ScienceAdvances

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Supplementary Materials for

Kinetic and structural comparison of a protein's cotranslational folding and refolding pathways

Avi J. Samelson, Eric Bolin, Shawn M. Costello, Ajeet K. Sharma, Edward P. O'Brien, Susan Marqusee

The PDF file includes:

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- fig. S2. Addition of the peptidyl-proline isomerase CypA does not affect HaloTag refolding or cotranslational folding rates.
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Other Supplementary Material for this manuscript includes the following:
(available at advances.sciencemag.org/cgi/content/full/4/5/eaas9098/DC1)

- table S4 (Microsoft Excel format). Normalized HX-MS data.
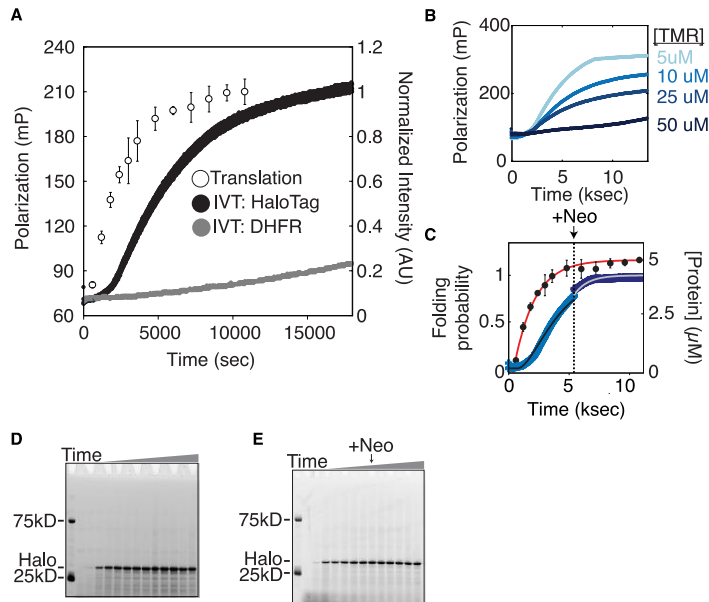
fig. S1. Cotranslational folding of HaloTag can be measured using FP. (A) Raw FP data (left axis) for IVT reactions initiated with HaloTag (black) and DHFR (gray) plasmids. Translation (right axis) of HaloTag as determined by gel. (**B**) Polarization as a function of TMR-ligand concentration during IVT of HaloTag. (**C**) Folding probability (left axis, blue lines) and HaloTag protein concentration (right axis, black dots with red line) as a function of time before and after the addition of neomycin. (**D**) and (**E**) Representative gels used to measure protein translation in figure S1A and figure S1C.
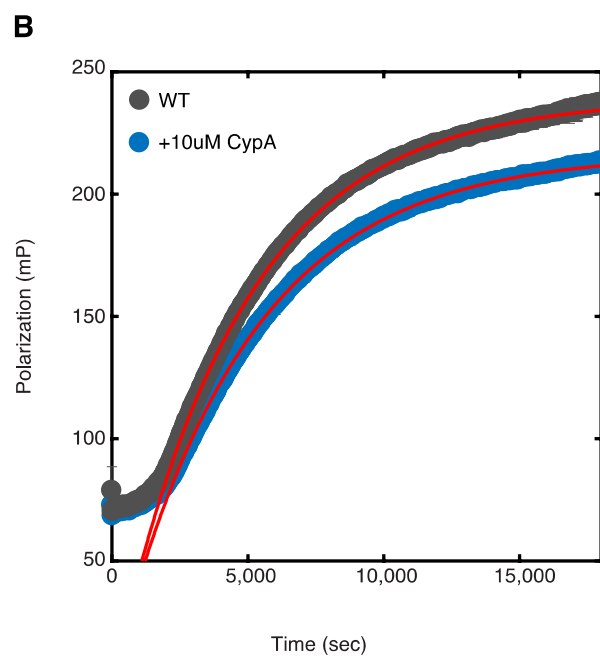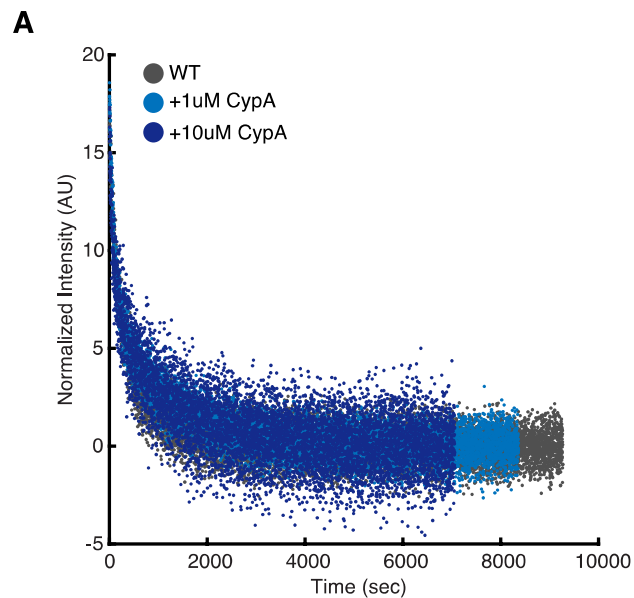
fig. S2. Addition of the peptidyl-proline isomerase CypA does not affect HaloTag refolding or cotranslational folding rates. (**A**)Refolding of HaloTag in increasing concentrations of CypA as monitored by CD. (**B**) FP of HaloTag in the presence of 10μM CypA (blue) and no CypA (gray).

