Supporting Information

Monitoring Hydrogen Exchange During Protein Folding by Fast Pressure Jump NMR Spectroscopy

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Materials and Methods

Protein expression and purification

A codon-optimized plasmid encoding residues 1-76 of human ubiquitin bearing the V17A/V26A mutations (ATUM) was transformed into *E. coli* BL21(DE3) competent cells (New England Biolabs). All bacterial media for protein expression contained 100 μg/L of ampicillin. Following overnight growth at 37 °C in 5 mL of LB media, the culture was spun down and transferred to 100 mL of M9 minimal medium. This culture was allowed to grow at 37 °C for 3-5 hours, and then the cells were pelleted and transferred to 1 L of M9 minimal media containing 1 g L^{-1} of $^{15}NH_4Cl$ and either 2 g L^{-1} of [*U*-^{13}C]-glucose or 6 g L^{-1} of natural abundance glucose. For perdeuteration, the M9 minimal media was prepared in 99.8% D_2O and [*U*-^{2}H,^{13}C]-glucose was used. All media for isotopic labeling was supplemented with 1 g L^{-1} of appropriately isotopically labeled ISOGRO (Sigma-Aldrich). When the optical density of these cultures reached *ca* 0.6-0.8 units, protein expression was initiated by addition of 1 mM IPTG and allowed to proceed for 4 hours at 37 °C. The cells were then harvested and stored at -80 °C until used.

Cell pellets were resuspended in 50 mM Tris-HCl buffer, pH 7.6, and then lysed using a Cell Disruptor (Constant Systems Ltd), with multiple passages of the cells at 2.2 kbar. The lysed cells were then spun at 50,000 x g for 20 minutes at 4 °C. The supernatant was collected and the pH was brought to 3.3 using acetic acid in order to precipitate contaminants. The pH 3.3 sample was then spun at 50,000 x g for 20 minutes at 4 °C, and the supernatant was concentrated and loaded onto a Superdex S75 26/60 column (GE Healthcare) equilibrated in 10 mM potassium buffer at pH 6.8. The ubiquitin-containing fractions were collected, concentrated, and then subjected to reverse-phase HPLC using a Vydac 214TP C4 column. Ubiquitin V17A/V26A eluted near 35% acetonitrile with 0.1% trifluoroacetic acid, and this sample was then dialyzed twice into four liters of 25 mM potassium phosphate buffer (pH 6.4), concentrated, and stored

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until use. All samples for NMR spectroscopy contained 7% D_2O in 25 mM potassium phosphate buffer at pH 6.4, 7.1, or 7.5. The ionic strength was slightly changed in the elevated pH samples due to the addition of NaOH.

NMR spectroscopy

The unfolded state of ubiquitin V17A/V26A was assigned using non-uniformly sampled 3D HNCA and HNCO spectra acquired on a Bruker Avance III 800 MHz spectrometer equipped with a cryogenically cooled probe. The pressure inside the NMR tube was maintained at 2.5 kbar (Daedalus Innovations, LLC), and NMR spectra were processed and NUS-reconstructed with NMRPipe[1] and SMILE.[2] All hydrogen exchange (HX) NMR spectra were collected on a Bruker Avance III 600 MHz spectrometer equipped with a 3-axis gradient, room temperature probe, modified with a home-built apparatus enabling fast pressure changes (see below). The pulse sequence to measure HX during the refolding process is analogous to that of Fitzkee *et al.*[3]

Pressure jump apparatus

The detailed design and performance characteristics of a home-built pressure-jump apparatus will be reported elsewhere. Briefly, a ceramic NMR tube[4] is connected via a high-pressure transfer tube to a "T" fitting mounted above the magnet. Pneumatic valves connected to the "T" fitting open to an adjustable high-pressure oil reservoir (≤3 kbar) or an atmospheric pressure vessel. Spectrometer-generated TTL signals trigger the valve openings to alternately pressurize and depressurize the protein sample. Pressure rise and fall times can be adjusted from ca 1 ms to several ms by restricting flow to the NMR tube. Reported data used switching times of ca 4 ms.

