SUPPLEMENTARY INFORMATION

Figure S1: Proteolysis of wtCBS under native conditions: analysis by native electrophoresis.

"N" refers to the uncleaved control, and the time points are depicted in minutes. The expected migraton of oligomers is shown by arrows in accordance with the previous report on the behavior of CBS in native electrophoretic gels *(Kozich et al. 2010).* Shifts in the migration of CBS during the proteolytic time course exhibit a dose-response pattern that was reported previously *(Kopecka et al. 2011)*. The analysis shows the absence any detectable monomeric or dimeric intermediate during the proteolytic time course.

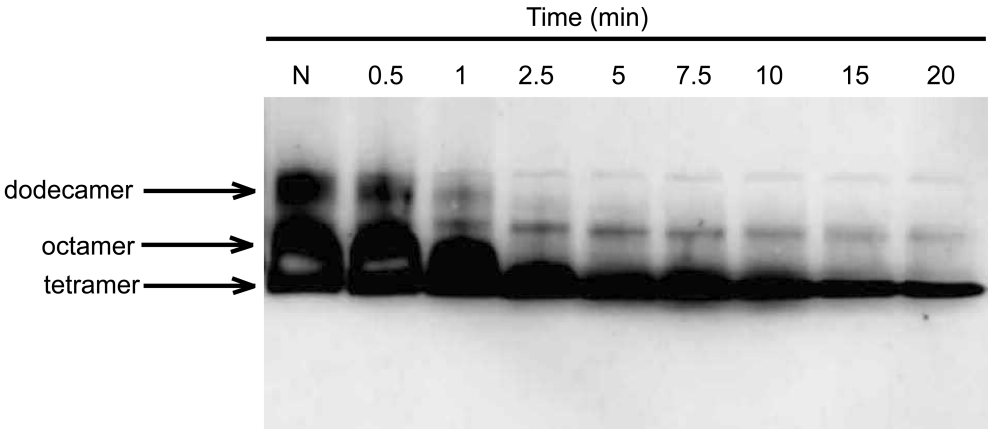


Figure S2: Nonlinear data fitting for wild-type CBS in bacterial crude extract. Points are the mean with standard deviations from four measurements. A. Proteolytic kinetics under native conditions; proteins in cell lysate (1 mg/ml) were cleaved by thermolysin (0.2 mg/ml). B and C. Dependence of k_{obs} (B) and the fraction of uncleaved protein B (C) on the concentration of thermolysin during proteolysis of wild-type CBS. D. Pulse proteolysis of wild-type CBS in bacterial crude extract. Proteins in cell lysate (1 mg/ml protein) were cleaved by thermolysin (0.2 mg/ml) for 60 seconds. The concentration of urea is plotted against f_{fold}, the fraction of folded proteins that remained uncleaved after the proteolytic pulse. The f_{fold} values at each point were calculated as a ratio of the remaining protein to the amount of uncleaved protein in the absence of urea.

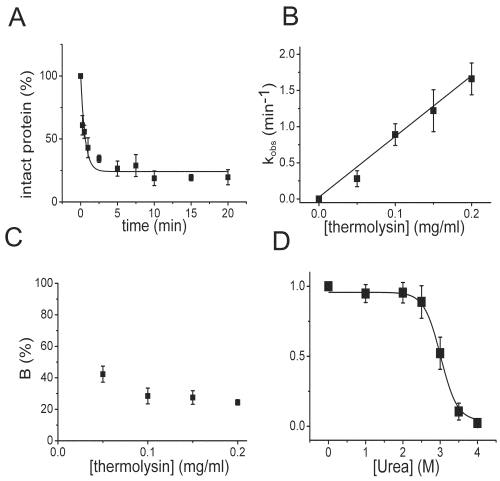


Figure S3: Proteolytic kinetics under native conditions of the wild-type and mutant CBS proteins at the thermolysin concentration that enabled the observation of the proteolytic time-course. Points are the mean with standard deviations from four measurements. Below each plot, the corresponding representative gels are shown. The values of k_p depicted in Table 2 were determined from these experiments. For cleavage of p.E302K and p.G307S, partially cleaved fragments were observed; this limited proteolysis was caused by the extremely low amounts of thermolysin in these experiments.

