Molecular Cell

Supplemental Information

Synonymous Codons Direct Cotranslational Folding toward Different Protein Conformations

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Figure S1

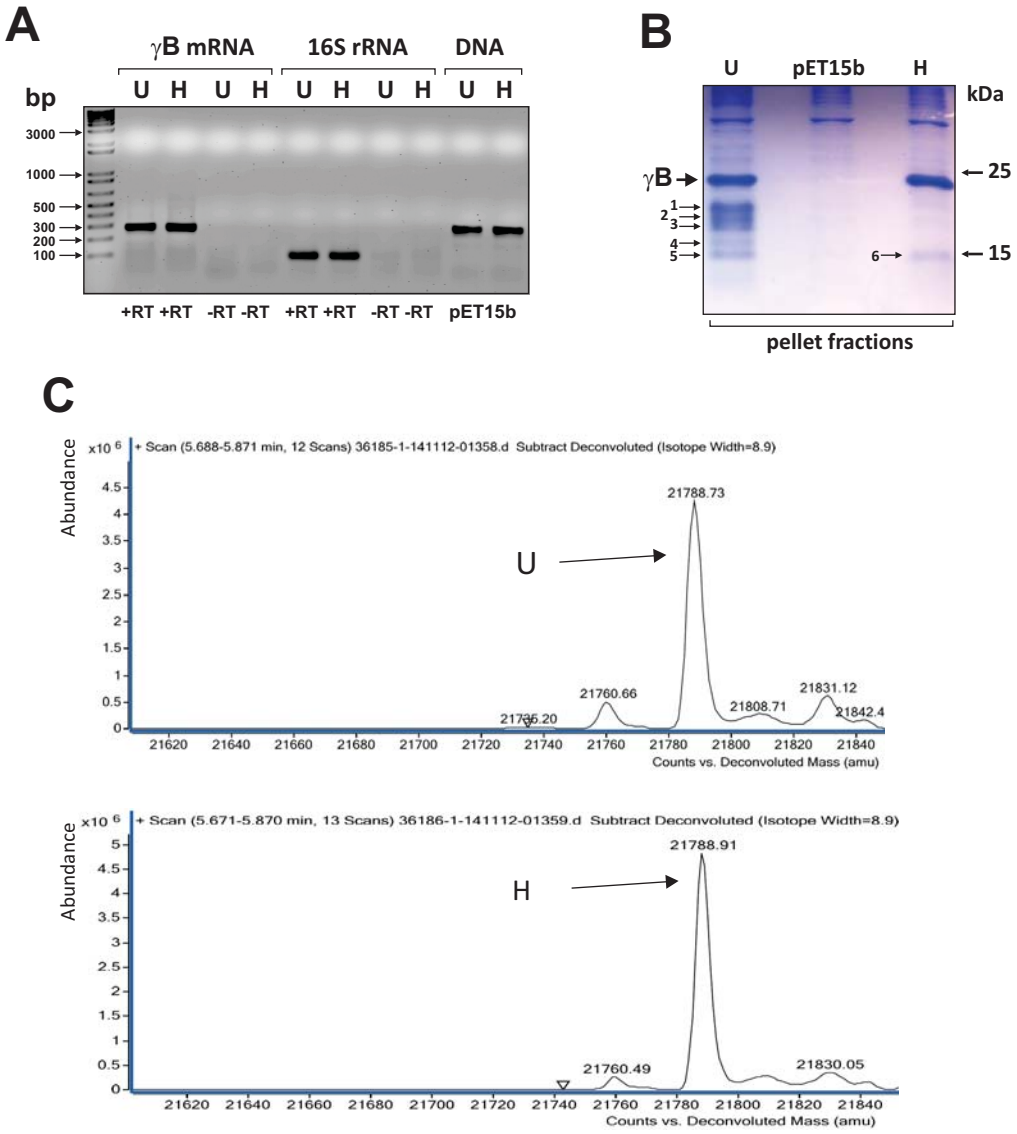


Figure S1, related to Figure 1. Analysis of gamma-B crystallin expression in *E. coli*

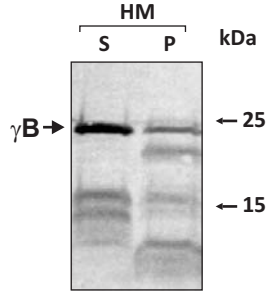
Figure S2

A

90.7% identity

```
                10        20        30        40        50        60        70        80
H       ATGGGAAAAATCACTTTCTATGAAGATCGCGGTTTTCAGGGTCATTGTTATGAATGTAGCAGCGATTGTCCGAACCTGCA
        :::::::::::::::::::: ::::: :: ::::: ::::: :: ::::: :::::::      :: ::::::::::::::
HM      ATGGGAAAAATCACTTTCTACGAAGACCGGGGTTTCCAGGGCCACTGTTACGAATGTTCTTCTGACTGTCCGAACCTGCA
                10        20        30        40        50        60        70        80

                90       100       110       120       130       140       150       160
H       GCCGTACTTTAGCCGCTGTAACAGCATCCGCGTTGATAGCGGTTGTTGGATGCTGTACGAACGCCCGAACTATCAGGGTC
        ::::::::::::::: ::::::   ::::::::::::    :: :::::::::::::::::::::::::::::::: ::::: :
HM      GCCGTACTTTAGCCGGTGTAACTCTATCCGCGTTGACTCTGGCTGTTGGATGCTGTACGAACGCCCGAACTACCAGGGCC
                90       100       110       120       130       140       150       160

                170       180       190       200       210       220       230       240
H       ATCAGTATTTTCTGCGCCGCGGTGATTATCCGGATTATCAGCAGTGGATGGGTTTTAACGATAGCATCCGCAGCTGTCGC
        : ::::: :: :::::::::::::::::: :::::::: :::::::::::::::::::::::::    ::::: ::::::::
HM      ACCAGTACTTCCTGCGCCGCGGTGATTACCCGGATTACCAGCAGTGGATGGGTTTTAACGATTCTATCCGGAGCTGTCGG
                170       180       190       200       210       220       230       240

                250       260       270       280       290       300       310       320
H       TTAATCCCACAACATACCGGTACTTTTCGGATGCGGATCTACGAACGGGACGATTTTCGGGGACAGATGTCCGAAATCAC
        :::::::: ::::: :: :: :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
HM      TTAATCCCCCAACACACTGGCACTTTTCGGATGCGGATCTACGAACGGGACGATTTTCGGGGACAGATGTCCGAAATCAC
                250       260       270       280       290       300       310       320

                330       340       350       360       370       380       390       400
H       TGATGACTGTCCGTCATTGCAAGATCGCTTTCATTTAACTGAAGTACATAGCTTAAACGTTCTGGAAGGGAGCTGGGTCT
        ::::::::::::::: :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
HM      TGATGACTGTCCGTCACTGCAAGATCGCTTTCATTTAACTGAAGTACATAGCTTAAACGTTCTGGAAGGGAGCTGGGTCT
                330       340       350       360       370       380       390       400

                410       420       430       440       450       460       470       480
H       TATACGAAATGCCGAGCTATCGCGGACGCCAGTATCTGCTGCGCCCGGGAGAATATCGCCGGTACTTGGATTGGGGAGCT
        :::::::::::::: ::::: :: ::::: ::::: :::::::: :: :::::::: :: ::::::::::::::::::::
HM      TATACGAAATGCCAAGCTACCGGGGACGGCAGTACCTGCTGCGACCAGGAGAATACCGGCGGTACTTGGATTGGGGAGCT
                410       420       430       440       450       460       470       480

                490       500       510       520
H       ATGAACGCAAAGGTAGGGTCACTACGGCGCGTTATGGACTTCTAC
        :::::::::::::::::::::::::::::: ::::::::::::::
HM      ATGAACGCAAAGGTAGGGTCACTACGGCGGGTTATGGACTTCTAC
                490       500       510       520
```

