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# C-terminal truncation of a bovine $B_{12}$ trafficking chaperone enhances the sensitivity of the glutathione-regulated thermostability

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**The human $B_{12}$ trafficking chaperone hCblC is well conserved in mammals and non-mammalian eukaryotes. However, the C-terminal ~40 amino acids of hCblC vary significantly and are predicted to be deleted by alternative splicing of the encoding gene. In this study, we examined the thermostability of the bovine CblC truncated at the C-terminal variable region (t-bCblC) and its regulation by glutathione. t-bCblC is highly thermolabile ($T_m$ = ~42°C) similar to the full-length protein (f-bCblC). However, t-bCblC is stabilized to a greater extent than f-bCblC by binding of reduced glutathione (GSH) with increased sensitivity to GSH. In addition, binding of oxidized glutathione (GSSG) destabilizes t-bCblC to a greater extent and with increased sensitivity as compared to f-bCblC. These results indicate that t-bCblC is a more sensitive form to be regulated by glutathione than the full-length form of the protein. [BMB Reports 2013; 46(3): 169-174]**

## INTRODUCTION

$B_{12}$ (vitamin $B_{12}$ derivatives, cobalamins) is being used as enzyme cofactors in the active forms of methylcobalamin (MeCbl) and adenosylcobalamin (coenzyme $B_{12}$, AdoCbl). Human cells contain two $B_{12}$-dependent enzymes, methionine synthase in cytosol and methylmalonyl-CoA mutase in mitochondria (1, 2). Methionine synthase is dependent on MeCbl to catalyze the methylation of homocysteine to form methionine, and methylmalonyl-CoA mutase is dependent on AdoCbl for the isomerization of L-methylmalonyl-CoA to succinyl-CoA. The enzyme cofactors, MeCbl and AdoCbl, are synthesized from exogenously derived $B_{12}$ derivatives by intracellular $B_{12}$ metabolism. Inborn errors of the $B_{12}$ metabolism lead to complex human diseases such as developmental delay, neurological disease and cognitive dysfunction (3), although the disease-causing mechanism of the defective $B_{12}$ metabolism is not fully understood.

The human protein hCblC (hMMACHC, methylmalonic aciduria and homocystinuria cblC type) is most often found to be defective in patients with inborn errors of intracellular $B_{12}$ metabolism (4, 5). The protein, as a $B_{12}$ trafficking chaperone, transports highly reactive $B_{12}$ in an early step of intracellular $B_{12}$ metabolism (6). In addition, the hCblC protein exhibits the catalytic activities of decyanation and dealkylation: reductive elimination of the cyanide ligand from CNCbl (cyanocobalamin, vitamin $B_{12}$) and glutathione-dependent elimination of the alkyl ligand from alkylcobalamins, respectively (6-8). These reactions generate cob(II)alamin or cob(I)alamin, that can be common intermediates for the subsequent synthesis of enzyme cofactors. The hCblC protein is extremely thermolabile *in vitro* with a $T_m$ = ~39°C that is close to the human body temperature of 37°C (9). We previously characterized bovine CblC protein (bCblC) that is also highly thermolabile with a $T_m$ = ~42°C (10) [bovine body temperature = 39-40°C (11)]. In addition, we demonstrated that the most abundant cellular non-protein thiol (1-10 mM) (12), glutathione, regulates the thermostability of bCblC (10, 13). Binding of the reduced form of glutathione GSH stabilizes bCblC by increasing the $T_m$, whereas binding of the oxidized form of glutathione GSSG destabilizes the protein by increasing the rate of unfolding at physiological temperature.

CblC proteins from mammals are highly conserved, and non-mammalian eukaryotes also contain homologous proteins. However, the C-terminal ~40 amino acids of the proteins are less conserved between mammalian proteins and are absent in the proteins from non-mammalian eukaryotes (Fig. 1A). In addition, the gene for the human protein hCblC is predicted to be alternatively spliced (14) to generate either the full-length protein with 282 amino acid residues or the C-terminal truncated protein with 245 amino acid residues. In the present study, we examined the thermostability of the bovine protein bCblC truncated at the C-terminus (t-bCblC, ΔC36) and its regulation by glutathione. The thermostability of t-bCblC is similar to that of the full-length bCblC (f-bCblC). However, binding of GSH stabilizes t-bCblC to a greater extent than f-bCblC, and with increased sensitivity. In addition, the binding of GSSG to t-bCblC demonstrates decreased stability of

