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Evidence for Involvement of the C-terminal Domain in the Dimerization of the CopY Repressor Protein from *Enterococcus hirae*

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Abstract

Metal binding to the C-terminal region of the copper-responsive repressor protein CopY is responsible for homodimerization and the regulation of the copper homeostasis pathway in *Enterococcus hirae*. Specific involvement of the 38 C-terminal residues of CopY in dimerization is indicated by zonal and frontal (large zone) size exclusion chromatography studies. The studies demonstrate that the attachment of these CopY residues to the immunoglobulin binding domain of Streptococcal protein G (GB1) promotes dimerization of the monomeric protein. Although sensitivity of dimerization to removal of metal from the fusion protein is smaller than that found for CopY (as measured by ultracentrifugation studies), the demonstration that an unrelated protein (GB1) can be induced to dimerize by extending its sequence with the C-terminal portion of CopY confirms the involvement of this region in CopY homodimerization.

Keywords

copper-responsive repressor protein; frontal size exclusion chromatography; self-association stoichiometry

1. INTRODUCTION

CopY is a copper-responsive homodimer repressor that regulates the expression of the “*cop* operon” [1]. Each monomer of the homodimer binds metal ions in a characteristic Cys–x–Cys–x–x–x–Cys–x–Cys metal-binding site [2–5]. One Zn(II) ion is bound in a tetrahedral

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geometry by four cysteine residues [6] to form the metalloprotein that is bound to the DNA. Under conditions of elevated copper concentrations, the displacement of the Zn(II) from the CopY metal-binding site by two Cu(I) ions leads to a decreased affinity of CopY for the DNA, which results in the expression of the copper homeostasis proteins of the “*cop* operon” [3,6,7].

The carboxy-terminal region of CopY contains a metal-binding motif as well as several leucine and isoleucine residues which repeat at approximately four residue intervals. Excluding the aliphatic repeats, the sequence does not have the features of a leucine-zipper motif [8]. Previous studies established the importance of metal-binding to the oligomeric state of the protein [4], but the direct involvement of the metal-binding region in the dimerization of CopY is still unclear. To better understand the dimerization process, the DNA sequence encoding for the C-terminal 38 residues of CopY (Ymbs38) was joined to the end of the gene coding for the protein GB1. This immunoglobulin-binding domain of the streptococcal protein G is a stable and soluble 56-residue monomeric protein for which the three-dimensional structure is known [9–13]. The results from zonal and frontal size exclusion chromatography studies demonstrate the ability of the fused CopY fragment to promote dimerization of the monomeric GB1 protein. Comparison of these results to ultracentrifugation studies of the full CopY protein indicates that metal binding affects the dimerization contact area in CopY, and that the contact area in the full protein may involve more than the C-terminal region immediately surrounding the metal binding site.

2. Materials and Methods

2.1 GB1–Ymbs38 fusion protein

A synthetic gene coding for the C-terminal 38 residues of CopY attached to the C-terminus of GB1 was purchased from GenScript Corporation. Codon usage was optimized for *Escherichia coli* expression. The GB1-Ymbs38 fusion gene was ligated into the pET-14b vector (Novagen) at the NdeI and HindIII sites. The resultant clone encoded the GB1-Ymbs38 fusion protein with a hexa-histidine tag attached to the N-terminus to facilitate purification.