B



C

Composite: trypsin only, chymotrypsin only,
trypsin and chymotrypsin **93% Sequence Coverage**

```
1    MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR
81   LIPQHTGTFR MKIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA
161  MNAKVGSLRR VMDFY
```

D

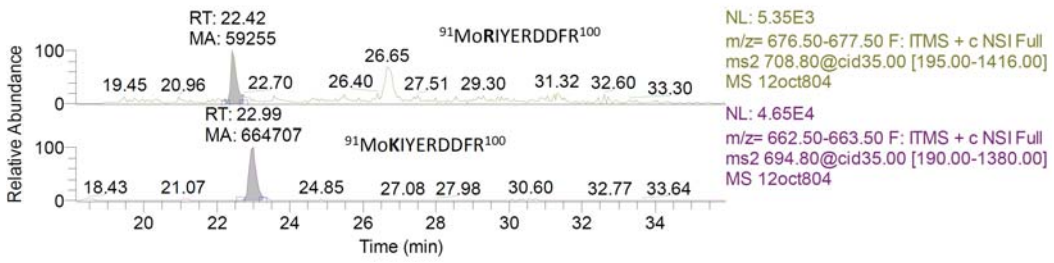


Figure S2, related to Figure 1. Analysis of gamma-B crystallin expression and sequence integrity in *E. coli*

Figure S3

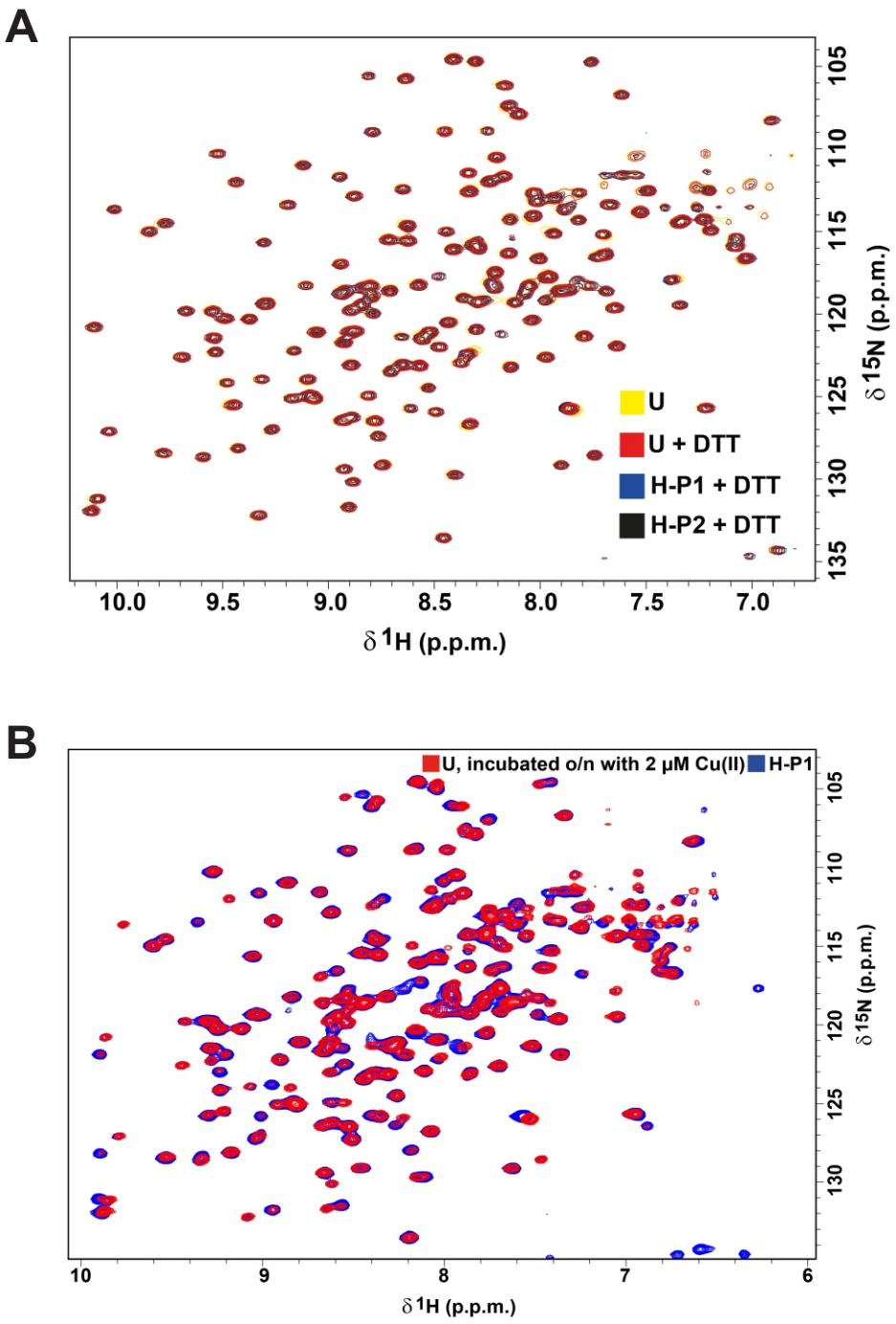


Figure S3, related to Figure 2. Overlay of 2D-1H-15N correlated NMR backbone spectra of gamma-B crystallin variants