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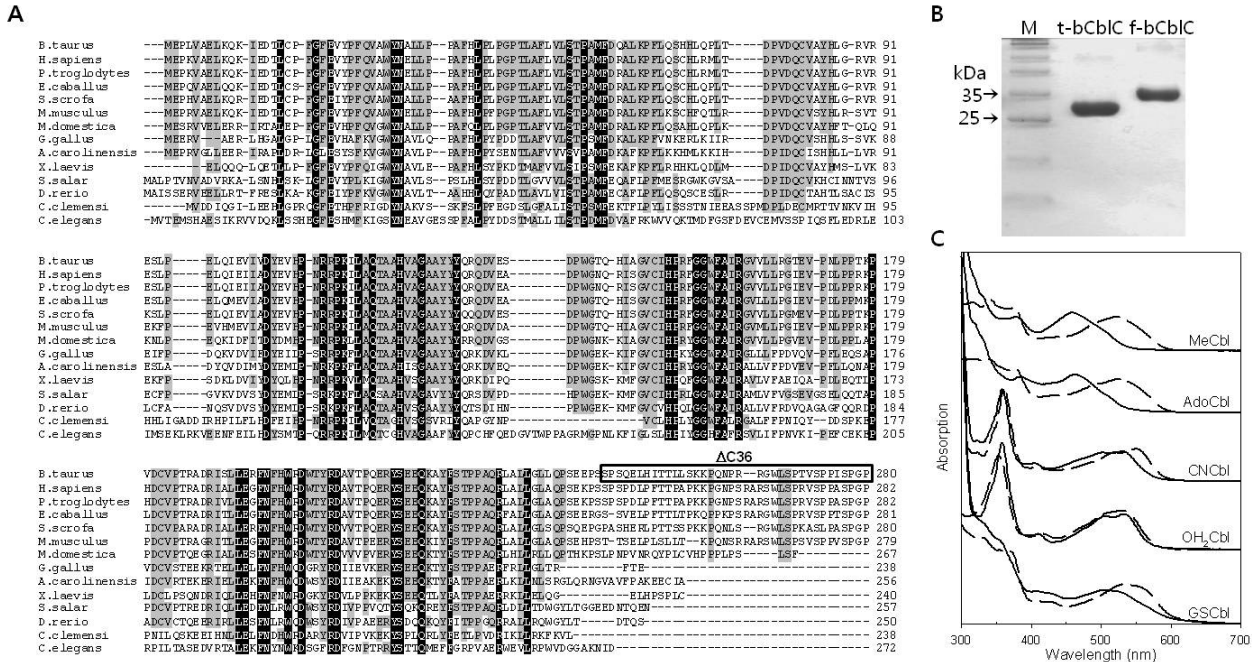


**Fig. 1.** Amino acid sequence alignment for CblC proteins (A) and preparation of t-bCblC. Identical amino acid residues in all CblC proteins, and only in mammalian proteins, are indicated on the black and gray backgrounds (A), respectively. The 36 C-terminal amino acid residues deleted in the t-bCblC are indicated in the box (ΔC36). Purified t-bCblC was analyzed by 12% SDS-PAGE (B) and was compared with the full-length bCblC (f-bCblC). The absorption spectra (C) for the indicated free cobalamins (dashed lines) were obtained in 50 mM Hepes pH 8.0, 150 mM KCl and 5% glycerol, and the spectra for t-bCblC-bound cobalamins (solid lines) were obtained by the addition of 10-fold molar excess protein to the free cobalamins.

the protein and increased sensitivity compared to f-bCblC. These results indicate that t-bCblC is more efficiently regulated by glutathione than f-bCblC and may also be applicable to the human protein hCblC.