The GB1-Ymbs38 fusion protein was overexpressed in HMS174(DE3) (Novagen) cells induced with 1.5 mM isopropyl- β -D-thiogalactopyranoside at a cell OD₆₀₀ of 0.9. The cells were harvested by centrifugation at $5000 \times g$ for 10 min. After resuspension of the cell pellet in 50 mM Tris, pH 8.0, 50 mM NaCl, 10% sucrose, 0.01% β -mercaptoethanol (2 mL/g of cells), lysis was promoted by incubating the suspension on ice for 30 min with 0.4 mg lysozyme per mL of resuspended cells. The suspension was sonicated to support cell lysis, then centrifuged at $34,800 \times g$ for 30 min. The clarified supernatant was diluted 1:1 with 50 mM Tris, pH 7.8, 300 mM NaCl before loading onto a Sigma® HIS-Select™ nickel affinity column equilibrated with the same buffer. After removal of unbound protein from the column by buffer washes, 250 mM imidazole was included in the buffer to elute the histidine-tagged fusion protein. Protein purity was assessed by SDS-PAGE on a 15% Tris-tricine gel [14]. Metal content was measured with a Perkin-Elmer 1100 Flame Atomic Absorption Spectrophotometer. The dithiodipyridine thiolate (DTDP) assay [15] was employed to quantify the cysteine content of the fusion protein. A molar extinction coefficient (ϵ_{280}) of $8250 \text{ M}^{-1} \text{ cm}^{-1}$ for GB1 was used to estimate the protein concentration, while metal and thiolate analyses were used to confirm the concentrations [13]. The 38-residue CopY-derived sequence lacks aromatic residues, and therefore does not contribute significantly to the extinction coefficient of the fusion protein at 280nm. GB1 is a monomeric protein that lacks cysteine residues and a metal-binding site. Measurement of metal content in the GB1-Ymbs38 fusion protein after modification of the cysteine residues with iodoacetamide showed that no metal was associated with the protein. Iodoacetamide

modifies the terminal sulfur of the cysteine residues with a carboxyamidomethyl group, blocking any potential interaction between the cysteines and metal ions, thus ensuring that the protein remains in a metal free “apo” form. With unmodified reduced cysteines, the protein can scavenge metals from buffers and is subject to oxidation and aggregation.

2.2 Site directed mutagenesis of the Ymbs38 sequence

The Stratagene QuikChange® Site-Directed Mutagenesis Kit was used to substitute serine for all leucine and isoleucine residues within Ymbs38. Mutagenesis oligonucleotide primers were designed with the aid of the Clone Manager Professional Suite software (Scientific & Educational Software). Positive mutants were verified by automated dideoxy DNA sequencing.

The mutant proteins were expressed and purified by the procedure outlined above. To prepare “apo” proteins, the four cysteine residues were again carboxyamidomethylated with iodoacetamide. The loss of free thiolates was monitored by DTDP analyses.

2.3 Size Exclusion Chromatography

Zonal chromatography on a 2.6×61 cm HiLoad 26/60 XK Superdex column (Pharmacia) equilibrated with 50 mM Tris, pH 7.8, 150 mM NaCl, 0.1% β -mercaptoethanol (4 °C) was used for preliminary molecular size characterization of the GB1–Ymbs38 fusion protein. Protein elution was monitored spectrophotometrically at 220 nm at a flow rate of 2 mL/min.

2.4 Frontal (large zone) size exclusion chromatography

A Shodex KW 803 HPLC column (0.8×30 cm; 15-mL volume) was used for quantitative studies of GB1–Ymbs38 self-association at 4°C by frontal chromatography [16–19]. After pre-equilibration of the column with 50 mM Tris, pH 7.8, 150 mM NaCl, 0.1% β -mercaptoethanol, solutions of purified protein in the same buffer (5 mL, 4–270 μ M) were applied at a flow rate of 1 mL/min before the resumption of elution with buffer. Continuous monitoring of the column effluent absorbance at 280 nm was used to record elution profiles. Studies of the self-association behavior of the holo- and apo- forms of GB1–Ymbs38 were followed by corresponding studies of the holo- and apo- forms of the mutants with serine substituted for all leucine and isoleucine residues within the Ymbs38 metal-binding motif.