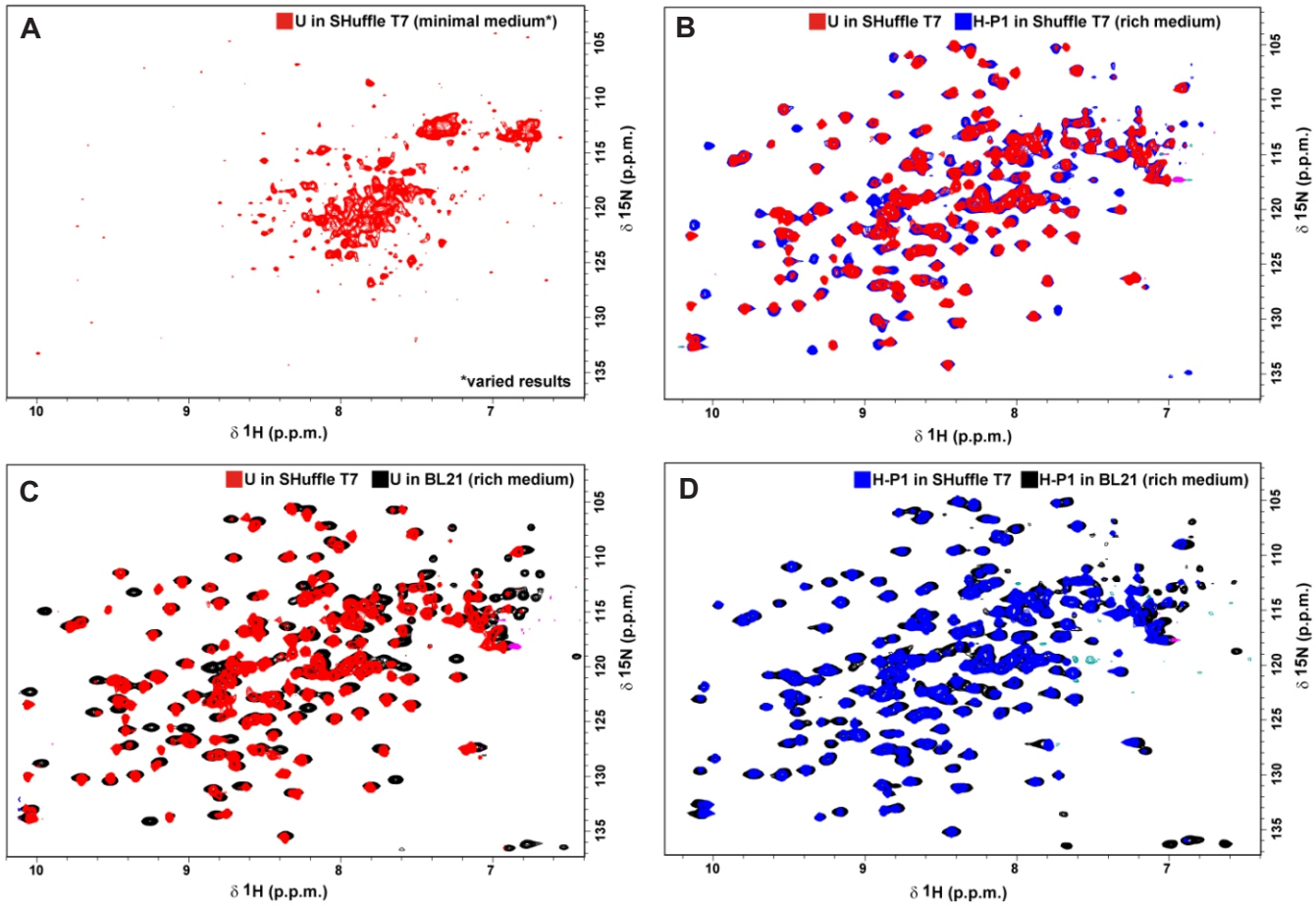


Figure S4, related to Figure 2. 2D-^{1}H-^{15}N correlated NMR backbone spectra of gamma-B crystallin variants U and H expressed in *E. coli* under normal (BL21 strain) and enhanced oxidizing conditions (SHuffle T7 strain)

Figure S5

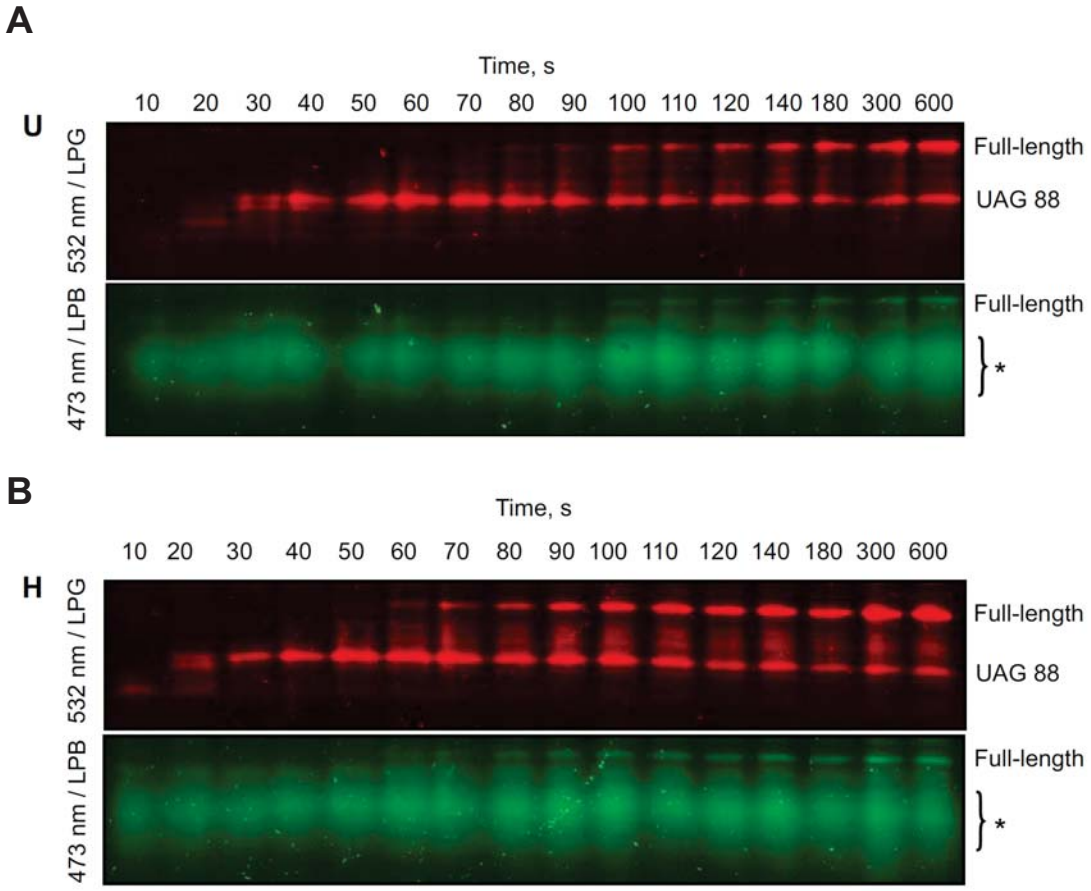


Figure S5, related to Figure 4. Kinetics of synthesis of U and H peptides with N-terminal BOP and BOF incorporated at the amber stop codon at position 88