## RESULTS

### Preparation and characterization of t-bCblC

The bovine protein bCblC is 280 amino acids in length and exhibits 88% amino acid sequence identity with the human $B_{12}$ trafficking chaperone hCblC (Fig. 1A). The C-terminal truncated bCblC (t-bCblC) was prepared, as described in the materials and methods, by deletion of the last 36 amino acids (Fig. 1A, ΔC36), which corresponds to the truncated human protein hCblC (1-245 aa), predicted as the product of alternative splicing of the encoding gene (14). The prepared t-bCblC showed a ~28 kDa single band by SDS-PAGE (Fig. 1B) that is in agreement with the calculated molecular mass (28.8 kDa) of the protein. The t-bCblC behaved as a monomer in size exclusion column chromatography (data not shown), as has been previously demonstrated for the full-length protein f-bCblC (15).

The binding of $B_{12}$ derivatives to t-bCblC was characterized by UV-Vis spectroscopy under dark conditions (Fig. 1C). As previously shown with f-bCblC (15, 16), the addition of t-bCblC to AdoCbl, MeCbl or CNCbl elicited changes in the absorption spectrum with a peak shift that is characteristic of the base-off transition of the Cbls: the α/β-peak of AdoCbl at 520 nm → 460 nm, the α/β-peak of MeCbl at 525 nm → 459 nm, and the α-peak of CNCbl at 550 nm → 531 nm. In addition, the binding of $OH_2$Cbl or glutathionylcobalamin (GSCbl) to t-bCblC (Fig. 1C) elicited changes in the absorption spectrum that are identical with those observed for f-bCblC (15). The dissociation constants for the complex of t-bCblC and cobalamins were determined to be $K_d$ = 8.1 ± 2.1 μM, 4.7 ± 1.0 μM, 11.9 ± 2.6 μM and 12.8 ± 2.3 μM, for AdoCbl, MeCbl, CNCbl and GSCbl, respectively, which were similar to the values previously reported for f-bCblC (15).

### Stabilization of t-bCblC by GSH binding

The thermostability of t-bCblC was examined by differential scanning fluorimetry (DSF) using the reporter dye Sypro orange that binds hydrophobic regions of unfolded protein and induces an increase in fluorescence intensity (17). Unfolding of t-bCblC exhibited a curve in a sigmoidal shape (Fig. 2A), as consistently observed for f-bCblC (10), indicating a cooperative two-state transition of the protein from the native to the unfolded state. The melting temperature of t-bCblC was determined to be $T_m$ = 41.5 ± 0.3°C and was similar to the $T_m$ = 42.1 ± 0.4°C of f-bCblC. In the presence of 10 mM GSH, the unfolding curve of t-bCblC shifted to a higher temperature with a $T_m$ = 45.2 ± 0.3°C ($\Delta T_m \approx 4.0$°C) (Fig. 2A), indicating the stabilization of the protein by GSH. While the

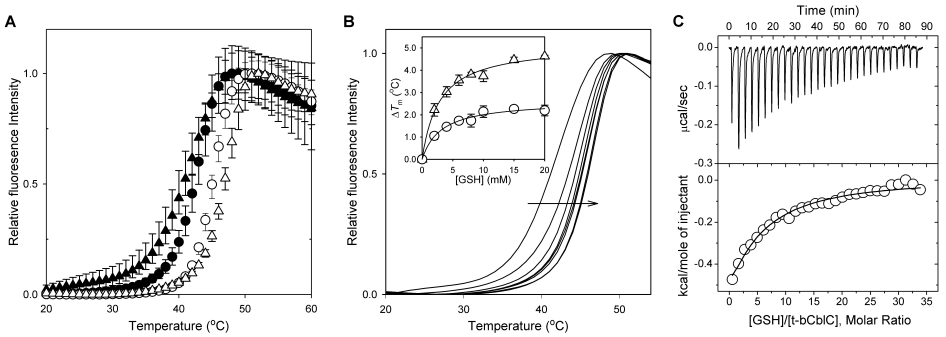


**Fig. 2.** Stabilization of t-bCblC by GSH. The unfolding curves (A) were obtained by DSF for t-bCblC (triangles) or f-bCblC (rounds) in the absence (closed symbols) and presence of 10 mM GSH (open symbols) (n = 6). Unfolding of t-bCblC at increasing GSH concentrations (B) showed shifts (arrow) of the unfolding curves towards higher temperatures. The plot of changes in $T_m$ ($\Delta T_m$ = $T_m$ at the indicated GSH concentrations - $T_m$ at no GSH, n = 6) versus the indicated GSH concentrations fits a hyperbolic saturation equation (solid lines). Representative isothermal titration (C) for binding of GSH to t-bCblC was conducted as described in the materials and methods.