Calculation of water magnetization transfer to folded protein

We derive the expression for the measured signal, $S_f = \Delta I_f / I_{f,ref}$, where $\Delta I_f = I_{f,ref} - I_{f,inv}$, and $I_{f,ref}$, $I_{f,inv}$ are the population scaled amide-spin z-magnetizations in the folded state, measured without (*ref*) and with (*inv*) water inversion, respectively. The time derivative of I_f is given by

$$dI_f / dt = -R_{1f}(I_f - p_f I_o) + \lambda I_u - k_f(I_f - p_f W) \qquad (1)$$

where,

R_{1f} is the amide proton spin - lattice relaxation rate in the folded state

$p_f = (1 - e^{-\lambda t})$, the fractional population of the folded state

I_o is the equilibrium amide spin magnetization

λ is the protein folding rate

I_u is the population scaled amide proton magnetization in the unfolded state

k_f is the amide HX exchange rate in the folded state

W is the water proton z - magnetization

Equation (1) accounts for changes in I_f due to relaxation (i) to (the population scaled) equilibrium magnetization, (ii) to transfer of magnetization from I_u due to folding, and (iii) to HX exchange with (the population scaled) water magnetization. The corresponding equation for ΔI_f, derived by

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using equation (1) to obtain the derivatives of $I_{f,ref}$ and $I_{f,inv}$, is given by

$$d\Delta I_f / dt + (R_{1f} + k_f)\Delta I_f = \lambda \Delta I_u + k_f p_f \Delta W = \lambda \Delta I_u + \Delta f I_o k_f (1 - e^{\lambda t}) e^{-R_{1W} t} \qquad (2)$$

where

$\Delta f = [W_{ref}(0) - W_{inv}(0)] / W_{ref}(0)$

R_{1W} = the spin relaxation rate of water

The final term on the right side of equation (2) was obtained using $\Delta W = \Delta f I_o \exp(-R_{1W} t)$.[3] In order to solve this equation, an expression for ΔI_u is required. This is obtained by solving the differential equation for I_u given by

$$dI_u / dt = -R_{1u}(I_u - p_u I_o) - \lambda I_u - k_u (I_u - p_u W) \qquad (3)$$

where,

R_{1u} is the amide proton spin-lattice relaxation rate in the unfolded state

$p_u = e^{-\lambda t}$ the fractional population of the unfolded state

k_u is the amide HX exchange rate in the unfolded state

Note that in the absence of HX, equations (1) and (3) are those given by Allard for non-equilibrium chemical exchange,[5] while in the absence of folding where p_u=1, equation (3) reduces to that of Fitzkee.[3] Using equation (3) we obtain the following differential equation for ΔI_u

$$d\Delta I_u / dt + (K_u + \lambda)\Delta I_u = I_o \Delta f k_u e^{-(\lambda + R_{1W})t} \qquad (4)$$

where,

$K_u = R_{1u} + k_u$

Solving this first-order linear equation yields

$$\Delta I_u = I_o \Delta f k_u e^{-\lambda t} (e^{-R_{1W} t} - e^{-K_u t}) / (K_u - R_{1W}) \qquad (5)$$

Inserting this expression for ΔI_u into equation (2) again yields a first-order linear equation whose solution is given by

$$\Delta I_f(T_{ex}) = I_o \Delta f \left(A_u + A_f \right) \qquad (6)$$

where,

$$A_u = [\lambda k_u / (K_u - R_{1W})] \left[(e^{-K_f T_{ex}} - e^{-(R_{1W} + \lambda) T_{ex}}) / (R_{1W} + \lambda - K_f) - (e^{-K_f T_{ex}} - e^{-(K_u + \lambda) T_{ex}}) / (K_u + \lambda - K_f) \right]$$

$$A_f = k_f \left[(e^{-K_f T_{ex}} - e^{-R_{1W} T_{ex}}) / (R_{1W} - K_f) - (e^{-K_f T_{ex}} - e^{-(R_{1W} + \lambda) T_{ex}}) / (R_{1W} + \lambda - K_f) \right]$$

$$K_f = R_{1f} + k_f$$

Note that A_u and A_f contain contributions to ΔI_f from HX in the unfolded and folded states respectively.