Figure S6

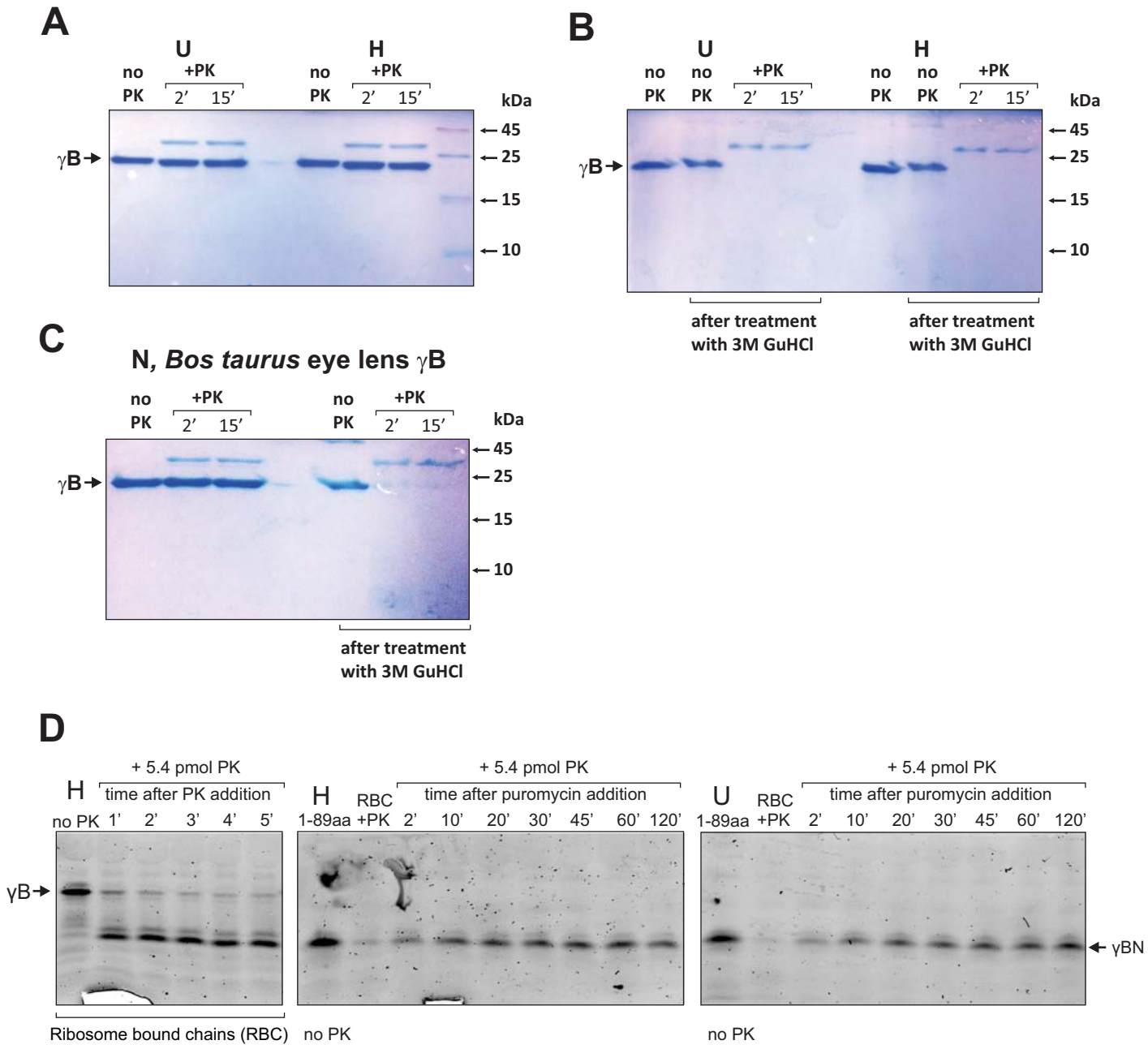


Figure S6, related to Figure 5. PK proteolysis of purified and *in vitro* translated gamma-B crystallin variants

SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. Analysis of gamma-B crystallin expression in *E. coli*
(A) Quantitative RT-PCR on RNA isolated from *E. coli* cells expressing either U or H variants. DNAse I pretreatment and PCR in the absence of RT served as a control (-RT). 16S rRNA PCR served as a reference. pET15b DNA was used to control for the size of the amplified fragments.
(B) SDS-PAGE of U and H pellet fractions (10 μg). CBB staining. Bands 1-6 were excised from the gel and subjected to MS analysis and microsequencing (*see* also Table S2).
(C) Mass spectra of the purified recombinant U and H proteins. The predicted mass of the 6xHis-tagged protein (U or H) is 21788.37 Da.

Figure S2, related to Figure 1. Analysis of gamma-B crystallin expression and sequence integrity in *E. coli*
(A) Sequence alignment of H and HM gamma-B crystallin variants. HM (Harmonized with Multiple additional synonymous substitutions) sequence was created by introducing additional synonymous changes to the H variant. Regions carrying synonymous substitutions are highlighted in red in H and blue in HM. HM is 90.7% identical to H.
(B) Expression of HM gamma-B crystallin (γB) variant in *E. coli*. Western blotting using polyclonal anti-γB antibodies; Soluble (S) and pellet (P) fractions are shown.
(C) Mascot Search Results showing sequence coverage and location of the MS-identified peptides in HM variant containing R92K substitution (K is enlarged).
(D) LC-MS/MS chromatogram showing that HM variant consists of a mixture of wild type and mutated peptides. Trypsin digest upper panel, chymotrypsin – bottom panel.

Figure S3, related to Figure 2. Overlay of 2D-^{1}H-^{15}N correlated NMR backbone spectra of gamma-B crystallin variants
(A) Addition of DTT resulted in full convergence of the backbone spectra of U (red), H-P1 (blue) and H-P2 (black) to a U-like spectrum (shown in yellow for comparison). These results corroborate the data presented in Figure 2D-F.
(B) Spectra of gamma-B crystallin U, incubated overnight with 2 μM Cu(II) under air supply and H-P1 variants
Both proteins were expressed in ^{15}N-rich labeling medium.

Figure S4, related to Figure 2. 2D-^{1}H-^{15}N correlated NMR backbone spectra of gamma-B crystallin variants U and H expressed in *E. coli* under normal (BL21 strain) and enhanced oxidizing conditions (SHuffle T7 strain)
(A) The spectrum of U expressed in SHuffle T7 cells in minimal medium.
(B) Overlay of U and H-P1 spectra, expressed in SHuffle T7 cells in rich medium.
(C) Overlay of spectra of U expressed in SHuffle T7 and BL21 cells (rich medium).
(D) Overlay of H-P1 spectra expressed in SHuffle T7 and BL21 cells (rich medium).

Figure S5, related to Figure 4. Kinetics of synthesis of U and H peptides with N-terminal BOP and BOF incorporated at the amber stop codon at position 88
(A) U variant. Translation products were resolved by SDS-PAGE and visualized by fluorescence scanning with different excitation wavelengths and emission filters for detection of BOP (top panel) and BOF fluorescence (bottom panel). Incomplete suppression of the amber stop codon (UAG88) and hydrolysis products of BOF-Cys-tRNA$^{Cys}_{CUA\ U32C}$ (asterisks) are indicated.
(B) Same as in **A,** except for the H variant. LPB, 510 nm long-pass filter; LPG, 575 nm long-pass filter.

Figure S6, related to Figure 5. PK proteolysis of purified and *in vitro* translated gamma-B crystallin variants
(A) PK proteolysis of U, H and N variants under native conditions (see Supplemental Experimental Procedures for details). CBB-stained Tris-tricine SDS-PAGE.
(B) After denaturation with 3M GuHCl, 24 h at 37°C.
(C) Natural purified bovine eye lens protein under native and denaturing (3M GuHCl, 24 h, 37°C) conditions.
(D) Proteolysis of *in vitro*-translated gamma-B crystallin H variant and the N-terminal domains (amino acids 1-89). Left panel: time course of PK proteolysis of ribosome-bound gamma-B crystallin chain (H variant; γB) obtained after 20 min of translation. A characteristic PK-resistant fragment has a size similar to that of isolated NTD (γBN). Middle and right panels: pulse proteolysis of ribosome-bound and puromycin-released gamma-B crystallin U and H, respectively.

SUPPLEMENTAL TABLES

Table S1. related to Figure 1. Nucleotide sequences of the U and H variants (gamma-B crystallin ORFs) and the respective codon usage frequencies in *B. taurus* and *E. coli*
***See* file Table S1.xls**

Table S2, related to Figure 1. Mascot Search Results showing sequence coverage and location of the MS-identified peptides (bands 1 to 6 from Figure S1A). All bands were digested in-gel using trypsin

Band #	Mascot Search Results Sequence Info	Trypsin digest Sequence coverage %
1	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B	**85%**
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
2	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B	**85%**
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
3	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B	**71%**
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
4	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B	**65.1%**
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
5	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B	**65.1%**
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
6	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B	**47.4%**
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	

Table S3, related to Figure 1 and S5. Mascot Search Results showing sequence coverage and location of the MS-identified peptides U, H and N (natural gamma-B crystallin isolated from bovine eye lenses) protein variants
Protein samples were digested using trypsin and/or chymotrypsin