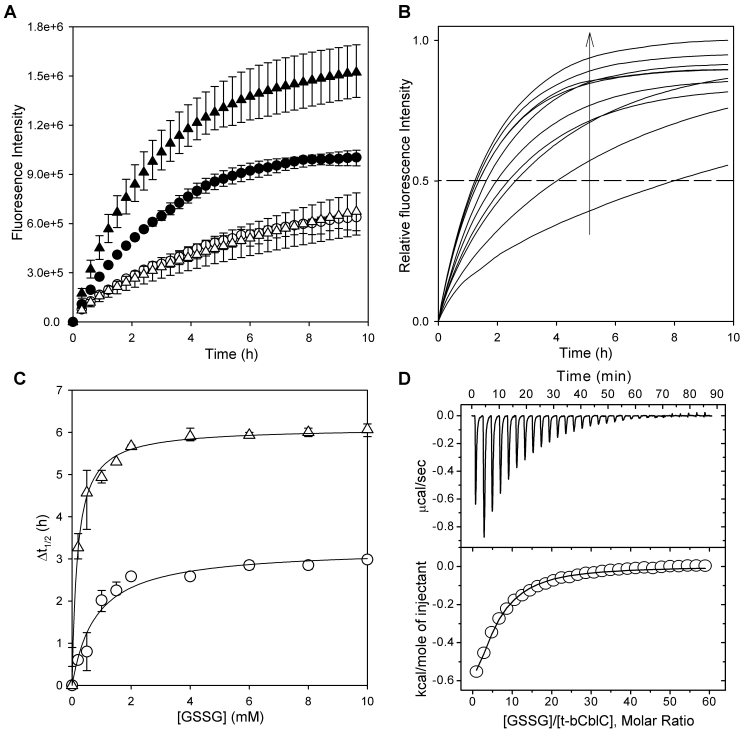


**Fig. 3.** Destabilization of t-bCblC by GSSG. Unfolding curves (A) were obtained by ITD of the t-bCblC (triangles) or f-bCblC (rounds) in the absence (open symbols) and presence of 10 mM GSSG (closed symbols) (n = 6). Unfolding curves for t-bCblC (B) showed increases (arrow) in the relative half-life of the protein ($t_{1/2}$, dashed line) as GSSG concentrations increased. The plot (C) of changes in $t_{1/2}$ ($\Delta t_{1/2}$ = $t_{1/2}$ at no GSSG - $t_{1/2}$ at the indicated GSSG concentrations, n = 6) versus the indicated GSSG concentrations fit a hyperbolic saturation equation (solid lines). Representative isothermal titration (D) for binding of GSSG to t-bCblC was conducted as described in the materials and methods.

unfolding of f-bCblC under the same conditions resulted in a lower $T_m$ = 44.2 ± 0.3°C ($\Delta T_m \approx$ 2.0°C) that is consistent with the previously reported value (10). The effect of GSH for the stabilization of t-bCblC was further examined at various GSH concentrations. The unfolding curves of t-bCblC were shifted toward higher temperatures with increases in $T_m$ by increasing GSH concentration (Fig. 2B). The plot (Fig. 2B inset) shows a hyperbolic relationship between changes in $T_m$ ($\Delta T_m$ = $T_m$ at the indicated GSH concentrations - $T_m$ at no GSH) and GSH concentration. The maximal $\Delta T_m$ was estimated to be $\Delta T_{m\ max}$ = 5.2 ± 0.2°C for t-bCblC which is

higher than the $\Delta T_{m\,max}$ = 2.8 ± 0.3°C estimated for f-bCblC. In addition, the concentration of GSH at the half-maximal stabilization level of t-bCblC was estimated to be $AC_{50}$ = 2.8 ± 0.3 mM, which is slightly lower than the $AC_{50}$ = 3.1 ± 0.3 mM obtained for f-bCblC. Isothermal titration calorimetry (ITC) consistently revealed that t-bCblC binds GSH tighter ($K_d$ = 206 ± 35 μM) (Fig. 2C) than the f-bCblC ($K_d$ = 355 ± 40 μM) (16). These results indicate that binding of GSH stabilizes t-bCblC to a greater extent than f-bCblC and occurs with increased sensitivity.

