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Disulfide bonds regulate binding of exogenous ligand to human cytoglobin

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ABSTRACT

Cytoglobin (Cgb) was discovered a decade ago and is a fourth member of the group of hexacoordinated globin-folded proteins. Although some crystal structures have been reported and several functions have been proposed for Cgb, its physiological role remains uncertain. In this study, we measured cyanide binding to the ferric state of the wild-type (WT) Cgb, and found that the binding consisted of multiple steps. These results indicated that Cgb may be comprised of several forms, and the presence of monomers, dimers, and tetramers was subsequently confirmed by SDS-PAGE. Remarkably, each species contained two distinguishable forms, and, in the monomer, analyses of alternative cysteine states suggested the presence of an intramolecular disulfide bond (monomer SS form) and a structure with unpaired thiol groups (monomer SH form). These confirmed that forms were separated by gel-exclusion chromatography, and that the cyanide binding of the separated fractions was again measured; they showed different affinities for cyanide, with the monomer fraction showing the highest affinity. In addition, the ferrous state in each fraction showed distinct carbon monoxide (CO)-binding properties, and the affinities for cyanide and CO suggested a linear correlation. Furthermore, we also prepared several variants involving the two cysteine residues. The C38S and C83S variants showed a binding affinity for cyanide similar to the value for the monomer SH form, and hence the fraction with the highest affinity for exogenous ligands was designated as a monomer SS form. We concluded that polymerization could be a mechanism that triggers the exertion of various physiological functions of this protein and that an appropriate disulfide bond between the two cysteine residues was critical for regulating the binding affinity of Cgb, which can act as a ROS scavenger, for exogenous ligands.

1. Introduction

Cytoglobin (Cgb) was discovered in 2001 as a fourth member of the vertebrate globin family. It is also known as histoglobin or stellate cell activated protein (STAP), since it is expressed ubiquitously, and was identified in rat stellate cells in fibrotic liver tissue [1,2]. The solved crystal structure suggests that Cgb has a typical globin-fold, generally similar to that of hemoglobin (Hb) and myoglobin (Mb) [3–5]. Although a histidine residue solely coordinates the heme iron in Hb and Mb, the His81 and His113 residues in Cgb are present as axial ligands. This structural feature is similar to triply mutated Mb [6] and neuroglobin (Ngb), which have bis-histidyl coordinations [7,8].

The amino acid sequence of Cgb is well conserved among other members of the globin family and is approximately 25% identical to that of Ngb [9]. In fact, Cgb absorption spectra in the ferric, ferrous, and carbon monoxide (CO)-bound states indicate typical hexacoordinated low-spin heme irons in all states, and the resonance Raman profiles are similar to those of Ngb [10–12]. Both Cgb and Ngb have cysteine residues, with three in human Ngb (Cys46, Cys55, and Cys120); it is thought that human Ngb can form an intramolecular disulfide bond between Cys46 and Cys55. Although a dimeric structure was reported for Cgb, with intermolecular disulfide bonds between two cysteine residues (Cys38 and Cys83) [3], previous studies have also suggested that Cgb could form an intramolecular disulfide bond that would permit several different configurations [13].

Since Cgb has structural features similar to those of other globins, it may have many of the same functions, such as oxygen transports and nitric oxide dioxygenase activities [14,15]. However, while upregulated Cgb and Ngb have been reported to protect against cell dysfunction

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under hypoxia/ischemic conditions, this is not the case for other globins [16–18]. Moreover, Cgb has been proposed to have several functions, *e.g.*, in collagen synthesis and suppression of oncogenic transformation [19,20]. Of note is that Cgb has been reported to be upregulated by tumor hypoxia and to be silenced by hypermethylation of its promoter [21]. Cgb-deficient mice were also shown to be more sensitive to carcinogens than wild-type (WT) mice [22], and hence, Cgb has been the focus of anti-tumor therapies. Although various functions have been suggested, the molecular mechanisms underlying these functions remain to be elucidated.

Cgb and Ngb were recently reported to react with reactive oxygen species (ROS) [23]. This implies that Cgb exhibits sensitivity to the oxidative status of its surroundings, and this has been proposed to occur *via* heme coordination and/or cysteine residues. In support of this proposition, an internal disulfide bond in Ngb has been reported to regulate conformational changes [24,25], implying that Cgb could also regulate its various functions by formation of disulfide bonds. In fact, the binding properties of exogenous ligands of Cgb were disturbed by the addition of dithiothreitol (DTT), a reductant for disulfide bonds, suggesting that conformational changes induced by inter- or intramolecular disulfide bonds could be critical for the physiological functions of Cgb [26].

In this study, we therefore examined the ability of certain conformations, with or without inter- or intramolecular disulfide bond(s) in detail. First, we newly report binding affinities for exogenous ligands of two conceivable Cgb forms, a monomer and a dimer. We also assigned the binding affinities for a solved dimeric structure with two intermolecular disulfide bonds and a monomer lacking a disulfide bond. Although these forms are presumed to be related to the functions of Cgb, the properties of each form have not yet been elucidated. Moreover, alternative polymerized forms were found to be constructed with such disulfide bonds. We explored other forms created by interaction of the cysteine residues and assigned exogenous ligand binding properties to these forms. The results of this investigation have revealed the contribution of disulfide bonds to the proposed or hypothesized physiological functions for Cgb, in particular to act as a superoxide scavenger.