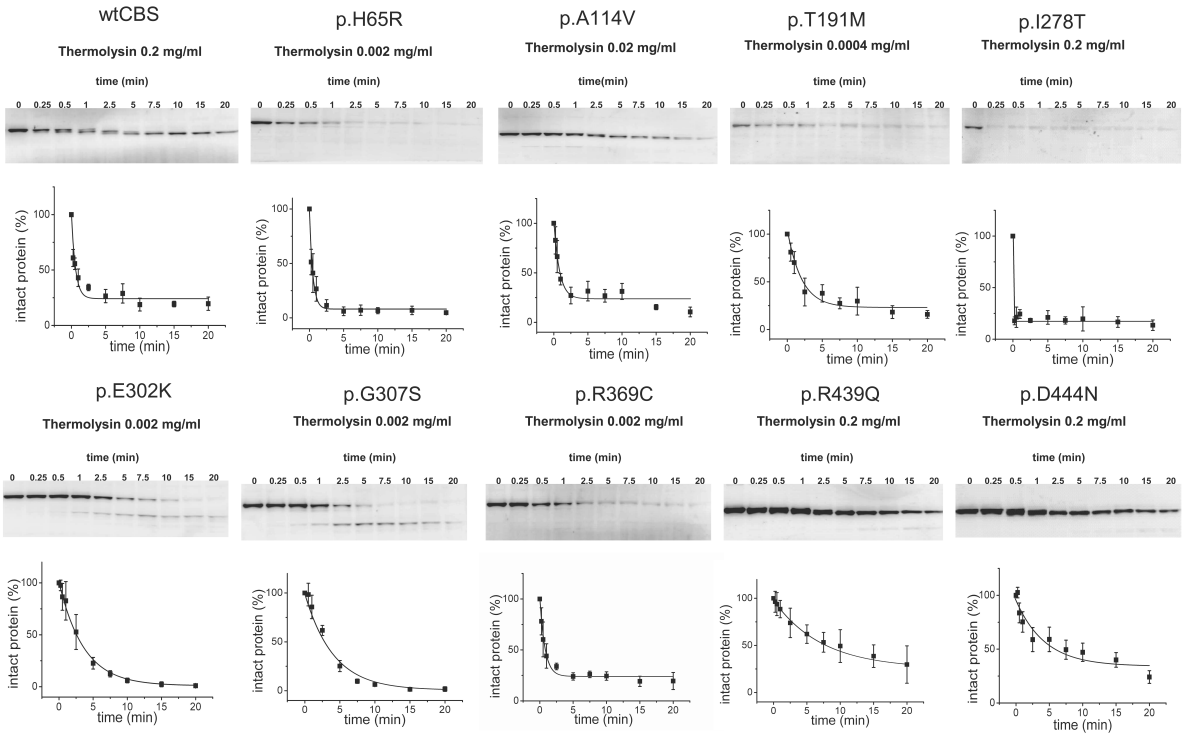


Figure S4: **Proteolytic cleavage of wild-type and mutant CBS in the concentration of urea that correspond to the beginning of the unfolding transition zone for each protein - plots with corresponding representative gels.** This experiment assessed the possible systematic error in the determination of the c_m value. Systematic error in determination of c_m value is negligible if kinetic constant of the cleavage is lower than 0.2 min^{-1} *(Park and Marqusee 2006)*.

A. The kinetic constant of wild-type CBS in 2.5M urea was determined to be 0.13 ± 0.01 min^{-1}.Points are the mean with standard deviations from four measurements. B. The kinetic constant of p.A114V in 2.5M urea was determined to be 1.14 ± 0.30 min^{-1}. Points are the mean with standard deviations from four measurements. Rapid cleavage indicates that c_m value for p.A114V may be determined with systematic error.

The cleavage of the unfolded mutants p.T191M, p.I278T and p.R369C (C) and the rigidified mutant and p.D444N (D) was negligible during the time intervals chosen. The mutant p.R439Q (D) was cleaved too slowly for reliable nonlinear data fitting during time interval chosen.

For wild-type CBS, p.R439Q and p.D444N, proteolytic fragments were observed; limited proteolysis was likely caused a by low catalytic activity of thermolysin in higher concentrations of urea.