We obtain $S_f(T_{ex})$ by dividing the right side of equation (6) by $I_{f,ref}(T_{ex}) = I_o$ (1-exp(-λT_{ex})) yielding,

$$S_f(T_{ex}) = \Delta f (A_u + A_f) / (1 - e^{-\lambda T_{ex}}) \qquad (7)$$

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In the limit that R_{1u}, R_{1f}, R_{1W}, k_f are all much less than $1/T_{ex}$ one recovers the approximate expression in the main text

$$S_f(T_{ex}) = \Delta f \{1 - [\lambda/(\lambda + k_u)][(1 - e^{-(\lambda + k_u)T_{ex}})/(1 - e^{-\lambda T_{ex}})]\} \qquad (8)$$

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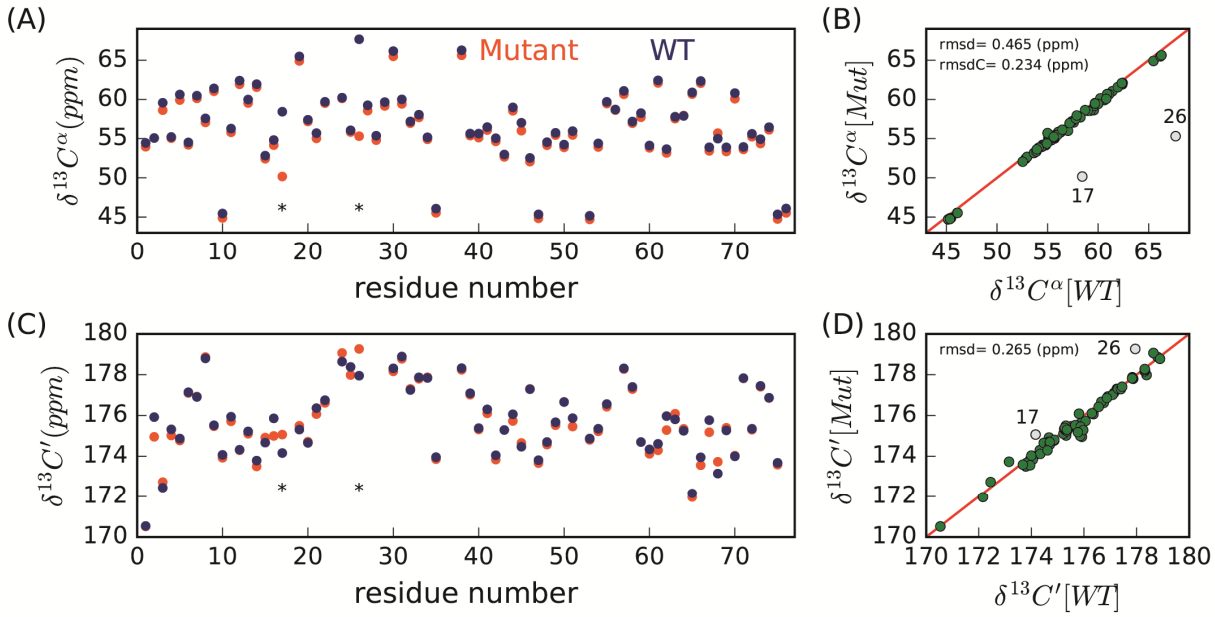

Figure S1. Comparison of $^{13}C^{\alpha}$ (A,B) and $^{13}C'$ (C,D) chemical shifts measured on a ^{1}H,^{13}C,^{15}N-labeled sample of WT ubiquitin[6] (*blue*) with those obtained here on a ^{2}H,^{13}C,^{15}N-labeled sample of the V17A/V26A ubiquitin double mutant (*orange*). The V17A/V26A ubiquitin chemical shifts were measured at 298 K on a 700 µM sample that was dissolved in 25 mM potassium phosphate buffer at pH 6.4. A small systematic difference in $^{13}C^{\alpha}$ shift is due to the isotope effect of ^{2}H, which was present in the V17A/V26A double mutant. The asterisks (*) correspond to V17 and V26, which have been mutated to Ala and thus show large chemical shift differences. In panels B and D, the RMSD is calculated for all residues, excluding A17 and A26, and the rmsd for $^{13}C^{\alpha}$ is dominated by the isotope shift of *ca* 0.4 ppm. Subtracting the average isotope shift decreased the $^{13}C^{\alpha}$ RMSD to rmsdC=0.23 ppm.