Protein	Mascot Search Results Sequence Info	Sequence coverage %
U	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B trypsin	55%
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
U	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B chymotrypsin	87%
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
U	Composite: trypsin only, chymotrypsin only, trypsin and chymotrypsin	95%
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
H	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B trypsin	61%
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
H	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B chymotrypsin	89%
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
H	Composite: trypsin only, chymotrypsin only, trypsin and chymotrypsin	95%
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
N	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B trypsin	60%
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
N	**Acc. #:** 61888870 **Species:** BOS TAURUS **Name:** gamma-crystallin B chymotrypsin	91%
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	
N	Composite: trypsin only, chymotrypsin only, trypsin and chymotrypsin	95%
Sequence	1 MGKITFYEDR GFQGHCYECS SDCPNLQPYF SRCNSIRVDS GCWMLYERPN YQGHQYFLRR GDYPDYQQWM GFNDSIRSCR 81 LIPQHTGTFR MRIYERDDFR GQMSEITDDC PSLQDRFHLT EVHSLNVLEG SWVLYEMPSY RGRQYLLRPG EYRRYLDWGA 161 MNAKVGSLRR VMDFY	

Table S4, related to Figure 2, S3 and S4. Summary of NMR data collection

NMR data in Figure	**2C**	**2D, 2E, 2F, S3, S4 (H-P1)**
Expression strain	BL21(DE3)	BL21(DE3)
Expression medium	Modified M9, supplemented with amino acids	Celltone Complete (rich medium)
Labelling	^{15}N cysteine-selective	^{15}N uniform
Sample concentrations	0.8 – 1.2 mM	300 - 350 μM
Spectrometer	Bruker AV600	Bruker AV600
Console	Bruker Avance II	Bruker Avance III
Software	Bruker TopSpin 3.1	Bruker TopSpin 3.2
Probe	5 mm TCI cryogenic probe ^{1}H,^{15}N,^{13}C Z-gradients	5 mm TXI cryogenic probe ^{1}H,^{15}N,^{13}C Z-gradients
B_0 field	600 MHz	600 MHz
Pulse Sequence	BEST-[^{15}N,^{1}H]-TROSY	BEST-[^{15}N,^{1}H]-TROSY
Number of Scans	64	384
Number of complex points (TD/2)	512 (^{1}H) 128 (^{15}N)	384 (^{1}H) 128 (^{15}N)
Relaxation Delay	500 ms	500 ms
Spectral width	14.0261 p.p.m. (^{1}H) 49.0822 p.p.m. (^{15}N)	13.0234 p.p.m. (^{1}H) 49.9982 p.p.m. (^{15}N)
B_1 field for ^{15}N hard pulses	6250 Hz	6410 Hz
$1/4J_{HN}$	3 ms	3.1 ms
Offset for amide band selective proton pulses	8.5 p.p.m.	8.5 p.p.m.
Lengths of selective proton pulses	3000 μs (PC9) 2000 μs (REBURP) 1920 μs (EBURP-2)	3000 μs (PC9) 2000 μs (REBURP) 1920 μs (EBURP-2)

Table S5, related to S3A and S4A. Summary of NMR data collection

NMR data in Figure	**S3A (U, oxidized)**	**S4A**
Expression strain	BL21(DE3)	Shuffle T7
Expression medium	M9 minimal medium	M9 minimal medium
Labelling	^{15}N uniform	^{15}N uniform
Sample concentrations	1.0 mM	0.8 mM
Spectrometer	Bruker AV700	Bruker AV700
Console	Bruker Avance I	Bruker Avance I
Software	Bruker TopSpin 2.1	Bruker TopSpin 2.1
Probe	5 mm TXI cryogenic probe ^{1}H,^{15}N,^{13}C Z-gradients	5 mm TXI cryogenic probe ^{1}H,^{15}N,^{13}C Z-gradients
B_0 field	700 MHz	700 MHz
Pulse Sequence	BEST-[^{15}N,^{1}H]-TROSY	BEST-[^{15}N,^{1}H]-TROSY
Number of Scans	32	32
Number of complex points (TD/2)	896 (^{1}H) 256 (^{15}N)	512 (^{1}H) 128 (^{15}N)
Relaxation Delay	500 ms	500 ms
Spectral width	16.0250 p.p.m. (^{1}H) 34.2614 p.p.m. (^{15}N)	13.0312 p.p.m. (^{1}H) 49.9759 p.p.m. (^{15}N)
B_1 field for ^{15}N hard pulses	6850 Hz	6667 Hz
$1/4J_{HN}$	2.5 ms	2.8 ms
Offset for amide band	8.5 p.p.m.	8.6 p.p.m.

selective proton pulses		
Lengths of selective proton pulses	2200 µs (PC9) 1500 µs (REBURP) 1400 µs (EBURP-2)	2570 µs (PC9) 1710 µs (REBURP) 1640 µs (EBURP-2)

Table S6, related to S4. Summary of NMR data collection

NMR data in Figure	**S4B, S4C - D (Shuffle T7)**	**S4C - D (BL21)**
Expression strain	Shuffle T7	BL21(DE3)
Expression medium	Celltone Complete (rich medium)	Celltone Complete (rich medium)
Labelling	^{15}N uniform	^{15}N uniform
Sample concentrations	250 µM	300 - 350 µM
Spectrometer	Bruker AV700	Bruker AV600
Console	Bruker Avance I	Bruker Avance III
Software	Bruker TopSpin 2.1	Bruker TopSpin 3.2
Probe	5 mm TXI cryogenic probe ^{1}H,^{15}N,^{13}C Z-gradients	5 mm TXI cryogenic probe ^{1}H,^{15}N,^{13}C Z-gradients
B_0 field	700 MHz	600 MHz
Pulse Sequence	BEST-[^{15}N,^{1}H]-TROSY	BEST-[^{15}N,^{1}H]-TROSY
Number of Scans	512	384
Number of complex points (TD/2)	608 (^{1}H) 128 (^{15}N)	384 (^{1}H) 128 (^{15}N)
Relaxation Delay	500 ms	500 ms
Spectral width	13.0312 p.p.m. (^{1}H) 49.9759 p.p.m. (^{15}N)	13.0234 p.p.m. (^{1}H) 49.9982 p.p.m. (^{15}N)
B_1 field for ^{15}N hard pulses	6667 Hz	6410 Hz
$1/4J_{HN}$	2.8 ms	3.1 ms
Offset for amide band selective proton pulses	8.5 p.p.m.	8.5 p.p.m.
Lengths of selective proton pulses	2570 µs (PC9) 1710 µs (REBURP) 1640 µs (EBURP-2)	3000 µs (PC9) 2000 µs (REBURP) 1920 µs (EBURP-2)

Table S7, related to Figure 2, S3 and S4. Backbone resonance assignment.

Backbone resonance assignment		
Expression strain	BL21(DE3)	
Expression medium	M9 minimal medium	
Labelling	^{15}N, ^{13}C uniform	
Sample concentrations	1.2 mM	
Spectrometer	Bruker AV800	Bruker AV900
Console	Bruker Avance I	Bruker Avance I
Software	Bruker TopSpin 2.1 Cara 1.9.0	Bruker TopSpin 2.1 Cara 1.9.0
Probe	5 mm TXI cryogenic probe ^{1}H,^{15}N,^{13}C Z-gradients	5 mm TXI cryogenic probe ^{1}H,^{15}N,^{13}C Z-gradients
B_0 field	800 MHz	900 MHz
Pulse sequences	BEST-TROSY HNCO BEST-TROSY-HNCA TROSY-HN(CO)CACB	BEST-TROSY TROSY-HNCACB

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Analytical scale protein expression