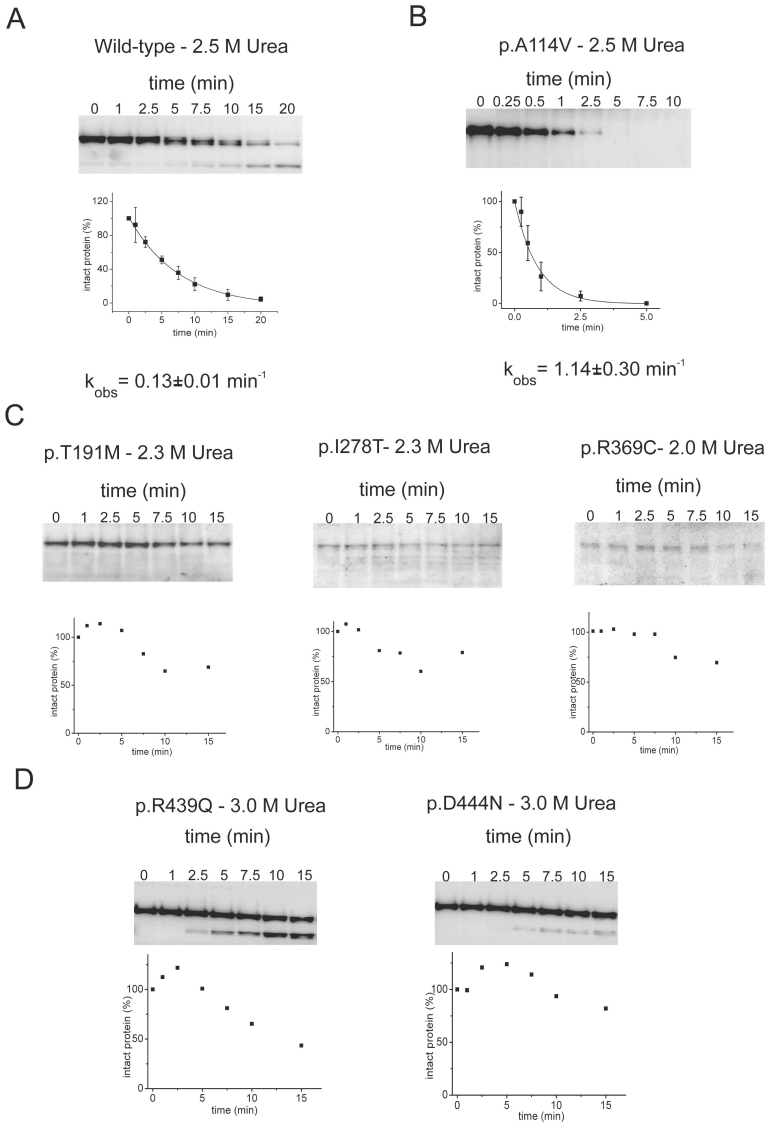


Figure S5: The amount of CBS proteins in crude cell extracts after expression in E. coli at 37 °C.

Bacterial cell lysates (20 µg total protein) were loaded to each lane after expression of CBS proteins using corresponding pHCS3 plasmids.

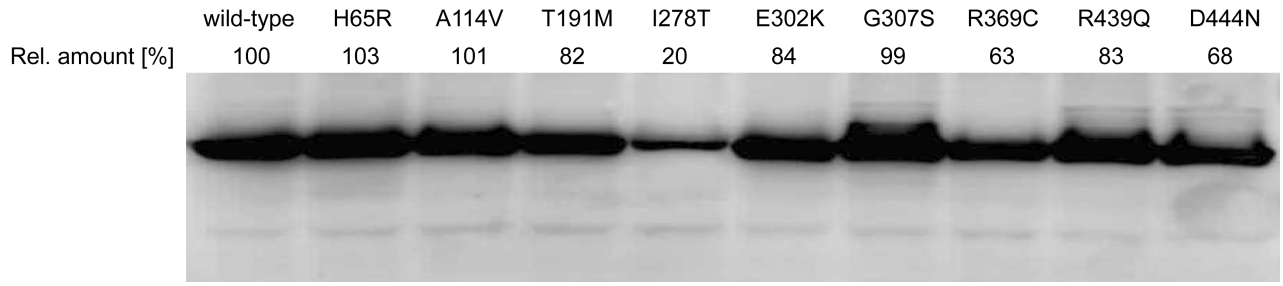


Figure S6: Pulse proteolysis of wild-type and mutant CBS proteins – plots with corresponding representative gels. The concentration of urea is plotted against f_{fold}, the fraction of folded proteins that remained uncleaved after the proteolytic pulse. The f_{fold} values at each point were calculated as a ratio of remaining protein to the amount of uncleaved protein in the absence of urea. Points are the mean with standard deviations from four measurements. Dashed lines in the plot of each mutant represent the curve determined for wild-type CBS. The presence of proteolytic fragments was observed only in several concentrations of urea for mutants p.A114V and p.R439Q; limited proteolysis was likely caused by a low catalytic activity of thermolysin in higher concentrations of urea.