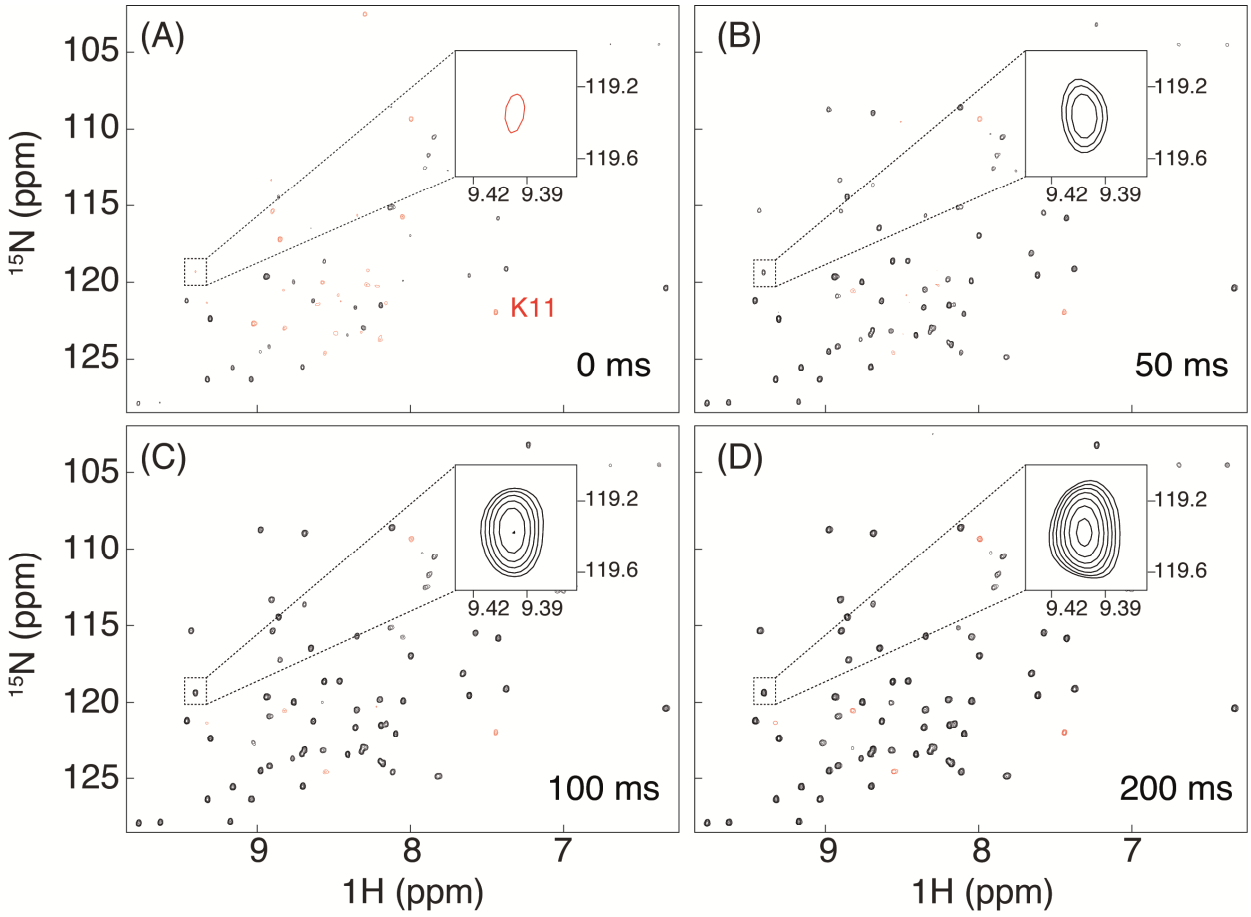

Figure S2. Ubiquitin 2D HSQC NMR spectra, measured at various refolding intervals, τ_r, with application of the water inversion pulses. The inset corresponds to the cross peak of H68. Negative signals are in red. The assignment of K11, representative of a residue with fast HX in the folded state ($k_f = 5.4\ s^{-1}$), is marked in (A).