2. Experimental

2.1. Preparation of WT and variant Cgbs

Recombinant WT and variant Cgbs were expressed and purified as described previously [11], except for a few modifications: *Escherichia coli* were cultured in 4 L of LB medium in a 5-L flask sealed with plastic wrap to maintain minimally aerobic conditions in order to avoid obtaining Cgb as a green protein [27]. When the culture attained an optical density of 0.5 at 600 nm, isopropyl β-(D) thiogalactopyranoside (IPTG) and aminolevulinic acid were each added to a final concentration of 0.5 mM to induce protein expression, and culturing was continued for a further 15 h. Cells were harvested by centrifugation and were frozen once at −80 °C, and the cell pellets were lysed in 150 mL of 50 mM Tris–HCl buffer (pH 8.0) with 0.1 M NaCl and 10 mM imidazole. The soluble fraction was isolated by ultracentrifugation (37,000 ×*g*), and then the supernatant was applied to Ni-NTA agarose (Qiagen). His-tagged Cgb was purified by stepwise imidazole concentration, and the imidazole was subsequently removed by dialysis. The His-tag domain was cleaved with a Thrombin kit (Novagen) and separated, again using Ni-NTA agarose. The purified Cgb was concentrated with Amicon Ultra (Merck Millipore), and then gel-filtration chromatography was performed using Sephacryl S-200 (2.5 cm × 120 cm) (GE Healthcare) to separate the presumed monomeric (*ca.* 20 kDa), dimeric (*ca.* 40 kDa), and tetrameric (*ca.* 80 kDa) forms. Flow rate was set to approximately 0.4 mL/min, and each reddish fraction was eluted after 10 h, 8 h, or 7 h of loading of the gel. Finally, the identities of the separated reddish Cgb fractions were confirmed by SDS-PAGE. Each fraction was also investigated in the presence and absence of β-mercaptoethanol (2-ME), in order to prepare a monomeric form lacking disulfide bonds as well as multimeric forms with inter- or intramolecular disulfide bonds.

2.2. Exogenous ligand affinities for Cgb in the ferric and ferrous states

The ferric Cgb was titrated with potassium cyanide in 10 mM sodium phosphate and 0.1 M NaCl (pH 7.0). The extent of HCN formation was not determined at this pH, but the free cyanide concentration ($[CN^-_{free}]$) was calculated, taking the amount bound to protein into account. A fraction of the cyanide-bound form (α) was evaluated by examining spectral changes of Cgb in the presence of cyanide, and dissociation constants were estimated by fitting Eq. (1), containing a Hill coefficient (n). Additionally, Eq. (2) was applied for fitting to multi-phasic bindings, where K_ds and *A* denote dissociation constants and amplitude for the first binding step, respectively.

$$\alpha = \frac{[CN^-_{free}]^n}{(K_d{}^n + [CN^-_{free}]^n)} \tag{1}$$

$$\alpha = \frac{[CN^-_{free}]^n}{K_{d1}{}^n + [CN^-_{free}]^n} + \frac{(1-A)\cdot[CN^-_{free}]^n}{(K_{d2}{}^n + [CN^-_{free}]^n)} \tag{2}$$

Moreover, CO titration was performed as described previously, with minor modifications [28]. A cuvette was filled with approximately 6 μM Cgb in 10 mM sodium phosphate, 0.1 M NaCl (pH 7.0), and 20 μM EDTA. The cuvette was extensively purged with nitrogen gas to create anaerobic conditions before addition of 2 mM glucose, 8 units/mL glucose oxidase, and 260 units/mL catalase to scavenge any remaining oxygen. Cgb was photoreduced by the addition of 400 μM NADH and 1 μM FMN photoexcited at 445.5 nm for about 30 min [29]. Full reduction was confirmed by absorption spectroscopy (SHIMADZU UV-2450). Aliquots of 10 mM sodium phosphate and 0.1 M NaCl (pH 7.0) saturated with CO were added to the protein solution, and the subsequent changes in absorption recorded. The CO concentrations were calibrated using horse Mb. Fractions of CO-bound states (α) were plotted against concentration of CO, and the plots were fitted by using Eq. (3) to estimate dissociation constants ($[Cgb]_0$ denotes total concentration of Cgb).

$$[CO_{total}] = \frac{\alpha}{1-\alpha}\cdot K_d + \alpha\cdot[Cgb]_0. \tag{3}$$

2.3. Resonance Raman spectroscopy

Resonance Raman spectra were measured as previously reported [10], with minor modifications. Samples were excited using a krypton ion laser (406.7 nm line, Coherent I-302), and spectra recorded using a triple monochromator (Jasco NR-1800) with a slit width of 6 cm^{-1}. A CCD detector (Andor DU420-BU) was used, and the frequencies were calibrated with indene. A spinning Raman cell was used throughout the measurements. Cgb concentration in the buffers was adjusted to 100 μM in 10 mM sodium phosphate and 0.1 M NaCl (pH 7.0).

2.4. Kinetics of exogenous ligand binding in the ferric state

Purified Cgb was diluted to 5 μM in several tubes; the various concentrations of potassium cyanide was added and thoroughly mixed, and the absorbance at the Soret band was monitored for 50 min. The absorbance changes were plotted against time, and either Eq. (4) or (5) was used to calculate observed rate constants, k_{obs}. Thus,