2.5 Ultracentrifugal studies of CopY

The procedure described by Portmann and coworkers [7] was used to isolate Zn(II)-CopY. Although a homogeneous protein preparation is ensured by the inclusion of a size-exclusion chromatography step, precautions need to be taken to minimize aggregation stemming from random crosslinking of the four cysteine residues within the monomer or between monomers. Prior to centrifugation, protein solutions were dialyzed against 50 mM Tris, pH 7.8, 150 mM NaCl, 0.1% β -mercaptoethanol. This dialysis step was carried out in a 5% hydrogen, 95% nitrogen-purged glovebox. The assembly and filling of the ultracentrifuge cells were also conducted in the same anaerobic environment.

Zn(II)-CopY and its apo- counterpart were examined by sedimentation equilibrium at 20°C in a Beckman XL-I ultracentrifuge operated at 11,000 and 20,000 rev./min for the holo- and apo-proteins respectively. After centrifugation for 24 hours the resulting sedimentation equilibrium distributions were recorded spectrophotometrically at 280 nm.

3. RESULTS

Analysis of the purified protein by flame atomic absorption spectroscopy revealed its isolation as a Zn(II)-metalloprotein. Comparison of the Zn(II) content with the thiol

concentration deduced by colorimetric assay [15] signified a ratio of 3.8 thiols per Zn(II). On the grounds that a value of unity for the ratio of zinc ion to protein applies to the GB1–Ymbs38 preparation, it is reasonable to presume that Zn(II)-binding is confined to the Ymbs38 fragment of the fusion protein.

3.1 Size-Exclusion Chromatography

The histidine-tagged GB1–Ymbs38 fusion protein (80 μ M, 2 mL) was subjected to zonal size-exclusion chromatography on a 2.6×61 cm HiLoad 26/60 XK Superdex 75 column. A resulting apparent molecular mass of 22.5 kDa was taken to signify the self-association of the fusion protein (calculated monomeric molecular mass of 12.7 kDa). A comparable sample of histidine-tagged GB1 eluted at an apparent molecular mass of 10 kDa (calculated value of 8.4 kDa).

3.2 Frontal Size-Exclusion Chromatography

The advancing elution profile obtained by frontal chromatography [16–22] of a 129 μ M solution of histidine-tagged GB1–Ymbs38 fusion protein on a Shodex KW 803 HPLC column is shown in Figure 1A. Also shown is the median bisector [23] of the boundary, this being an elution volume (V_w) that refers to the weight-average composition of the solution (with concentration C_t) applied to the column. Because of species re-equilibration in accordance with the law of mass action, the measured elution volume (V_w) decreases with increasing C_t (Figure 1B) as the result of an increase in the weight-fraction of polymer at the expense of monomer.

The dependence of V_w upon C_t allows quantification of the association equilibrium between monomer (M) and a single polymeric state (P), provided the elution volumes of monomer (V_M) and polymer (V_P) can be assigned. From Figure 1B the former (V_M) was taken as 12.24 mL by extrapolating the results (Δ) for the weakly self-associating Leu/Ile-to-Ser variant of apoGB1–Ymbs38 to zero protein concentration. The elution volume for the polymeric species (V_P) was determined to be 11.10 mL, as indicated in Figure 2 (see Discussion).

Quantitative assessment of the strengths of dimerization for the four variants of the GB1–Ymbs38 fusion protein is summarized in Table 1. Association constants (K) were determined from least-squares analysis of the (C_p, f_M) data sets according to eqn 4 (see Discussion) with $n = 2$ to obtain K as the sole curve-fitting parameter. Also shown is the standard free energy change, $\Delta G^\circ = -RT \ln K$ where R is the universal gas constant and T the absolute temperature (277 K). Mutation of the leucine and isoleucine residues within the metal-binding motif had little effect on dimerization of the holoprotein. The removal of the Zn(II) weakened the strength of dimerization, particularly in the mutant lacking the aliphatic residues. The Zn(II) removal was a dominant effect.