### Destabilization of t-bCblC by GSSG binding

The effect of GSSG on the thermostability of t-bCblC was examined by isothermal denaturation (ITD). Only partial unfolding of t-bCblC was detected by incubation of the protein for 10 h at 37°C, which was similar to the unfolding observed with f-bCblC (Fig. 3A). However, in the presence of 10 mM GSSG, unfolding of t-bCblC and f-bCblC was more significant, and the unfolding rates were determined to be $k_{ob}$ = 0.37 ± 0.02 $h^{-1}$ and a $k_{ob}$ = 0.25 ± 0.01 $h^{-1}$, respectively. These results indicate that GSSG destabilizes the proteins, and considering the unfolding rates, the destabilizing effect of GSSG is greater for t-bCblC than f-bCblC. To confirm this observation, the ITD of t-bCblC was further conducted at various GSSG concentrations, and resulted in an increase in the unfolding rate as GSSG concentration increased (Fig. 3B). The destabilizing effect of GSSG for t-bCblC and f-bCblC was compared by determining the relative half-life ($t_{1/2}$) of the proteins (see discussion). The plot (Fig. 3C) shows a hyperbolic relationship between changes in $t_{1/2}$ ($\Delta t_{1/2}$ = $t_{1/2}$ at no GSSG - $t_{1/2}$ at the indicated GSSG). The maximal change in the half-life of t-bCblC was estimated to be $\Delta t_{1/2\,max}$ = 6.1 ± 0.1 h, which is higher than the $\Delta t_{1/2\,max}$ = 3.3 ± 0.2 h of f-bCblC. In addition, the concentration of GSSG at half-maximal destabilization of t-bCblC was estimated to be $AC_{50}$ = 0.18 ± 0.1 mM which was lower than the $AC_{50}$ = 0.81 ± 0.17 mM for f-bCblC. ITC also revealed that t-bCblC binds GSSG tighter ($K_d$ = 184 ± 24 μM) (Fig. 3C) than f-bCblC ($K_d$ = 303 ± 74 μM) (13). These results indicate that binding of GSSG destabilizes t-bCblC to a greater extent than f-bCblC and with increased sensitivity.

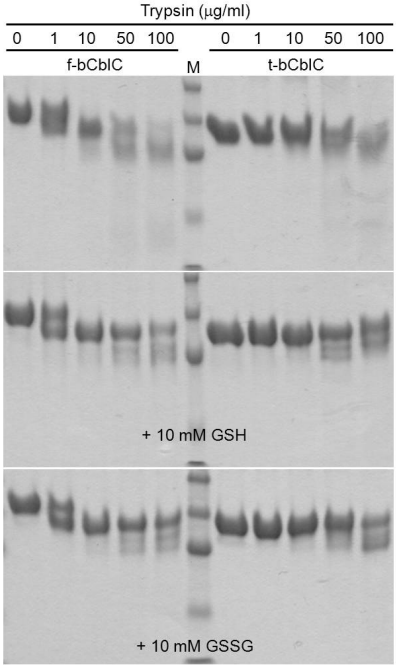


**Fig. 4.** Conformational changes of t-bCblC and f-bCblC by binding of glutathione. Limited proteolysis was conducted, as described in the materials and methods, by addition of the indicated concentrations of trypsin to the proteins in the absence and presence of 10 mM GSH or GSSG. Proteolyzed proteins were analyzed by 12% SDS-PAGE (M, size marker).

### Conformational changes of t-bCblC by glutathione binding

Changes in the thermostability of f-bCblC induced by binding of GSH or GSSG were shown to be derived from conformational changes of the protein (10, 13, 16). Limited proteolysis of f-bCblC in the absence or presence of 10 mM GSH or GSSG showed differences in the cleavage patterns and resistance against trypsin (Fig. 4), consistently indicating conformational changes of the protein induced upon binding of the ligand. Similar proteolysis of t-bCblC in the absence of glutathione also exhibited a different cleavage pattern, with increased resistance to trypsin, as compared to those observed in the presence of GSH or GSSG. These results indicate that changes in the thermostability of t-bCblC are likely to be derived from conformational changes of the protein upon binding of GSH and GSSG.