0.4M Urea   0.6M Urea   0.8M Urea
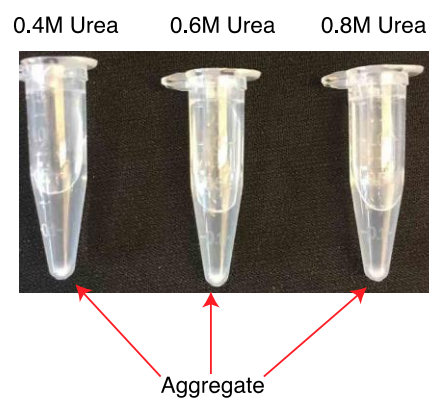
Aggregate

fig. S3. Aggregation of HaloTag. HaloTag aggregates after refolding via dilution from 8.0M urea to the indicated final concentrations of urea.
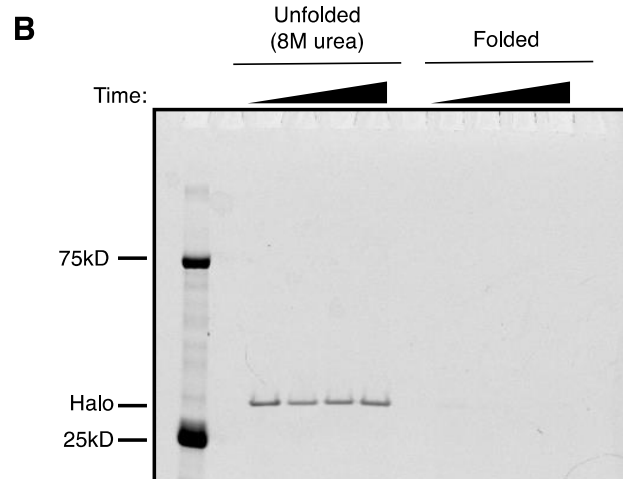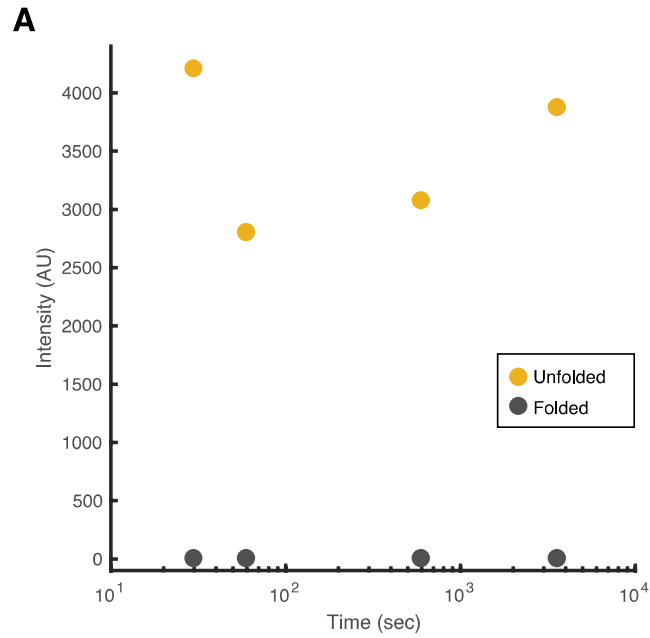
**fig. S4. Cysteine accessibility of WT HaloTag.** (**A**) Cysteine accessibility as a function of time as measured by fluorescein-maleimide fluorescence for unfolded (yellow circles) and folded (grey circles) HaloTag. (**B**) Raw data for plot in (A)
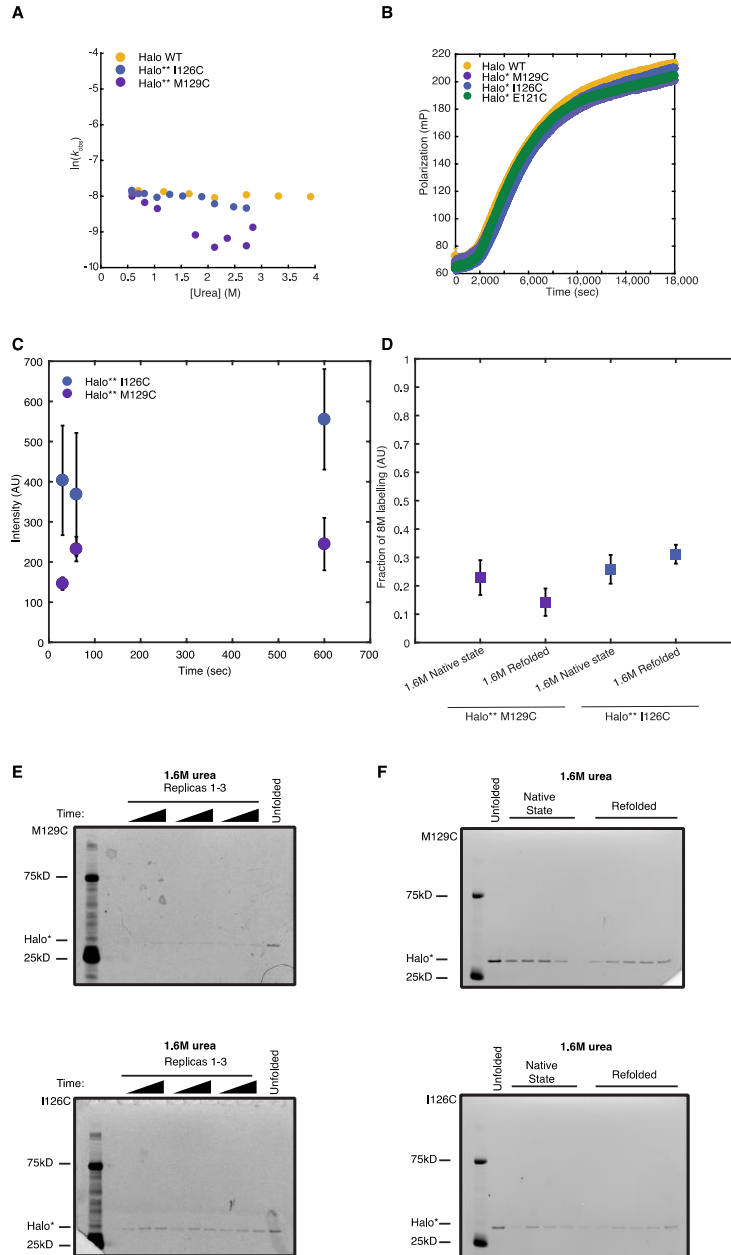