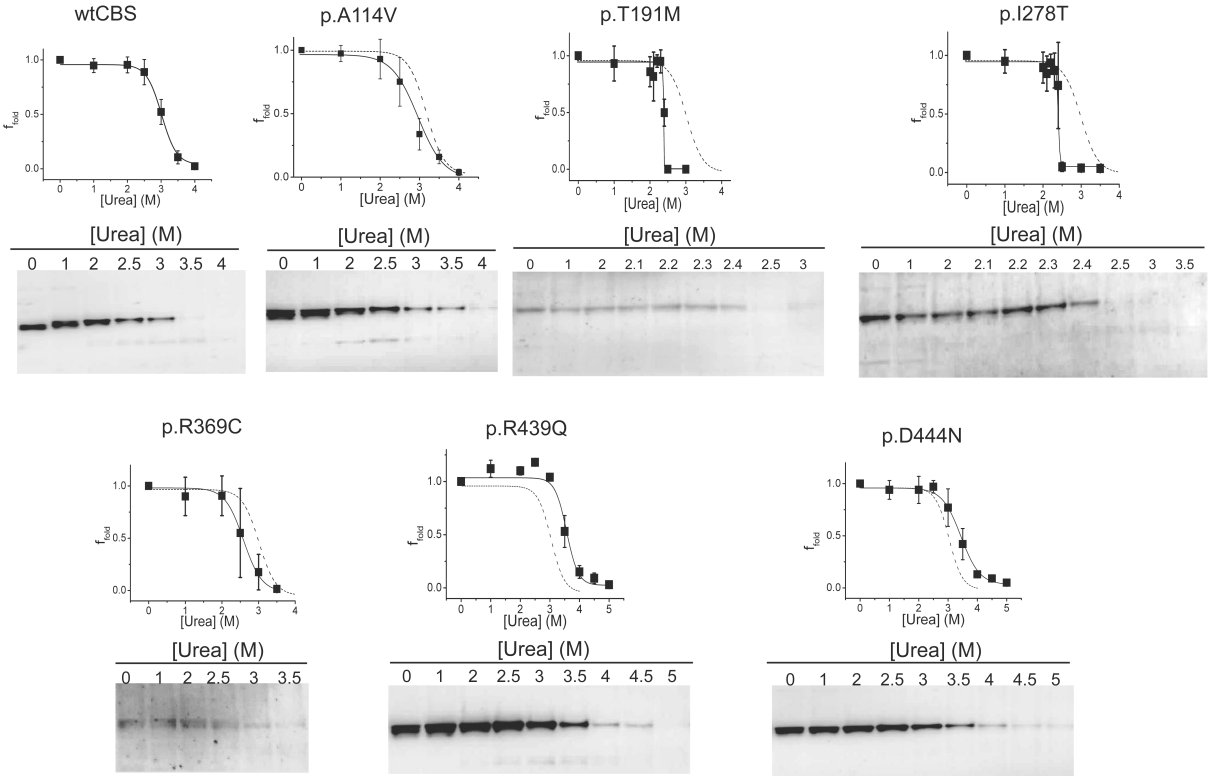


Figure S7: Evaluation of the pulse proteolysis of p.A114V , which calculates the correction on cleavage of partially unfolded proteins.

Table. For each concentration of urea, the following values were calculated: i, "*estimated* k_{obs}" is a kinetic constant for the proteolysis of p.A114V in urea concentrations if urea-induced unfolding did not occur. The values are calculated according to observed k_{obs} in the absence of urea together with theoretical decrease in k_{cat} of thermolysin in the urea gradient that was previously published *(Park and Marqusee 2004b)*. ii, "*estimated amount of uncleaved protein in the absence of urea-induced unfolding*" represents the amount of p.A114V that will be uncleaved after the pulse if urea-induced unfolding did not occur. It is calculated according to values of "*estimated* k_{obs}." iii, "*fraction of globally unfolded protein*" was calculated as the difference between the estimated and observed amount of uncleaved protein. iv, the fraction of partially unfolded protein is *100 -fraction of globally unfolded protein.* Using this estimation, we assessed the degree of urea-induced unfolding of the protein.