## DISCUSSION

The gene for hCblC was identified through a study of patients with defective synthesis of both AdoCbl and MeCbl, which are the enzyme cofactors of methylmalonyl-CoA mutase and methionine synthase, respectively (5). The alternative splicing of hCblC gene has been predicted to produce the hCblC protein with 282 amino acid residues, or the C-terminal truncated version of the protein with 245 amino acid residues (14). Interestingly, only three benign mutations are found beyond the residue 245, although >400 hCblC defective patients have been characterized (4). This aspect prompted us to examine the difference between a CblC protein in the full-length and the C-terminal truncated form.

In this study, we used the bovine $B_{12}$ trafficking chaperone bCblC that exhibits 88% amino acid sequence identity with hCblC. The C-terminal truncation of bCblC (ΔC36) did not cause a significant change in binding of cobalamins in terms of the conformational transition of the cobalamins and the binding affinities (Fig. 1C). These results indicated that the C-terminal end region of the protein is not directly involved in cobalamin binding, which is consistently revealed in the crystal structure of hCblC (18, 19). The thermostability of the C-terminal truncated protein t-bCblC is similar to the full-length protein f-bCblC, however, the stabilizing effect of GSH for each protein is different (Fig. 2A, B). The increased

sensitivity of GSH for the stabilization of t-bCblC is due to the tighter binding of GSH to t-bCblC than to f-bCblC (Fig. 2C). Stabilization of t-bCblC by GSH binding is likely derived from conformational changes of the protein (Fig. 4). Binding of GSH also induced changes in the conformation of f-bCblC to a more stable form, which is consistent with previously reported results (10, 16). However, the conformation of GSH-bound t-bCblC appeared to be different from that observed for GSH-bound f-bCblC, as compared the different cleavage patterns of the ligand-bound proteins observed by limited proteolysis (Fig. 4). This difference may explain the higher thermostability of GSH-bound t-bCblC than GSH-bound f-bCblC.

The destabilizing effect of GSSG could not be examined using the DSF method due to the detection limit ($\Delta T_m \approx \pm 0.5^oC$) (20, 21). ITD is a more sensitive method to examine ligand-induced changes in the stability of proteins, by measuring the kinetics of protein unfolding at a constant temperature (usually a few degrees below $T_m$). GSSG destabilized t-bCblC and the destabilizing effect of GSSG appeared to be greater for t-bCblC than for f-bCblC, as measured by the comparison of the protein unfolding rates (Fig. 3A). Although ITD reactions contained the same amount of protein, the fluorescence intensity detected after complete unfolding of t-bCblC was higher than that measured for f-bCblC (Fig. 3A). This may be due to the higher molar concentration of t-bCblC (28.8 kDa), as compared to f-bCblC (32.6 kDa), in unfolding reactions. Therefore, the destabilizing effects of GSSG were compared by using the relative half-life of the proteins (Fig. 3C) instead of the protein unfolding rates. Clearly, the effect of GSSG is increased, and is more sensitive to t-bCblC than f-bCblC. The increased sensitivity was consistent with the higher binding affinity of t-bCblC for GSSG. Destabilization of t-bCblC and f-bCblC by GSSG binding is also likely derived from conformational changes of the protein, as shown by limited proteolysis (Fig. 4). The cleavage pattern of GSSG-bound t-bCblC was different from that observed for GSSG-bound f-bCblC, which indicates difference in the conformations of the ligand-bound proteins, and may explain the decreased thermostability of GSSG-bound t-bCblC.

In summary, we demonstrated that t-bCblC is a more sensitive form than f-bCblC in terms of glutathione-regulated thermostability. Shifts up or down in the thermostability of t-bCblC are likely to be derived from conformational changes of the protein induced upon binding of GSH and GSSG, respectively. Glutathione is the most abundant cellular non-protein thiol (1-10 mM) and, in normal cells, exists predominantly in the reduced form of GSH (12). Under oxidative stress, the level of the oxidized form, GSSG, is elevated, and the molar ratio of GSH/GSSG in cells decreases from ~100 to ~1 (22). Although, to what extent the *in vitro* thermostability parameters obtained in this study applied *in vivo*, the molar ratio of GSH/GSSG would regulate the cellular stability of t-bCblC more sensitively than that of f-bCblC. Furthermore, our results indicate that t-bCblC is a more sensitive form to be regulated by glutathione than the full-length form of the protein.