fig. S5. Characterization of Halo* cysteine mutants. (**A**) Refolding rate as a function of [urea] as measured by FP for different HaloTag constructs. WT – yellow; Halo* M129C – purple; Halo* I126C – blue. (**B**) Cotranslational folding of HaloTag variants measure by FP. WT – yellow; Halo* M129C – purple; Halo* I126C – blue; Halo*E121C – green. (**C**) Cysteine accessibility as a function of time at 1.6M urea for Halo variants. (**D**) Cysteine accessibility as a fraction of unfolded intensity for refolded and native state Halo variants at 1.6M urea. (**E**) And (**F**) Gels used for plots in (**C**) and (**D**) respectively
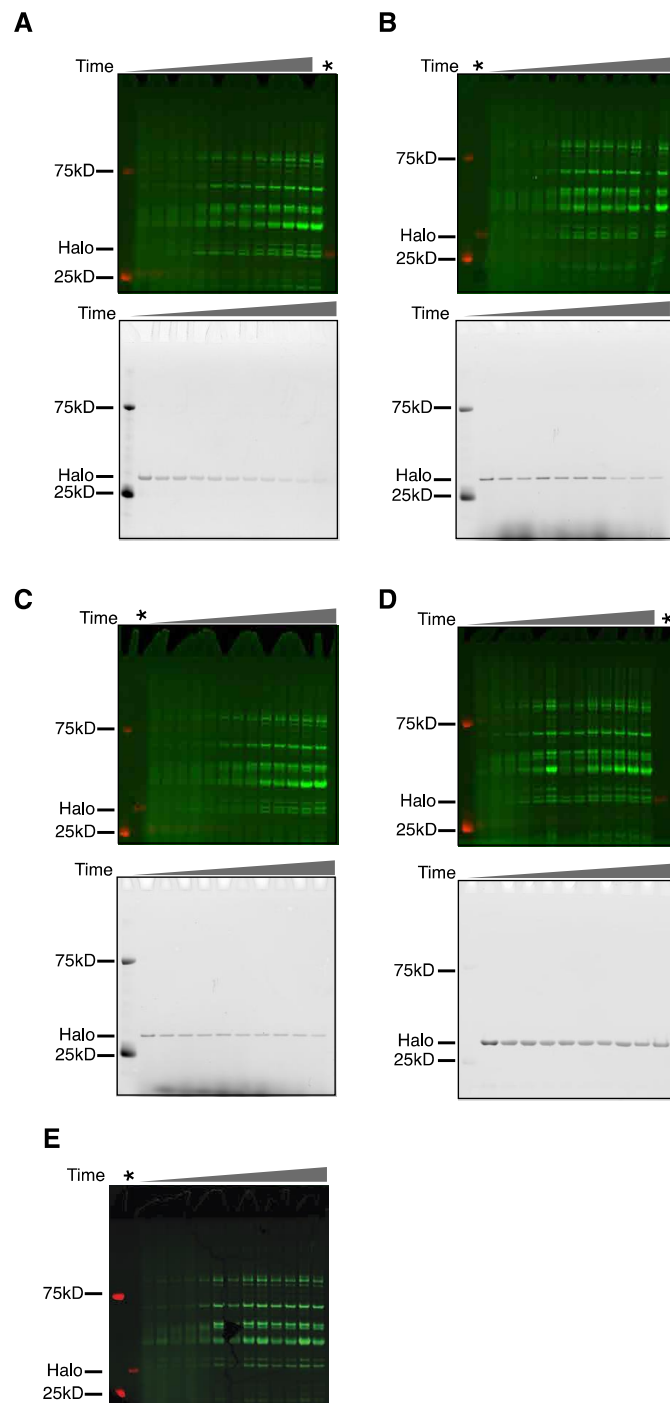