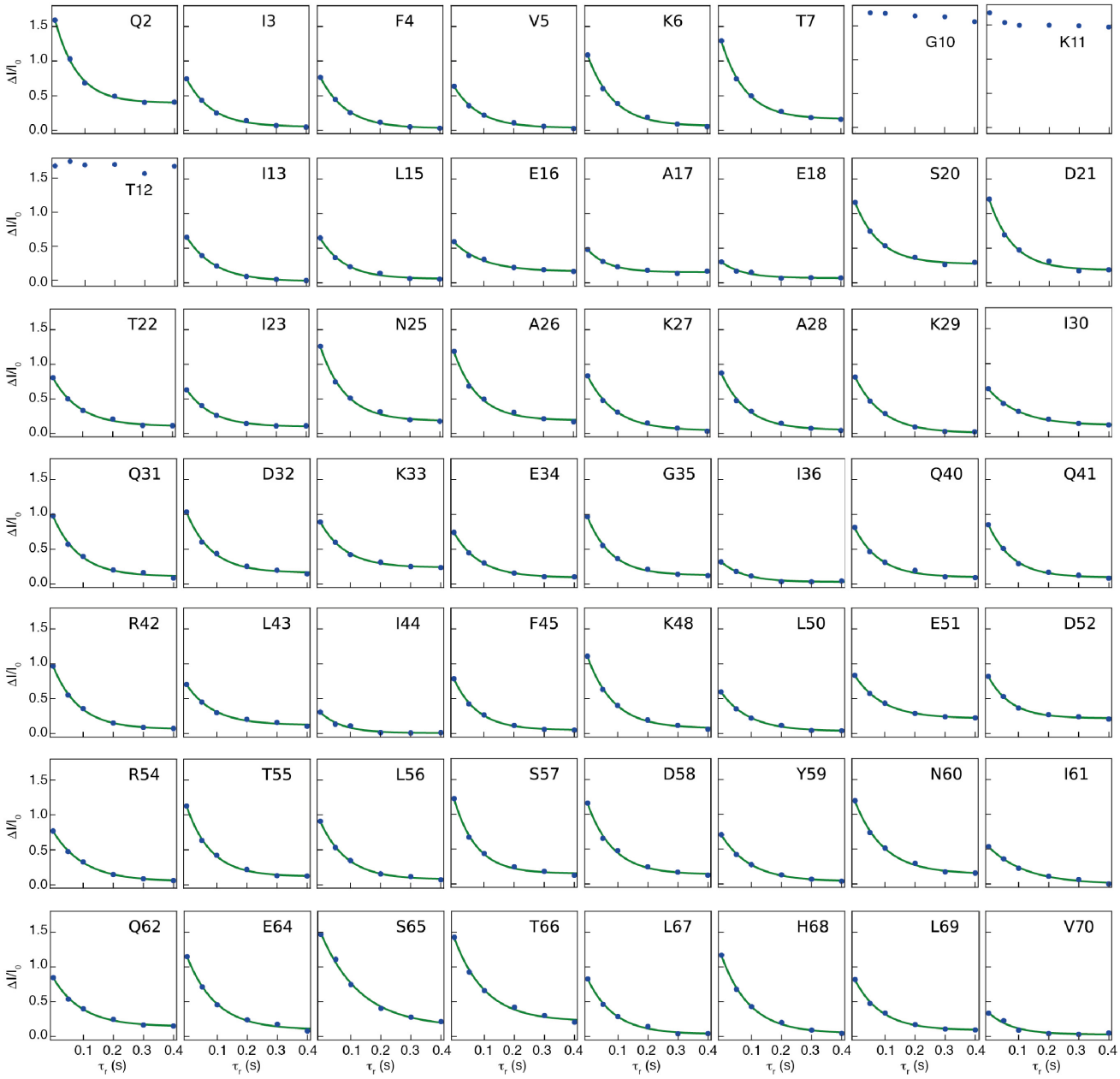

Figure S3. Fits of $S_f(\tau_r,T_{ex}) = \Delta I(\tau_r)/I_0$ to $Ae^{-\tau r/\tau f} + B$ for all of the residues analyzed. The sample contained 300 µM of V17A/V26A ubiquitin (^{2}H,^{13}C,^{15}N) dissolved at pH 7.5 thermostated at 298 K when at 2.5 kbar. An exchange time (T_{ex}) of 125 ms was used in this experiment, with τ_r values of 0, 50, 100, 200, 300, and 400 ms. Intensities of residues with fast HX in both the unfolded and folded states (G10, K11, T12) show no significant decay and were not fitted.