## MATERIALS AND METHODS

### Preparation of t-bCblC

The gene t-b*cblc* for the C-terminal truncated form of bCblC (t-bCblC, 1-244 aa, ΔC36) was amplified by PCR using the template gene that was previously obtained for full-length bCblC (f-bCblC, 1-280 aa) (15) and the following primers: 5′-ATGGTACCATGGAGCCGCTAGTCGCAGAGCTGAAGC-3′ (forward) and 5′-ATGGTACTCGAGGCTAGGCTCCTCTGAGGGTGAAG-3′ (reverse). The amplified t-b*cblc* was cloned into the pET28a(+) vector (Novagen) between the NcoI and XhoI restriction sites and the authenticity of the gene was confirmed by DNA sequencing. The recombinant t-bCblC (6x His-tagged at the C-terminus) was prepared by over-expression of t-bCblC in *E. coli* as previously described (15). Protein concentrations were determined using a Bradford assay (23).

### Differential scanning fluorimetry (DSF)

DSF was conducted as previously described (10, 13) with 0.2 mg/ml t- or f-bCblC in 50 mM Hepes pH 8.0, 150 mM KCl and 5% glycerol containing the 10× fluorescent reporter dye Sypro orange (Invitrogen). The protein was incubated in the presence of the indicated GSH concentrations with increasing temperatures (5-75°C and 1°C/min) using an ABI 7500 Real-Time PCR system (Applied Biosystems). The protein unfolding was monitored by following the increase in the fluorescence of the Sypro orange. Unfolding curves were normalized by setting the minimum and maximum fluorescence to 0 and 1.0, respectively. The melting temperatures of proteins ($T_m$) were determined by fitting the unfolding curves to the Boltzmann equation. The concentration of GSH to achieve half-maximal protein stabilization ($AC_{50}$) was determined by fitting the plot of changes in $T_m$ ($\Delta T_m$) vs. GSH concentrations ([GSH]) to the equation of $\Delta T_m = \Delta T_{m\ max}[GSH]/(AC_{50} + [GSH])$, where $\Delta T_{m\ max}$ is the maximal change in $T_m$ at saturation.

### Isothermal denaturation (ITD)

ITD was conducted with the protein prepared as above by incubation in the presence of the indicated GSSG concentrations at a constant temperature of 37°C. Protein unfolding rates ($k_{ob}$) were determined by fitting the resulting unfolding curves to a single exponential equation. The relative half-life of the protein at 37°C ($t_{1/2}$) was estimated from the normalized unfolding curves by setting the minimum and maximum fluorescence obtained at the highest GSSG concentration to 0 and 1.0, respectively. The concentration of GSSG to achieve the half-maximal protein destabilization ($AC_{50}$) was determined by fitting the plot of changes in $t_{1/2}$ ($\Delta t_{1/2}$) vs. GSSG concentrations ([GSSG]) to the equation of $\Delta t_{1/2} = \Delta t_{1/2\ max}[GSSG]/(AC_{50} + [GSSG])$, where $\Delta t_{1/2\ max}$ is the maximal change in $\Delta t_{1/2}$ at saturation.

### Isothermal titration calorimetry (ITC)

ITC was carried out at 4°C as previously described in (16, 24) using a VP-ITC microcalorimeter (1.44-ml cell volume) (Microcal Inc.). The ligand GSH or GSSG (2.0-4.5 mM) dissolved in 50 mM Hepes

pH 8.0, 150 mM KCl and 5% glycerol was injected into 20-40 μM bCblC in the same buffer. The $K_d$ for GSH and GSSG were determined by analyzing three independent sets of binding isotherm data using a single-site binding model.

### Limited trypsin proteolysis

Proteolysis was conducted with 5 μg (16 μM) t- or f-bCblC in 50 mM Hepes pH 8.0, 150 mM KCl and 5% glycerol in the absence or presence of 10 mM GSH or 10 mM GSSG. The reaction was initiated by the addition of the indicated concentrations of trypsin. After incubation for 30 min at 15°C, proteolysis was terminated by mixing the sample with SDS-PAGE sample buffer and immediate boiling at 95°C for 5 min. Proteolyzed proteins were subsequently analyzed by 12% SDS-PAGE stained with coomassie brilliant blue.

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