3.4 Ultracentrifugal analysis of CopY

Sedimentation equilibrium distributions were analyzed in accordance with the expression

$$A_{280}(r) = A_{280}(r_F) \left\{ f_M \exp \left[M_M \left(1 - \bar{v} \rho_s \right) \omega^2 (r^2 - r_F^2) / (2RT) \right] + (1 - f_M) \exp \left[2M_M \left(1 - \bar{v} \rho_s \right) \omega^2 (r^2 - r_F^2) / (2RT) \right] \right\}$$

(1)

in which $A_{280}(r)$ is the absorbance at radial distance r in an experiment conducted at angular velocity ω and absolute temperature T (293 K) in a buffer with density ρ_s . R is the universal gas constant. $A_{280}(r_F)$ denotes the absorbance at reference radial position r_F , taken as the

hinge point – the position at which the protein composition reflects that of the loaded solution. Nonlinear regression analysis in terms of eqn (1) has the potential to yield f_M , the weight-fraction of monomer, in a solution with concentration corresponding to $A_{280}(r_F)$. The monomer molecular mass (M_M) and partial specific volume ($\bar{v}$) of CopY were taken as 16 kDa and 0.740 mL/g respectively, the latter value having been deduced from the amino acid composition. A buffer density at 20 °C of 1.0066 g/mL was obtained by the standard procedure in an Anton Paar density meter.

Traditional analyses of sedimentation equilibrium distributions for apo- and holo-CopY are shown in Figures 3A and 3B. Much of the distribution for apo-CopY conformed with that predicted (broken line in Figure 3A) for a 16 kDa species, the molecular mass of CopY monomer. Nonlinear curve-fitting of the $[r, A_{280}(r)]$ data set for holo-CopY with $A_{280}(r_F) = 0.437$ yielded a value (± 2 SD) of $0.09 (\pm 0.03)$ for f_M . These sedimentation studies signify that the apo-form of CopY is essentially monomeric but that Zn(II)CopY is predominantly dimeric (about 10% monomer as judged by approximate analysis). A more definitive characterization in terms of reversible dimerization was precluded by protein aggregation, a phenomenon that has persisted despite attempts to maintain an anaerobic environment. Nevertheless, the presence of a heavier contaminant does not affect the conclusion that zinc removal destabilizes the dimeric state of CopY, confirming the earlier inference [8].

4. DISCUSSION

4.1 Size-Exclusion Chromatography

The observance of apparent molecular masses that indicated GB1 was monomeric and the GB1-Ymb38 fusion was dimeric provided an early indication that the 38 C-terminal residues of CopY constitute a dimerization domain. Because of the inherent ambiguity in the interpretation of molecular mass from size exclusion chromatography data, self-association was studied by additional means.

4.2 Frontal Size-Exclusion Chromatography

The frontal form of size exclusion chromatography was used to quantify self-association of the fusion protein. Because the elution profile in Figure 1A comprises a single, reasonably symmetrical boundary, it was concluded that any self-association reaction must reflect monomer and polymer(s) in rapid association equilibrium. The association equilibrium was quantified in terms of stoichiometry (n) and association constant (K). Assignment of magnitudes to the elution volumes of monomer for two-state self-association ($nM \rightleftharpoons P$) allowed the weight-fraction of monomer, f_M , in each applied solution to be calculated as

$$f_M = (V_w - V_p) / (V_M - V_p) \quad (2)$$

The reaction stoichiometry (n) was deduced [24] by analyzing the $[C_t, f_M]$ data set in terms of the logarithmic transform of the law of mass action for two-state self-association ($nM \rightleftharpoons P$) governed by association constant K , namely

$$\log [(1 - f_M) C_t] = \log (nK) + n \log (f_M C_t) \quad (3)$$

where C_t is the base-molar concentration (weight-concentration divided by monomer molecular mass). To avoid the bias of statistical weighting imposed by use of the linear transform, K was obtained by direct analysis of the $[C_t, f_M]$ data set in terms of the untransformed relationship

$$(1 - f_M) C_t = nK(f_M C_t)^n \quad (4)$$

using the integer value of n deduced from the slope of the linear dependence of $\log [(1 - f_M)C_t]$ upon $\log (f_M C_t)$.