Analytical-scale protein expression was performed in BL21 (DE3) *Δtig::Kan* strain (Deuerling et al., 1999) (B, F^-,*dcm, ompT, hsd*S(r_B^-,m_B^-), *gal*, (DE3) *Δtig::Kan*, a gift of Dr. Bernd Bukau, ZMBH, Heidelberg, Germany), which lacks the ribosome-associated chaperone Trigger Factor. Freshly transformed *E. coli* cells harboring either U or H gamma-B crystallin variants were grown in flasks with 50 mL of liquid Luria Bertani (LB) medium containing 100 µg/mL ampicillin at 37°C with shaking at 230 rpm until the cultures reached an OD_{600} of 0.8. Protein expression was induced by addition of 1 mM IPTG (isopropyl-β-d-thiogalactopyranoside) and incubation was continued for an additional 1.5 h at 37°C with shaking. Cells were harvested by centrifugation (3200×g, 15 min). Cell pellets were re-suspended in 1 mL of 50 mM Tris-HCl buffer, pH 8.0, containing 500 mM NaCl and 1 tablet of EDTA-free protease inhibitor cocktail (Roche), and sonicated (20 s, 3 times) using an ultrasonic processor (Fisher Scientific). Lysates were pelleted at 17,000×g for 30 min, using a TLA-55 fixed-angle rotor (Beckman Coulter) to separate insoluble/aggregated proteins (pellet) from soluble protein (supernatant). The supernatant was transferred into a clean tube. The pellet was re-suspended in an equal (to that of supernatant) volume of 50 mM Tris-HCl buffer, pH 8.0, containing 500 mM NaCl and EDTA-free protease inhibitor cocktail (Roche).

Quantitative RT-PCR

For analysis of relative mRNA expression levels, total RNA was isolated from *E. coli* BL21 (DE3) *Δtig::Kan* cells expressing U and H variants using Trizol Reagent (Life Technologies). Total RNA (2 µg) was subjected to DNAse treatment (Promega) and 1 µg of DNAse-treated RNA was used for cDNA amplification with the Invitrogen Superscript III RT kit (Life Technologies). PCR reactions were carried out for 21 cycles (to avoid saturation) using the following gamma-B crystallin U/H variant-specific primers:

U forward: 5'-AAAAACCATGGGGAAGATCACTTTTTAC-3'
U reverse: 5'-GAAAGTGCCGGTGTGTTGCGG-3
H forward: 5'-AAAAACCATGGGAAAAATCACTTTCTATG-3'
H reverse: 5'-CCGAAAAGTACCGGTATGTTGTGG-3',

Both the U and H primer pairs generated products 264 bp length. PCR without RT, using DNAse-treated RNA as the template was used to control for plasmid DNA contamination. Amplified fragments of 16S rRNA were used as reference standards (16S rRNA forward primer: 5'- GCTACAATGGCGCATACAAA-3'; 16S rRNA reverse primer: 5'-TTCATGGAGTCGAGTTGCAG-3'; 101 bp PCR product).

Analysis of recombinant protein expression

Protein concentrations in samples were determined using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific) with bovine serum albumin (BSA) as a reference standard and equal total protein amounts were analyzed for each sample. For Western blotting, 2 µg total protein (soluble and/or insoluble fractions) was separated under reducing conditions using Tris-Tricine SDS-PAGE using a 4% stacking and a 16.5% separation gel (49.5% T, 3% C). Proteins were transferred to a 0.45 µm pore size PVDF membrane (EMD Millipore) using a wet Mini Trans-Blot blotting system (Bio-Rad Laboratories) at 110V for 1.5 h. The membrane was blocked (7.5% non-fat dry milk, TBS, 0.1% Tween) for 1 h at room temperature, washed and probed with either anti-gamma-B crystallin antibody (1:1000 dilution, P-18, Santa Cruz Biotechnology) (Figure 1C) or with mouse monoclonal anti-poly-histidine antibody (1:3000, H1029, Sigma) (Figure 1E). Membranes were then incubated with secondary donkey anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology) or goat anti-mouse IgGH&L(HRP) (1:4000, ab6789, Abcam) antibodies. Signals were detected using ECL Plus reagent (GE Healthcare) and a Typhoon 9410 imaging scanner (GE Healthcare). Band intensities were quantified using either Image Quant (v. 2005) or ImageJ software. For ELISA, 2 µg of total cellular protein in 200 mM sodium carbonate/bicarbonate buffer, pH 9.5 was coated on 96 well plates and incubated overnight, at 4°C. Protein was blocked using 300 µL blocking buffer containing 7.5% non fat dry milk (Promega) in 0.1% Tris-Buffered Saline (TBS) per well and incubated for 1 hr at room temperature. After 1 hr, blocking buffer was removed and 200 µL of polyclonal anti-gamma-B crystallin antibody (P-18, Santa Cruz Biotechnology, 1:1000) diluted in 7.5% non fat dry milk, 0.1% tween TBS was added to each well and incubation was continued on shaking platform for 1 hr at room temperature. Wells were washed with 300 µl wash buffer (0.1% tween TBS) for 5 min (×3). After washing, secondary donkey anti-goat IgG-HRP antibody (1:2000) (Santa Cruz Biotechnology) diluted in 7.5% non fat dry milk, 0.1% tween TBS was added and incubation was continued for 1 hr on shaking platform followed by washing with 300 µL of wash buffer (0.1% tween TBS) for (5 min x 3 times). For detection, 100 µL of TMB One Solution (Promega) was added to each well and the samples were incubated for 10 min with shaking in the dark at room temperature. Reactions were stopped by adding 100 µL of 1N hydrochloric acid (HCl) to each well. Protein was detected by recording the absorbance at 450 nm on Victor3™ Plate reader (PerkinElmer). Percentages of the soluble and pellet fractions were calculated as a fraction of the total protein.

Recombinant protein purification

Gamma-B crystallin protein variants expressed as described above were isolated using immobilized metal-affinity chromatography (IMAC) on Ni-Nitrilotriacetic Acid Agarose (Ni-NTA) (Qiagen) followed by size-exclusion

chromatography (SEC) on a Superdex 75 gel-filtration column (1.6 cm×60 cm) (GE Healthcare) using an ÄKTA purifier liquid chromatography system (GE Healthcare). Gamma-B crystallin fractions were collected and concentrated using an Amicon Ultra-4 centrifugal device (EMD Millipore). Aliquots were flash frozen in liquid nitrogen and stored at -80°C. The purified protein samples were subjected to MS analysis and microsequencing at either the Cleveland Clinic proteomics core facility or the Harvard Mass Spectrometry facility.

Gamma-B crystallin purification from bovine eye lenses

Gamma-B crystallin from bovine eye lenses (native protein, N) was purified generally as described (Slingsby and Miller, 1983) with the following modifications. Frozen lenses collected from young cows (less than 30 months old) were purchased from Animal Technologies (Tyler, TX, USA). Lenses were thawed, decapsulated and homogenized in 50 mM Tris-HCl buffer, pH 7.2, containing 1 tablet of EDTA-free protease inhibitor cocktail (Roche). After the homogenate was centrifuged at 27,000×g for 30 min, the pellet was discarded and the supernatant was filtered and used for further purification. Gamma crystallins were separated from other proteins (including alpha- and beta-crystallins) by SEC using a Superdex 75 gel-filtration column (1.6 cm×60 cm, GE Healthcare) on an ÄKTA purifier liquid chromatography system (50 mM Tris-HCl buffer, pH 7.2, flow-rate 0.75 mL/min). Fractions containing gamma crystallins were collected and dialyzed against 20 mM Tris-acetate, pH 6.0 (buffer A). Proteins were further separated by cation exchange chromatography on a Mono S 5/50 Column (GE Healthcare). The column was equilibrated and washed with buffer A and a gradient and stepwise elution of buffer B (20 mM Tris-Acetate, 0.4 M sodium acetate, pH 6.0) was used. Elution was done by running a 5-10% buffer B gradient for 2.5 min, 10% isocratic buffer B for 10 min, followed by a 10-40% buffer B gradient for 15 min (flow rate 0.5 mL/min). To identify fractions containing gamma-B crystallin, proteins from each isolated peak were run on Tris-tricine SDS-PAGE gels, Coomassie stained, and submitted for MS analysis and microsequencing (Table S3).