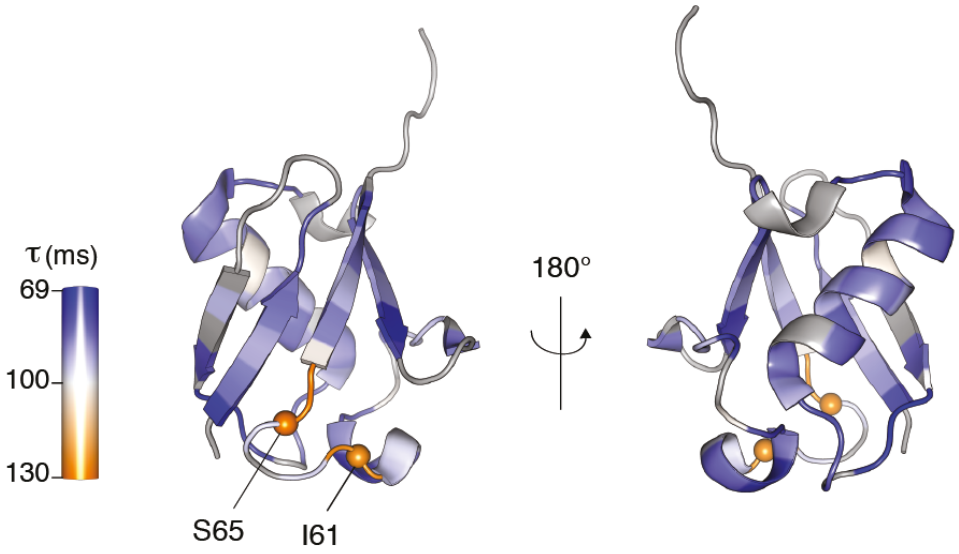

Figure S4. Protection times, $\tau_f = 1/\lambda$, for HX during protein folding mapped on the ribbon diagram of the ubiquitin structure (PDB entry 2MJB). Grey indicates residues for which no protection times were obtained.

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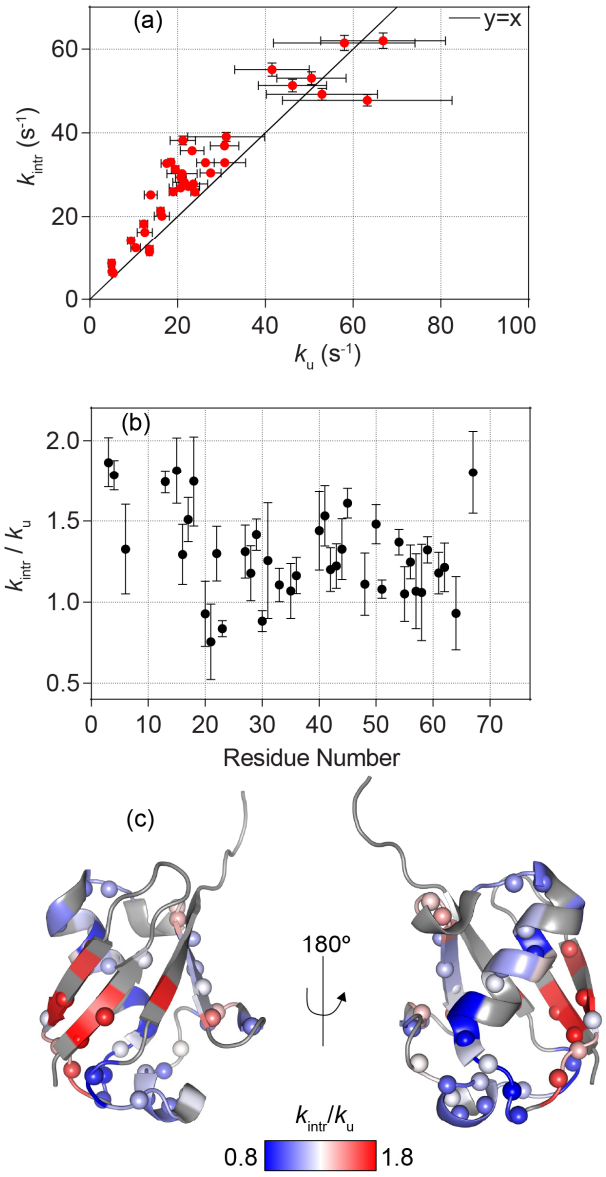