fig. S6. Gels for data shown in Fig. 4. For (A)-(D), gel in color is during translation and gel shown in black and white is during refolding. * marks HaloTag bound to TMR-ligand for the purpose of finding the HaloTag band during analysis. (**A**) HaloTag WT. (**B**) Halo* M129C. (**C**) Halo* I126C. (**D**) Halo* E121C. (**E**) No template added.
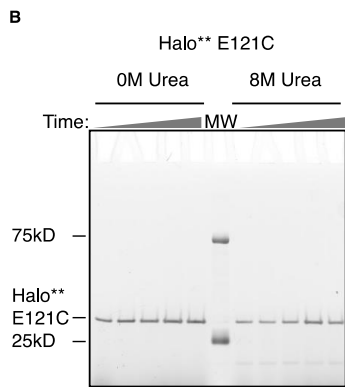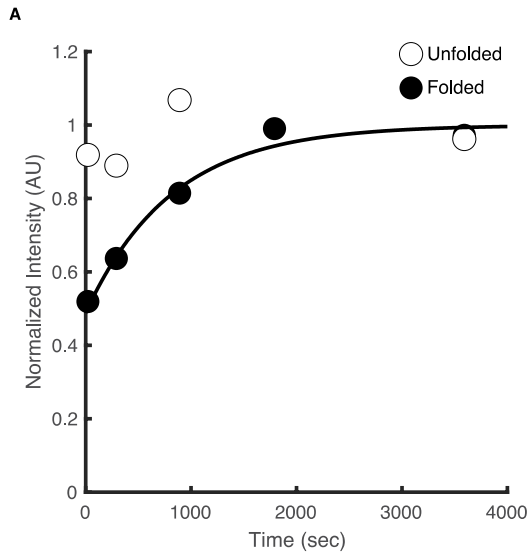
fig. S7. Characterization of Halo* E121C cysteine accessibility. (**A**) Cysteine accessibility of folded (black circles) and unfolded (white circles) Halo* E121C as measured by fluorescein-maleimide reactivity. (**B**)Gel used in (A)