Proteolysis of purified gamma-B crystallin

For proteinase K (PK) proteolysis under native/non-denaturing conditions, 190 pmol (4 μg) of purified gamma-B crystallin (U, H and/or N) in 50 mM Tris-HCl pH 8.0, 200 mM NaCl were treated with 38 pmol (1.1 μg) PK at 37°C. Reactions were stopped by PMSF addition (10 mM final concentration) and samples were immediately flash frozen in liquid nitrogen, thawed, mixed with 2X Tris-tricine loading buffer and resolved on Tris-tricine SDS-PAGE gels (Schägger and von Jagow, 1987). For proteolysis under denaturing conditions, gamma-B crystallin (U, H and/or N) in 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 3M GuHCl was first denatured at 37°C for 24 h and then subjected to PK treatment as described above.

Mass spectrometry

Identification of gamma-B crystallin protein products was carried out by LC-MS/MS analysis at the Cleveland Clinic Mass Spectrometry Laboratory for Protein Sequencing (Cleveland, OH) and the Harvard Mass Spectrometry facility (Cambridge, MA). Finnigan LTQ-Obitrap Elite hybrid mass spectrometer systems were used. Purified proteins or gel-separated protein products were subjected to MS analysis. For in-gel protein digestion, bands were excised, washed with water, and dehydrated in acetonitrile. The bands were then reduced with DTT and alkylated with iodoacetamide prior to in-gel digestion. Bands were digested in-gel by adding either 5 μL 10 ng/μL trypsin or 10 ng/μL chymotypsin in 50 mM ammonium bicarbonate and incubating overnight at room temperature to achieve complete digestion. Peptides were extracted with a 50% acetonitrile, 5% formic acid solution, evaporated to <10 μL using a Speedvac and resuspended in 1% acetic acid to a final volume of 30 μL for LC-MS/MS analysis.

mRNA for *in vitro* translation

mRNAs coding for full-length U and H protein variants, or the N-terminal fragment (amino acids 1-89) of U and H, were prepared by T7 transcription using pET15b-based constructs containing the corresponding sequences. Neither construct contained the C- terminal 6xHis tag or a stop codon. The T7 forward primer (5'- GATCCCGCGAAATTAATACGACTC-3') was used with the following reverse primers:

U (full-length): 5'-ATAAAAATCCATCACCCGTCTTAAAGAACC-3';
U (1-89): 5'- GAAAGTGCCGGTGTGTTGCG-3';
H (full-length) 5'- GTAGAAGTCCATAACGCGCCGTA-3'; and
H (1-89): 5'-AAAAGTACCGGTATGTTGTGGGATTAAG-3'.

For FRET experiments, the threonine codon at position 88 (counting from the initiator Met) of full-lengths U or H mRNA was replaced by an amber stop codon UAG. The position 88 was chosen because it is located (i) close to the end of the NTD (position 80), which should allow for monitoring the NTD folding as soon its synthesis is completed, and (ii) at a distance that would allow efficient FRET, and because the linker can be replaced without a change in protein stability (Mayr et al., 1994).

Fluorescence-labeled tRNAs

To obtain BOP-Met-tRNA^{fMet}, [^{3}H]Met-tRNA^{fMet} (15 μM) was labeled with Bodipy576/589-NHS (5 mM) (MolecularProbes) in 100 mM sodium acetate pH 5.0, 80% DMSO for 18 h at 4°C. Unreacted fluorophore was removed by extraction with 50:50 phenol:chloroform mixture followed by ethanol precipitation. BOP-Met-tRNA^{fMet} was purified by HPLC on a

LiChrospher WP300 RP-18 column using a gradient of 5 to 40% ethanol in 20 mM ammonium acetate, 10 mM $MgCl_2$, 400 mM NaCl pH 4.5.
The gene coding for *E. coli* $tRNA^{Cys}$ under a T7 promotor was generated by primer overlap PCR and was ligated into the SmaI site of pUC19. Genes coding for amber, opal and ochre suppressor $tRNA^{Cys}$ variants were constructed by site-directed mutagenesis of the anticodon. $tRNA^{Cys}$ variants were generated by *in vitro* transcription and purified on a HiTrapQ column (GE healthcare) using a linear gradient of 0 to 1.1 M NaCl in 50 mM sodium acetate pH 5.0 and 10 mM $MgCl_2$. Aminoacylation was carried out in 100 mM HEPES pH 7.5, 15 mM KCl, 7 mM $MgCl_2$, 5 mM DTT with 2 mM ATP, 150 µM L-cysteine, 35 A_{260} units/mL of $tRNA^{Cys}$ variant, and 6 µM CysRS-His_6 for 45 min at 37°C. The yield of aminoacylation was 50% for all variants. Opal suppressor Cys-$tRNA^{Cys}$ from *E. coli* was reported to be active in translation in rabbit reticulocyte and wheat germ extracts (Gubbens et al., 2010). In contrast, in the *E. coli* system, only wt $tRNA^{Cys}$ and the amber suppressor $tRNA^{Cys}_{CUA}$ were active. To improve the decoding properties of $tRNA^{Cys}_{CUA,}$ we introduced a further replacement into the anticodon loop, U32 to C, which restored the rate of decoding to the level of wt $tRNA^{Cys}$ (Olejniczak and Uhlenbeck, 2006). Cys-$tRNA^{Cys}_{CUA\ U32C}$ (30 µM) was labeled with BodipyFL-C_1-IA (Molecular Probes) (1 mM) in 50 mM Hepes pH 7.5, 50% DMF for 45 min at 25°C. Unreacted fluorophore was removed by extraction with 50:50 phenol:chloroform mixture followed by ethanol precipitation. BOF-Cys-$tRNA^{Cys}_{CUA\ U32C}$ was separated from deacylated and unlabeled tRNA by HPLC on a LiChrospher WP300 RP-18 column using a gradient of 5 to 40% ethanol in 20 mM ammonium acetate, 10 mM $MgCl_2$, 400 mM NaCl.