Figure S5. Comparison of the intrinsic HX rates (k_{intr}) in the pressure-denatured state of V17A/V26A ubiquitin with the HX rates in the pre-transition state (k_u) measured during the pressure-jump experiments. (a) Correlation plot of the pressure-corrected k_{intr} rates (rates of Table S2, multiplied by 2.0 (to account for the 1 unit decrease in the pH of phosphate buffer, prepared at pH 7.5, when pressure is raised to 2.5 kbar, an effect that is partially offset by a five-fold increase in OH^- concentration resulting from pressure-induced water ionization) and k_u rates determined from the pressure-jump experiments. In both cases, the sample contained 300 μM of ^2H,^{13}C,^{15}N-labeled sample, in 25 mM sodium phosphate buffer, pH 7.5 at 1 bar. Static experiments at 2.5 kbar were carried out at 295 K (k_{intr}). Pressure jump experiments (k_u) were carried out with the 2.5 kbar sample thermostated at 298 K, corresponding to 295 K after the pressure drop to 1 bar. (b) The ratio of k_{intr} over k_u shown as a function of residue number. (c) Data from panel (b) shown on the structure of ubiquitin (PDB 2mjb). Residues that are colored gray have no data available.

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Table S1. Protection times ($\tau_f = 1/\lambda$), in units of ms, for HX during protein folding. The time constants below were obtained at 295 K for the refolding of V17A/V26A ubiquitin using a sample prepared at pH 7.5 (at 1 bar) in 25 mM potassium phosphate buffer. Refolding delays (τ_r) of 0, 50, 100, 200, 300, and 400 ms were employed with a constant exchange time (T_{ex}) of 125 ms. Uncertainties in the reported values are those obtained from the covariance matrix of the fit.

Q2	75	±	4
I3	84	±	6
F4	88	±	3
V5	85	±	6
K6	86	±	7
T7	80	±	4
I13	94	±	2
L15	83	±	9
E16	100	±	15
A17	69	±	9
E18	74	±	19
S20	82	±	6
D21	78	±	9
T22	91	±	8
I23	86	±	4
N25	84	±	6
A26	81	±	8
K27	92	±	7
A28	86	±	8
K29	93	±	4
I30	104	±	7
Q31	87	±	9
D32	83	±	8
K33	84	±	4
E34	86	±	3
G35	79	±	4
I36	78	±	11

Q40	83	±	9
Q41	80	±	6
R42	86	±	3
L43	90	±	10
I44	74	±	16
F45	79	±	4
K48	86	±	5
L50	93	±	7
E51	94	±	3
D52	75	±	5
R54	102	±	5
T55	80	±	4
L56	87	±	4
S57	74	±	6
D58	85	±	9
Y59	97	±	5
N60	95	±	6
I61	126	±	14
Q62	96	±	7
E64	96	±	9
S65	131	±	14
T66	103	±	10
L67	87	±	8
H68	94	±	6
L69	87	±	6
V70	78	±	20

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Table S2. Intrinsic HX rates (k_{intr}) in units of s^{-1} at 2.5 kbar, 295 K, of the unfolded state of V17A/V26A ubiquitin for a sample prepared at pH 7.5 (at 1 bar) in 25 mM potassium phosphate buffer.[a]

I3	17	±	1
F4	17	±	1
V5[b]	30	±	1
K6	29	±	2
T7	44	±	3
L8	54	±	3
T9	77	±	4
G10	88	±	5
K11	53	±	3
T12	53	±	3
I13	13	±	0.6
T14	19	±	0.9
L15	13	±	0.6
E16	8.3	±	0.4
A17	7.2	±	0.4
E18	4.4	±	0.3
S20	32	±	2
D21	25	±	1.2
T22	14	±	0.6
I23	5.8	±	0.3
E24	8.1	±	0.4
N25	42	±	3
A26[b]	12.2	±	0.3
K27	15	±	1
A28	14	±	1
K29	15	±	1
I30	6.1	±	0.3
Q31	20	±	1
D32	23	±	1
K33	16	±	1
E34[b]	12	±	1
G35	17	±	1
I36	3.2	±	0.2
D39	14	±	1
Q40	16	±	1
Q41	18	±	1
R42	19	±	1
L43	10.3	±	0.5
I44	3.4	±	0.2
F45	16	±	1
A46	39	±	2
G47	32	±	2
K48	27	±	2
Q49	20	±	1
L50	9.3	±	0.5
E51	13.3	±	0.6
G53	24	±	1
R54	13	±	1
T55	27	±	1
L56	17	±	1
S57	40	±	2
D58	32	±	2
Y59	10.9	±	0.5
N60	39	±	2
I61	6.3	±	0.3
Q62	14	±	0.6
K63	26	±	1
E64	25	±	1
S65	71	±	4
T66	80	±	5
L67	20	±	1
H68[b]	183	±	5
L69	36	±	2
V70	6.6	±	0.3
L71	10	±	0.5
R72	26	±	1
L73	23	±	1
R74	40	±	2
G75	82	±	5
G76	3.9	±	0.3

