomeric species for <sup>15</sup>N and <sup>13</sup>C $\alpha$  nuclei, respectively;  $R_{2,E}$  is <sup>15</sup>N transverse relaxation rate of the monomeric species in the absence of exchange;  $\varphi^N$ ,  $\varphi^{CA}$  are offsets that account for small errors in the reference value of the chemical shift,  $\delta_{ref}$  (measured for 50 µM htt<sup>ex1</sup>);  $\sigma$ are the errors of the measurements, assumed to be 0.5 Hz for  $\delta_{ex}$  and 0.5 s<sup>-1</sup> for 15N- $R_{2,eff}$ . The uncertainties in the values of the optimized parameters, corresponding to confidence intervals of ±1 S.D., were determined from the Jacobian variance-covariance matrix of the nonlinear fit. Uncertainties in the rate constants recalculated from the optimized parameters ( $k_1$  and  $k_2$ ) were determined by standard error propagation. All calculations were performed using an in-house program written in MATLAB (MathWorks Inc, MA).

**Data availability.** The experimental data in digital format, together with MatLab scripts for simulations and global best-fitting, have been deposited in Figshare: doi:10.35092/yhjc.12536015.



**Figure S1.** Full set of concentration-dependent <sup>15</sup>N and <sup>13</sup>C $\alpha$  exchange-induced chemical shifts ( $\delta_{ex}$ ) for U-[<sup>15</sup>N/<sup>13</sup>C]-labeled htt<sup>ex1</sup> (5 °C and 800 MHz). The experimental data are shown as circles, and the continuous lines are the best-fit curves obtained global fit to the  $\delta_{ex}$  and <sup>15</sup>N- $R_{2,eff}$  data using the scheme in Figure 3A (main text). The overall reduced  $\chi^2$  of the global fit is 2.25.

S4



**Figure S2.** Full set of concentration-dependent <sup>15</sup>N- $R_{2,eff}$  data for U-[<sup>15</sup>N/<sup>13</sup>C]-labeled htt<sup>ex1</sup> (5 °C and 800 MHz). at three spin-lock (SL) RF fields (700, 1500 and 3000 Hz). The experimental data are shown as circles, and the continuous lines are the best-fit curves obtained from the global fit to the  $\delta_{ex}$  and <sup>15</sup>N- $R_{2,eff}$  data using the scheme in Figure 3A (main text). The overall reduced  $\chi^2$  of the global fit is 2.25.



**Figure S3.** Correlation plots comparing <sup>15</sup>N- $\Delta\omega$  and <sup>13</sup>C $\alpha$ - $\Delta\omega$  values (ppm) between the oligometric (E<sub>2</sub>/E<sub>4</sub>) and monometric (E) species of htt<sup>ex1</sup> obtained for a three-state fit to the <sup>15</sup>N/<sup>13</sup>C $\alpha$ - $\delta_{ex}$  data only in this work (y-axis) with those obtained previously using the full four-state model of exchange, x-axis. The correlation coefficients are indicated in the right lower corner of the plots. Error bars (± 1 S.D.) for the x and y axes are shown in blue and red, respectively.

Residue	$\Delta \omega$ (ppm)			
	<sup>15</sup> N	<sup>13</sup> Ca		
4	$\textbf{-5.17} \pm 0.13$	$3.63 \pm 0.15$		
5	$\textbf{-3.42}\pm0.09$	$3.00\pm0.12$		
7	$\textbf{-4.01} \pm 0.10$	$2.88 \pm 0.12$		
8	$\textbf{-3.93}\pm0.10$	$2.70\pm0.11$		
9	$\textbf{-3.04}\pm0.09$	$2.08\pm0.09$		
10	b	$2.53\pm0.10$		
11	$\textbf{-3.91}\pm0.10$	$2.49\pm0.10$		
12	$-2.54\pm0.09$	$2.45\pm0.10$		
14	$\textbf{-2.36} \pm 0.08$	$2.51\pm0.10$		
15	$\textbf{-2.37} \pm 0.09$	$2.42\pm0.10$		

**Table S1**. <sup>15</sup>N and <sup>13</sup>C $\alpha$  chemical shift differences ( $\Delta\omega$ ) between dimer/tetramer (E<sub>2</sub>/E<sub>4</sub>) and monomer (E) species of htt<sup>ex1</sup> obtained from the global fit of the  $\delta_{ex}$  and <sup>15</sup>N- $R_{2,eff}$  data using the scheme in Figure 3A (main text).<sup>a</sup>

<sup>a</sup> No backbone chemical shift changes were observed for residues 16-23, 35-51 and 62-73. Residues 24-34 and 52-61 are prolines; as the backbone nitrogen of proline is not bonded to a proton, the two polyproline tracts are not detectable in  ${}^{1}\text{H}{-}^{15}\text{N}$  HSQC correlation maps. No  ${}^{13}\text{C}\alpha$  chemical shifts are observed for the two polyproline tracts.

<sup>b</sup> Not determined for Phe10.