In the plots below the table, dashed lines represent the curve of wild-type CBS. Points are the mean with standard deviation from four measurements. *Left*. the default analysis neglects the cleavage of the partially folded mutant at the beginning of the apparent transition zone. *Right*. the plot shows the corrections due to the rapid cleavage of the partially unfolded mutant protein based on the data from the table.

Urea (M)	estimated k_{obs} (min^{-1})	estimated amount of uncleaved protein in absence of urea-induced unfolding (%)	observed amount of uncleaved protein (%)	fraction of globally unfolded proteins (%)	fraction of partially unfolded proteins (%)
0	0.80	44.9	44.9	0	100
1	0.38	68.1	52.4	15.7	84.3
2	0.18	83.2	44.6	38.6	61.4
2.5	0.13	88.0	40.0	48.0	52.0
3	0.09	91.6	16.2	75.4	24.6
3.5	0.06	93.8	7.7	86.1	13.9
4	0.03	97.2	2.5	94.7	5.3

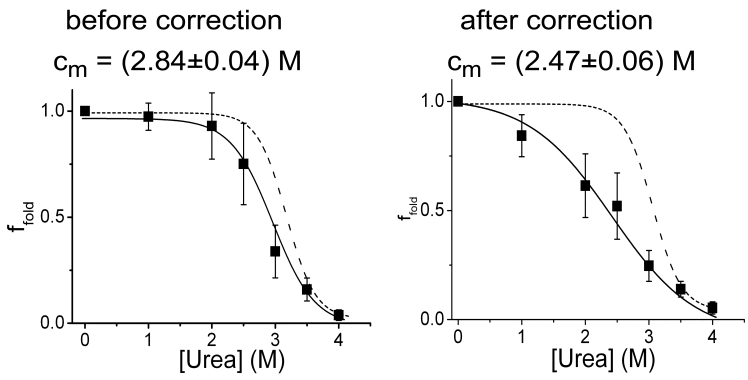
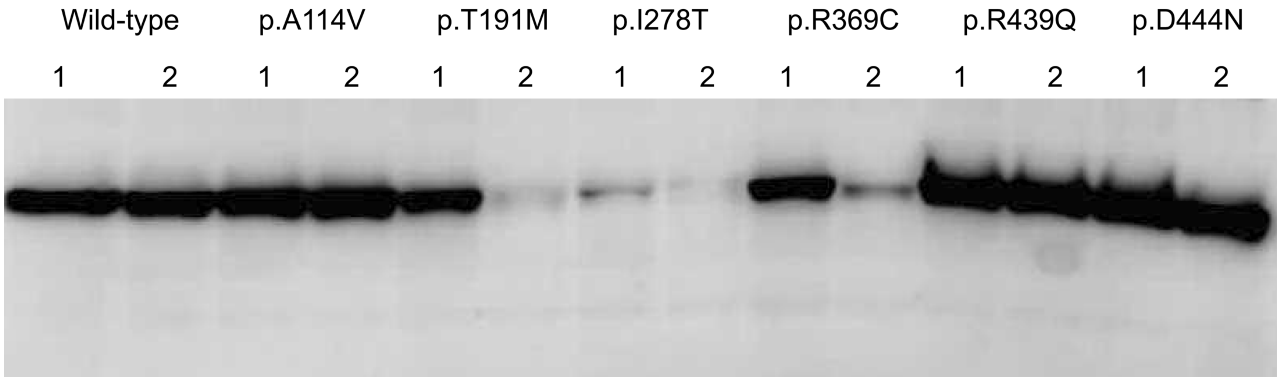


Figure S8: Differences in CBS signal after overnight incubation in the absence of urea at 4 °C. Bacterial lysates containing CBS proteins (20 µg total protein per lane) are shown with identical numbering: 1 – freshly thawed sample; 2 – freshly thawed sample after overnight incubation at 4 °C. Extensively unfolded mutants (p. T191M, p.I278T and p.R369C) showed a significant decrease in the amount of CBS antigen after the overnight incubation, indicating the formation of aggregates.

Table. The residual amount of the CBS antigen was determined using densitometry. The values are related to the amount of freshly thawed samples containing the corresponding CBS protein.



Protein	Residual amount of the CBS antigen after overnight incubation [%]
Wild-type	94
p.A114V	120
p.T191M	10
p.I278T	66
p.R369C	20
p.R439Q	118
p.D444N	113