While the elution volume of monomer (V_M) was easily extrapolated from the data at low protein concentration, the elution volume of polymer (V_P) from the data at high protein concentration was more difficult to estimate. V_P must be less than the V_w observed for GB1-Ymbs38 (●) and its Leu/Ile-to-Ser mutant (○). The approach used to identify V_P is illustrated in Figure 2, which plots the results for GB1-Ymbs38 in the manner suggested by eqn 3. A value of 11.30 mL was precluded by the curvilinear form of the two-species plot (▲,). A decrease in the assigned value of V_P to 11.20 mL led to a reasonably linear plot (○, ---) with a slope ($\pm$ 2SD) of 2.31 ($\pm$ 0.38). A further decrease in the assigned V_P to 11.10 mL maintained essential linearity of the plot (●, —) and improved the precision of the best-fit slope, 1.98 ($\pm$ 0.24). Additional manipulation of V_P provided no further improvement. The V_P was thus deemed 11.10 mL, which, from the slope (n) of the solid line in Figure 2 is now identified unequivocally as dimer for the zinc-containing GB1-Ymbs38. Similar conclusions emanated from analyses of results for apoGB1-Ymbs38 fusion protein as well as for a Leu/Ile-to-Ser mutant of the apoGB1-Ymbs38 metalloprotein. All cysteine residues of both apo protein preparations were carboxyamidomethylated to protect against any metal binding and disulfide induced aggregation. Although weaker self-association of the mutant of apoGB1-Ymbs38 (see Figure 1B) precludes delineation of n by such analysis of the chromatographic data, those results also conformed reasonably well with interpretation in terms of a monomer-dimer model.

The zonal and frontal size-exclusion chromatography methods show evidence of induced dimerization. Having evidence from the frontal method of rapid association equilibrium between monomeric and polymeric states, the observation of an apparent molecular mass of 22.5 kDa for the holo-fusion protein (25.6 kDa for dimer) from the zonal method may signify migration of the protein as a reaction zone rather than as a single (dimeric) species. Continual dilution of the migrating protein solution would cause continual readjustment of species proportions to maintain the monomer-dimer equilibrium.

Previous ESI mass spectroscopy experiments provide additional support for this finding [25]. The ESI mass spectrum of GB1-Ymbs38 under soft ionization conditions [26] exhibits ions from which a dimeric molecular mass of 25.3 kDa can be derived, in addition to monomeric ions with a derived molecular mass of 12.6 kDa. The GB1-Ymbs38 dimer must possess sufficient stability to withstand the ion generation conditions in the mass spectroscopy process. The ESI mass spectrum of the GB1 protein without the 38 C-terminal CopY residues exhibits only ions with a derived molecular mass equal to that of the monomeric protein.

The critical point to emerge from the frontal zone chromatography investigation is the unequivocal demonstration that fusion of the 38 C-terminal residues of CopY to GB1 promoted dimerization of this monomeric protein. It therefore seemed reasonable to invoke direct involvement of this C-terminal fragment in the dimerization of CopY, a postulate stemming from an earlier correlation between Zn(II)-binding and the dimeric state of the protein [4,8]. The present finding of a relatively minor effect of Zn(II) removal on the energetics of dimerization only refers to the GB1-Ymbs38 fusion protein. Sedimentation equilibrium studies on the holo- and apo- forms of CopY signify a greater sensitivity of dimerization to the presence of metal ion.

4.3 Ultracentrifugal Analysis of CopY

A curvilinear dependence of $\ln A_{280}(r)$ upon the square of radial distance was noted for both forms of CopY (Figure 3A and 3B), a feature signifying sample heterogeneity. However, the extent of curvilinearity is too great to reflect solely the effects of reversible dimerization described by eqn (1). On the basis that a molecular mass of 115 kDa for a second component was deduced from corresponding analysis of the residual absorbance, we concluded that the curvilinear dependence of $\ln A_{280}(r)$ upon r^2 for the apo-protein reflected the presence of a small amount of much larger contaminant (presumably aggregated CopY resulting from random cross-bridging of sulfhydryl groups despite precautions to minimize such oxidation). Reasonable agreement between experimental and predicted sedimentation equilibrium distributions was obtained by setting the proportion of contaminant at 5% (solid line in Figure 3A).