[a] Note that these values have not been corrected for the effect of pressure, and the 1 bar rates will be *ca* 2-fold faster, due to the lower pH at 2.5 kbar, which is partially offset by the increase in $[OH^-]$.[7] Measurements were carried out at three pH values (6.4, 7.1, and 7.5), with reported values pertaining to pH 7.5. Residues for which accurate values were only available at pH 7.1 and 6.4, or 6.4 only, were converted to pH 7.5 using the known dependence of k_{intr} on $[OH^-]$ observed for residues with measurements at all three pH values.

[b] Values measured only at pH 6.4 but converted to pH 7.5 using the known pH dependence of HX.

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Table S3. HX rates (k_f) in units of s^{-1} for the folded V17A/V26A ubiquitin mutant at 1 bar, pH 7.5, 295 K

Q2	1.5 ± 0.1
I3	0.06 ± 0.1
F4	0.03 ± 0.1
V5	0.03 ± 0.1
K6	0.06 ± 0.1
T7	0.5 ± 0.1
L8	4.0 ± 1.3
T9[a]	56 ± 3
G10	4.5 ± 1
K11	5.4 ± 1
T12	4.1 ± 1
I13	0.11 ± 0.1
T14	1.6 ± 0.1
L15	0.07 ± 0.1
E16	0.55 ± 0.1
V17	0.57 ± 0.1
E18	0.07 ± 0.1
S20	0.82 ± 0.1
D21	0.13 ± 0.1
T22	0.27 ± 0.1
I23	0.15 ± 0.1
N25	0.43 ± 0.1
V26	0.06 ± 0.1
K27	0.05 ± 0.1
A28	0.06 ± 0.1
K29	0.02 ± 0.1
I30	0.04 ± 0.1
Q31	0.07 ± 0.1
D32	0.24 ± 0.1
K33	0.72 ± 0.1
E34	0.19 ± 0.1
G35	0.17 ± 0.01
I36	0.08 ± 0.1
D39	2.4 ± 0. 2
Q40	0.32 ± 0.1
Q41	0.16 ± 0.1
R42	0.11 ± 0.1
L43	0.39 ± 0.1
I44	0.05 ± 0.1
F45	0.08 ± 0.1
A46	2.8 ± 2.2
G47	3.3 ± 0.3
K48	0.21 ± 0.1
Q49	3.5 ± 0.3
L50	0.12 ± 0.1
E51	0.79 ± 0.1
D52	0.53 ± 0.1
R54	0.20 ± 0.1
T55	0.30 ± 0.1
L56	0.12 ± 0.1
S57	0.29 ± 0.1
D58	0.50 ± 0.1
Y59	0.14 ± 0.1
N60	0.41 ± 0.1
I61	0.07 ± 0.1
Q62	0.50 ± 0.1
K63	2.31 ± 0.1
E64	0.23 ± 0.1
S65	0.60 ± 0.1
T66	0.58 ± 0.1
L67	0.05 ± 0.1
H68	0.03 ± 0.1
L69	0.06 ± 0.1
V70	0.04 ± 0.1
L71	0.34 ± 0.1
R72	2.3 ± 0.2
L73	4.8 ± 0.8
R74	3.2 ± 2.3
G75[a]	59 ± 15
G76	4 ± 0.2

[a] Values measured only at pH 6.4 but converted to pH 7.5 using the known pH dependence of HX

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