**Table S2.** Impact of number of RF field strengths used to measure the concentration dependence of <sup>15</sup>N- $R_{2,eff}$  on the optimized values of the equilibrium dissociation constants ( $K_1^{diss}$ ,  $K_2^{diss}$ ) and dissociation rate constants ( $k_{-1}$ ,  $k_{-2}$ ) obtained from the global fit to the <sup>15</sup>N/<sup>13</sup>C $\alpha$ - $\delta_{ex}$  and <sup>15</sup>N- $R_{2,eff}$  data for htt<sup>ex1</sup>.<sup>a</sup>

RF field strengths (Hz) <sup>b</sup>	Equilibrium dissociation		Dissociation rate constants	
	const	ants		
	$K_1^{diss}(mM)$	$K_2^{diss}(\mu M)$	$k_{-1}(s^{-1})$	$k_{-2}(s^{-1})$
[750, 1500, 3000]	65 ± 2	34 ± 2	4.4 (± 0.4) $x10^4$	2.3 (± 0.2) $x10^4$
[750, 3000]	$66 \pm 3$	$36 \pm 4$	4.1 (± 0.6) $x10^4$	$2.3 (\pm 0.5) \times 10^4$

<sup>a</sup> The full concentration range from 0.1 to 1.4 mM was employed.

<sup>b</sup>The values reported in the text for the full set of three RF field strengths is highlighted in bold. Comparison of the results obtained using all three RF field strengths or just the outer two RF field strengths (750 and 3000 Hz), indicates that two RF field strengths are sufficient to define the optimized parameters.

**Table S3.** Impact of concentration range on the optimized values of the equilibrium dissociation constants ( $K_1^{\text{diss}}$ ,  $K_2^{\text{diss}}$ ) and dissociation rate constants ( $k_{-1}$ ,  $k_{-2}$ ) obtained from the global fit to the  ${}^{15}\text{N}/{}^{13}\text{C}\alpha$ - $\delta_{\text{ex}}$  and  ${}^{15}\text{N}$ - $R_{2,\text{eff}}$  data for htt<sup>ex1</sup>.<sup>a</sup>

Concentration range (mM)	Equilibrium dissociation		Dissociation rate constants	
	constants			
	$K_1^{diss}(mM)$	$K_2^{diss}(\mu M)$	$k_{-1}(s^{-1})$	$k_{-2}(s^{-1})$
[0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1,1, 1.2, 1.3, 1.4]	65 ± 2	34 ± 2	4.4 (± 0.4) $\times 10^4$	2.3 (± 0.2) $x10^4$
$ \begin{bmatrix} 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, \\ 0.8, 0.9, 1.0 \end{bmatrix} $	$69 \pm 4$	$39 \pm 4$	4.7 (± 0.4) $x10^4$	2.1 (± 0.2) x10 <sup>4</sup>
[0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8]	65 ± 6	$39 \pm 7$	4.2 (± 0.5) x10 <sup>4</sup>	$2.2 (\pm 0.5) \text{ x} 10^4$
[0.1, 0.2, 0.3, 0.4, 0.5, 0.6]	$65 \pm 13$	$70 \pm 41$	3.7 (± 0.5) $x10^4$	$1.8 (\pm 1.6) \times 10^4$
[0.1, 0.2, 0.3, 0.4]	$70 \pm 20$	9 ± 7	4.7 (± 0.7) $\times 10^4$	113 ( $\pm$ 381) x10 <sup>4</sup>

<sup>a</sup>The values obtained from the global fit over the full range of concentrations (0.1 to 1.4 mM) are highlighted in bold; the values obtained from fitting smaller concentration ranges (0.1 to 0.6 and 0.1 to 0.4 mM) are shown in red. The values of the equilibrium and rate constants are defined by the data for a concentration range of 0.1 to 0.8 mM or higher. When the data are only used up to a concentration of 0.6 mM,  $K_2^{\text{diss}}$  is poorly defined. If the concentration only extends up to 0.4 mM, only  $K_1^{\text{diss}}$  and  $k_{-1}$  are defined, albeit with relatively large uncertainties.

## **Supplementary References**

1. Ceccon, A.; Tugarinov, V.; Ghirlando, R.; Clore, G. M., Abrogation of Prenucleation, Transient Oligomerization of the Huntingtin Exon 1 Protein by Human Profilin I. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 5844-5852.

2. Chen, S.; Wetzel, R., Solubilization and Disaggregation of Polyglutamine Peptides. *Protein Sci.* **2001**, *10*, 887-91.

3. Anthis, N. J.; Clore, G. M., Sequence-Specific Determination of Protein and Peptide Concentrations by Absorbance at 205 nm. *Protein Sci.* 2013, *22*, 851-8.

4. Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A., Nmrpipe: A Multidimensional Spectral Processing System Based on Unix Pipes. *J. Biomol. NMR* **1995**, *6*, 277-93.

5. Ying, J.; Delaglio, F.; Torchia, D. A.; Bax, A., Sparse Multidimensional Iterative Lineshape-Enhanced (Smile) Reconstruction of Both Non-Uniformly Sampled and Conventional NMR Data. *J. Biomol. NMR* **2017**, *68*, 101-118.

6. Ceccon, A.; Tugarinov, V.; Bax, A.; Clore, G. M., Global Dynamics and Exchange Kinetics of a Protein on the Surface of Nanoparticles Revealed by Relaxation-Based Solution NMR Spectroscopy. *J. Am. Chem. Soc.* **2016**, *138*, 5789-92.

7. Yuwen, T.; Brady, J. P.; Kay, L. E., Probing Conformational Exchange in Weakly Interacting, Slowly Exchanging Protein Systems Via Off-Resonance  $R_{1\rho}$  Experiments: Application to Studies of Protein Phase Separation. *J. Am. Chem. Soc.* **2018**, *140*, 2115-2126.

8. Ceccon, A.; Clore, G. M.; Tugarinov, V., Decorrelating Kinetic and Relaxation Parameters in Exchange Saturation Transfer NMR: A Case Study of N-Terminal Huntingtin Peptides Binding to Unilamellar Lipid Vesicles. J. Phys. Chem. B 2018.