Because the upward curvature of the experimental data for Zn(II)CopY (Figure 3B) also signified the presence of material with molecular mass greater than the dimeric value (32 kDa), the $A_{280}(r)$ values were first corrected for sample contamination by the impurity detected in apoCopY (5% of material with a molecular mass of 115 kDa). Despite the presence of the contaminant, the results clearly illuminate the influence of Zn(II) on the dimerization of CopY.

The aim of this investigation has been to obtain experimental evidence for direct involvement of the C-terminal region of CopY in homodimerization. This region of the CopY sequence is of interest because it contains the binding site for a single Zn(II) that is displaced by two Cu(I) ions in the course of its biological function. Involvement of the C-terminal region in CopY dimerization is indicated by the demonstration that attaching the 38 C-terminal residues of CopY to the small immunoglobulin binding domain of Streptococcal protein G (GB1) promotes dimerization of a non-associating protein. Frontal size exclusion chromatography has been used to establish the existence of a rapid association equilibrium between the two oligomeric states of the fusion protein (Figures 1 and 2) and to provide magnitudes for the association constant governing the dimerization equilibrium. Although there is some sensitivity of the strength of dimerization upon metal ion removal (Table 1), the effect is less than that found between Zn(II)CopY and apoCopY (Figure 3). These observations signify that the C-terminal region is only part of the contact area involved in the dimerization of CopY, and that the organization of structure within the C-terminal region as the result of Zn(II)-coordination to the four cysteine residues induces the formation of a contact surface with additional and/or stronger residue interactions. Nevertheless, the demonstration that dimerization of an unrelated, non-associating protein (GB1) can be induced by extending its sequence with the 38-residue C-terminal sector of CopY points strongly to the involvement of this region in homodimerization.

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Abbreviations used

CopY	copper-responsive repressor protein from <i>Enterococcus hirae</i>
ESI mass spectrometry	electrospray ionisation mass spectrometry
GB1	immunoglobulin binding domain of streptococcal protein G