***In-vitro* translation**

Translation in the fully reconstituted single-turnover *in vitro* translation system was carried out as described (Doerfel et al., 2013; Mittelstaet et al., 2013). 70S initiation complexes (IC) were prepared by incubating 70S ribosomes (purified from *E. coli* MRE 600 strain (Rodnina, and Wintermeyer, 1995) (0.75 µM) with mRNA (1.5 µM), BOF- or BOP-labeled [^{3}H]Met-$tRNA^{fMet}$ (0.5 µM), a mixture of initiation factors IF1, IF2, IF3 (1.5 µM each), and GTP (2 mM) in $TAKM_7$ buffer (50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$ 2 mM DTT) for 1 h at 37°C. Initiation efficiency was verified by nitrocellulose filtration and [^{3}H]-radioactivity counting (Milon et al., 2007); typically, all added fluorescence-labeled [^{3}H]Met-$tRNA^{fMet}$ was found in the complex with the ribosome. IC stock (0.125 µM) was diluted to HiFi translation buffer (50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 3.5 mM $MgCl_2$, 0.5 mM spermidine, 8 mM putrescine, 2 mM DTT). Factor mix (FM) was formed by incubating elongation factor (EF) EF-Tu–GDP (250 µM), GTP (2 mM), phosphoenolpyruvate (6 mM), pyruvate kinase (2%), and EF-G (7.5 µM) at 37°C for 15 min, followed by addition of purified total *E. coli* aa-tRNA (100 µM) aminoacylated with the mixture of amino acids containing [^{14}C]Phe and additional incubation for 2 min at 37°C. The concentrations of the components in the *in vitro* system were optimized to achieve the speed and fidelity similar to that *in vivo* (Wohlgemuth et al., 2010; Rudorf et al., 2014). *In vitro* translation was initiated by rapid mixing of IC (0.015 µM) with FM (40 µM ternary complex) at 37°C. At indicated time points after the start of translation, aliquots (25 µl) were withdrawn and the reaction was stopped by adding 5 µl of 2 M NaOH and digested for 30 min at 37°C. The efficiency of translation was tested by precipitating the peptides by cold trichloroacetic acid (5%) and measuring [^{3}H]Met and [^{14}C]Phe incorporation into proteins; typically, 70-80% of the IC carried peptides of expected length. 2 M Hepes acid (5.5 µL) and 2X loading buffer (50 mM Tris-HCl, pH 6.8, 12% (w/v) glycerol, 2% β-mercaptoethanol, 4% SDS) was added to each sample and the translation products were separated by Tris-tricine SDS-PAGE using 4% stacking, 10 % spacer and 16.5 % separation gels (49.5% T, 3% C). The gel was washed with water and scanned on a FLA-9000 fluorescence imager (Fujifilm Lifescience). Fluorescence was excited at 473 nm and monitored after passing a LPB (510LP) filter. Because the mRNAs used for translation did not have a stop codon, translation was limited to a single round per ribosome and the nascent peptides did not dissociate from the ribosome, unless released by puromycin treatment.
For FRET experiments, translation was carried out with an IC containing BOP-Met-$tRNA^{fMet}$ and an mRNA variant containing a UAG stop codon at position 88. The stop codon was decoded by an amber suppressor $tRNA^{Cys}_{CUA\ U32C}$ (Gubbens et al., 2010) aminoacylated with Cys and thiol-modified by BOF. The fluorophores BOF or BOP were exited at 470 nm and 540 nm, respectively. The emission of BOF was monitored after passing a 500 nm cut-off filter; the emission of BOP after a 570 nm cut-off filter. As the acceptor-only control, unlabeled Cys-$tRNA^{Cys}_{CUA\ U32C}$ was used. Cys-$tRNA^{Cys}_{CUA\ U32C}$ was separated from deacylated tRNA by purifying the ternary complex EF-Tu•GTP•Cys-$tRNA^{Cys}_{CUA\ U32C}$ on a Superdex 75 10/300 size exclusion column.

Protein expression and purification for NMR spectroscopy

Aliquots of Invitrogen OneShot BL21(DE3) *E. coli* cells (Life Technologies) or SHuffle T7 *E. coli* cells (NEB) were freshly transformed with the pET15b plasmid (U or H) and incubated overnight in 50 mL LB medium containing 100 mg/L ampicillin (37°C, 220 rpm shaking). Pre-cultures were pelleted (15 min, 5000×g, 4°C) and resuspended in a small volume of the final expression medium, which was then used to inoculate main cultures to a starting OD_{600} of 0.2 – 0.3 for BL21(DE3) and 0.5 for SHuffle T7 cells.
All expression media contained 100 mg/L ampicillin. A rich medium for uniform ^{15}N-labeling, Celltone Complete (98% ^{15}N, Cambridge Isotope Labs) was used (incubation conditions: 250 mL culture volume in 1 L baffled flasks, 37°C, 160-180 rpm). ^{15}N/^{13}C–labeled gamma-B crystallin for backbone assignment was expressed in M9 minimal medium containing 1 g/L [^{15}N]NH_4Cl and 2 g/L [U-^{13}C]glucose (incubation conditions: 2 L culture volume in 5 L baffled flasks, 37°C, 120 rpm). For

selective ^{15}N-cysteine labeling, a modified M9 medium containing [^{15}N]cysteine was used, as described by Muchmore et al., 1989 (incubation conditions: 1 L culture volume in 5 L baffled flasks, 37°C, 120 rpm). Expression cultures were incubated at 37°C until an OD_{600} of 0.8 was reached, at which point protein expression was induced by addition of 1 mM IPTG and allowed to continue for 3 h before harvesting the cells by centrifugation (15 min, 5000×g, 4°C). Per 1 L of culture volume, cell pellets were resuspended in 35 mL of lysis buffer (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 20 mM imidazole, complete EDTA-free protease inhibitor tablet (Roche)). For resuspension volumes smaller than 50 mL, cells were lysed by sonication (6 × 1 min, 5 min cooling on ice, 40% cycle time, 60% power, Bandelin Sonoplus HD 2070). For resuspension volumes greater than 50 mL, lysis was performed using an M-110P microfluidizer (3 cycles, 1000 Bar, Microfluidics). Using a divided reference expression, we demonstrated that no artificial structural changes in gamma-B crystallin were introduced by different lysis conditions (data not shown). Cell lysates were cleared by centrifugation (2 × 30 min, 20.000×g, 4°C) and supernatants were loaded onto a 5 mL HisTrap HP Ni-NTA column (GE Healthcare) that was pre-equilibrated with NTA washing buffer (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 20 mM imidazole). Columns loaded with lysate were washed to A_{280} baseline levels and 6xHis-tagged gamma-B crystallin was eluted with NTA elution buffer (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 250 mM imidazole). Protein fractions were pooled and concentrated to a volume of 10 mL using a Vivaspin 20 centrifugal concentrator (Sartorius), followed by SEC using a HiLoad 26/60 Superdex 75 prep grade gel-filtration column (GE Healthcare) in IEX buffer A (50 mM Tris, pH 9.0). Fractions corresponding to monomeric gamma-B crystallin were pooled and loaded onto a 5 mL HiTrap Q XL anion exchange column (GE Healthcare), which was subsequently washed with 5 column volumes of IEX buffer A. pH gradient elution over 30 column volumes to 100% IEX buffer B (50 mM Tris, pH 6.0, 50 mM NaCl) was performed and corresponding peak fractions were pooled. Concentration to a final volume of 600 µL and buffer exchange into NMR buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 10% D_2O, 0.1% DSS) was performed using Vivaspin 20 and Vivaspin 2 centrifugal concentrators (Sartorius). All chromatographic steps were performed on an ÄKTA purifier liquid chromatography system (GE Healthcare) at 25°C. The combination of protein concentrations above 5 mg/ml and temperatures below 20°C was avoided at all times during purification in order to prevent potentially structurally relevant phase transitions and precipitation. 10 µM samples for SDS-page and native-PAGE were taken after each purification step. Samples for C-4 reverse-phase HPLC were taken before and after ion exchange chromatography.

Oxidation of U with catalytic amounts of Cu(II)

Gamma-B crystallin U variant (10 µM in NMR buffer) isolated for NMR analysis was incubated overnight with 2 µM $CuCl_2$ under air supply and continuous stirring, essentially as described by Cavallini et al., (1969). The protein was then re-purified by SEC in NMR buffer, concentrated to 1 mM and subjected to NMR analysis.

Analytical HPLC

Analytical HPLC runs were performed on a Jasco HPLC system using a PerfectSil 300 C4-RP column (250 × 4.6 mm). Gradient: 10-35% B (20 min), 35% B isocratic (10 min), 45-50% B (15 min). Buffer A: H_2O, 0.1% TFA; Buffer B: Acetonitrile, 0.1% TFA.

Supplemental References

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