table S1. Kinetic data obtained for HaloTag folding using FP.

	Lag Time (sec)	Rate (x10^{-4} sec^{-1})
Refolding (polarization)	NA	4.8±0.6
Folding (*in vitro* translation)	811.29±8.57	4.42±0.02
Translation	251.29±34.6	4.50±0.10
Folding$_{before +neo}$	724.18±17.0	5.49±0.07
Folding$_{after +neo}$	NA	9.13±0.19
Folding$_{[TMR] = 5uM}$	1065.89±17.32	4.39±0.04
Folding$_{[TMR] = 10uM}$	811.29±8.6	4.42±0.02
Folding$_{[TMR] = 12.5uM}$	780.05±8.9	6.13±0.04

table S2. Determination of HaloTag folding efficiency under different conditions.

	Fraction Folded	Number of samples
Native	1.04±0.01	3
Unfolded	0.0006±0.0003	3
0.8M refolded	0.73±0.10	15
0.8M dialysis	0.74±0.11	15
IVT refolded	0.69±0.06	15
IVT native	0.91±0.03	15

table S3. Folding rates of HaloTag and variants measured by FP.

	Rate (x10^{-4} sec^{-1})	t_0 (sec)
WT (10µM TMR)	4.40±0.02	811.3±9
+10µM CypA	2.12±0.02	2007±70
Halo** I126C	2.08±0.02	612±33
Halo** M129C	2.20±0.08	848±10
Halo** E121C	2.32±0.11	755±57

table S4. Normalized HX-MS data. Normalized HX/MS peptides used in Figure 4. Peptides in blue were excluded from analysis.