Ymbs38

the 38 C-terminal residues comprising the metal binding site region of CopY

REFERENCES

1. Strausak D, Solioz M. CopY is a copper-inducible repressor of the *Enterococcus hirae* copper ATPases. *J. Biol. Chem.* 1997; 272:8932–8936. [PubMed: 9083014]
2. Odermatt A, Solioz M. Two trans-acting metalloregulatory proteins controlling expression of the copper-ATPases of *Enterococcus hirae*. *J. Biol. Chem.* 1995; 270:4349–4354. [PubMed: 7876197]
3. Cobine P, Wickramasinghe WA, Harrison MD, Weber T, Solioz M, Dameron CT. The *Enterococcus hirae* copper chaperon CopZ delivers copper (I) to the CopY repressor. *FEBS Letters.* 1999; 445:27–30. [PubMed: 10069368]
4. Cobine PA, Jones CE, Dameron CT. Role for zinc(II) in the copper(I) regulated protein CopY. *J. Inorg. Biochem.* 2002; 88:192–196. [PubMed: 11803039]
5. Solioz M, Stoyanov JV. Copper homeostasis in *Enterococcus hirae*. *FEMS Microbiol Rev.* 2003; 27:183–195. [PubMed: 12829267]
6. Cobine P, George GN, Jones CE, Wickramasinghe WA, Solioz M, Dameron CT. Copper transfer from the Cu(I) chaperone, CopZ, to the repressor, Zn(II)CopY: metal coordination environments and protein interactions. *Biochemistry.* 2002; 41:5822–5829. [PubMed: 11980486]
7. Portmann R, Magnani D, Stoyanov J, Schmechel A, Multhaup G, Solioz M. Interaction kinetics of the copper-responsive CopY repressor with the Cop promoter of *Enterococcus hirae*. *J. Biol. Inorg. Chem.* 2004; 9:396–402. [PubMed: 15057514]
8. Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M. Classification of human B-ZIP proteins based on dimerization properties. *Mol. Cell. Biol.* 2002; 22:6321–6335. [PubMed: 12192032]
9. Gronenborn AM, Filpula DR, Essig NZ, Achari A, Whitlow M, Wingfield PT, Clore GM. A novel, highly stable fold of the immunoglobulin binding domain of Streptococcal Protein G. *Science.* 1991; 253:657–661. [PubMed: 1871600]
10. Barchi JJ, Grasberger B, Gronenborn AM, Clore GM. Investigation of the backbone dynamics of the IgG-binding domain of Streptococcal Protein G by heteronuclear two-dimensional ^1H – ^{15}N nuclear magnetic resonance spectroscopy. *Protein Sci.* 1994; 3:15–21. [PubMed: 8142892]
11. Idiyatullin D, Nesmelova L, Daragan VA, Mayo KH. Comparison of $^{13}\text{C}\alpha\text{H}$ and ^{15}NH backbone dynamics in protein GB1. *Protein Sci.* 2003; 12:914–922. [PubMed: 12717014]
12. Frank MK, Dyda F, Dobrodumov A, Gronenborn AM. Core mutations switch monomeric protein GB1 into an intertwined tetramer. *Nature Struct. Biol.* 2002; 9:877–885. [PubMed: 12379842]
13. Byeon IL, Louis JM, Gronenborn AM. A protein contortionist: core mutations of GB1 that induce dimerization and domain swapping. *J. Mol. Biol.* 2003; 333:141–152. [PubMed: 14516749]
14. Schaegger H, Von Jagow G. Tricine–sodium dodecyl sulphate –polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 1987; 166:368–379. [PubMed: 2449095]
15. Grassetti DR, Murray JF Jr. Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiopyridine. *Arch. Biochem. Biophys.* 1967; 119:41–49. [PubMed: 6052434]
16. Winzor DJ, Scheraga HA. Studies of chemically reacting systems on Sephadex. I. Chromatographic demonstration of the Gilbert theory. *Biochemistry.* 1963; 2:1263–1267. [PubMed: 14093900]
17. Winzor DJ, Scheraga HA. Studies of chemically reacting systems on Sephadex. II. Molecular weights of monomers in rapid association equilibrium. *J. Phys. Chem.* 1964; 68:338–343.
18. Ackers GK, Thompson TE. Determination of stoichiometry and equilibrium constants for reversibly associating systems by molecular sieve chromatography. *Proc. Natl Acad. Sci. USA.* 1965; 53:342–349. [PubMed: 14294067]
19. Ackers GK. Analytical gel chromatography of proteins. *Adv. Protein Chem.* 1970; 24:343–446. [PubMed: 4916268]

20. Beckett D, Koblan KS, Ackers GK. Quantitative study of protein association at picomolar concentrations: the lambda phage c1 repressor. *Anal. Biochem.* 1991; 196:69–75. [PubMed: 1888038]
21. Darling PJ, Holt JM, Ackers GK. Coupled energetics of lambda *cro* repressor self-assembly and site-specific DNA operator binding. I. Analysis of *cro* dimerization from nanomolar to micromolar concentrations. *Biochemistry.* 2000; 39:11500–11507. [PubMed: 10985796]
22. Winzor DJ. Analytical size-exclusion chromatography. *J. Biochem. Biophys. Methods.* 2003; 56:15–52. [PubMed: 12834967]
23. Winzor, DJ. Analytical gel filtration. In: Leach, SJ., editor. *Physical Principles and Techniques in Protein Chemistry.* Academic Press; New York: 1969. p. 451-495.
24. Winzor DJ, Loke JP, Nichol LW. Quantitative studies of rapid polymerization equilibria by gel filtration: the z-average elution volume of a reaction boundary. *J. Phys. Chem.* 1967; 71:4492–4498.
25. Pazehoski KO, Collins TC, Boyle RJ, Jensen-Seaman MI, Dameron CT. Stalking metal-linked dimers. *J. Inorg. Biochem.* 2008; 102:522–531. [PubMed: 18177941]
26. Li Y-T, Hsieh Y-L, Henion JD, Senko MW, McLafferty FW, Ganem B. Mass spectrometric studies on noncovalent dimers of leucine zipper peptides. *J. Am. Chem. Soc.* 1993; 115:8409–8413.

Figure 1. Frontal size exclusion chromatography of GB1-Ymbs38

A Shodex KW 803 HPLC column (0.8×30 cm) pre-equilibrated at 4°C with 50 mM Tris–150 mM NaCl–0.1% β -mercaptoethanol, pH 7.8, was used. (A) Advancing elution profile for the carboxyamidomethylated apo-GB1-Ymbs38 ($129\ \mu\text{M}$) showing the weight-average elution volume (V_w) obtained as the median bisector (first moment of the boundary with limiting absorbance A_l). (B) Concentration dependence of V_w for Zn(II)GB1-Ymbs38 (●) and three variants under the same conditions: ▲, carboxyamidomethylated apo-GB1-Ymbs38; ○, the Zn(II) bound form of the Leu/Ile-to-Ser mutant; △, the carboxyamidomethylated apo form of the Leu/Ile-to-Ser mutant.

Figure 2. Evaluation of the reaction stoichiometry for GB1–Ymbs38 self-association

Deduction of the polymer elution volume (V_p) and reaction stoichiometry (n) for self-association of GB1–Ymbs38 fusion protein in 50 mM Tris–150 mM NaCl–0.1% β -mercaptoethanol, pH 7.8, from the plot of size-exclusion chromatography results according to eqn (3) with monomer elution volume (V_M) taken as 12.24 mL and values of 11.30 mL ($\blacktriangle$), 11.20 mL ($\circ$) and 11.10 mL ($\bullet$) assigned to V_p .

Figure 3. Effect of zinc removal on the macromolecular state of CopY

Analyses of sedimentation equilibrium distributions for (A) the apo-form of CopY obtained at 20,000 rev./min and (B) Zn(II)CopY at 20,000 rev./min. The broken line in (A), which is the best-fit description of data in the region $48 < r^2 < 50 \text{ cm}^2$, describes the distribution of monomeric CopY, whereas the solid line is the predicted description after allowance for the presence of a small amount (5%) of a 115-kDa contaminant. The solid line in (B) is the predicted distribution for a similarly contaminated monomer-dimer mixture of Zn(II)CopY with dimer the predominant (91%) species.

Table 1
Self-association parameters for GB1–Ymbs38 fusion protein mutants by frontal size exclusion chromatography

Reaction stoichiometries (n) were determined by analyzing the concentration dependence of the weight-fraction of monomer (f_M) according to eqn 3, after which the association constant (K) was obtained by nonlinear regression analysis of the same dependence according to eqn 4 with n taken as the appropriate integer ($n = 2$). Numbers in parentheses denote the uncertainties ($\pm 2SD$) of the estimates.

Species	Stoichiometry	$10^{-4}K$ (M ⁻¹)	ΔG° (kcal mol ⁻¹)
Zn(II)GB1–Ymbs38	1.98 (± 0.24)	2.26 (± 0.20)	–5.52 (± 0.08)
apoGB1–mbs38	1.87 (± 0.30)	1.59 (± 0.34)	–5.32 (± 0.12)
Zn(II)Ile/Leu mutant	1.86 (± 0.22)	2.44 (± 0.22)	–5.56 (± 0.06)
apoIle/Leu mutant	2 (assumed)	0.14 (± 0.03)	–3.99 (± 0.12)