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Coupling of disulfide bond and distal histidine dissociation in human ferrous cytoglobin regulates ligand binding

Penny Beckerson, Brandon J. Reeder *, Michael T. Wilson

School of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, United Kingdom

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ABSTRACT

Earlier kinetics studies on cytoglobin did not assign functional properties to specific structural forms. Here, we used defined monomeric and dimeric forms and cysteine mutants to show that an intramolecular disulfide bond (C38–C83) alters the dissociation rate constant of the intrinsic histidine (H81) (~1000 fold), thus controlling binding of extrinsic ligands. Through time-resolved spectra we have unequivocally assigned CO binding to hexa- and penta-coordinate forms and have made direct measurement of histidine rebinding following photolysis. We present a model that describes how the cysteine redox state of the monomer controls histidine dissociation rate constants and hence extrinsic ligand binding.

1. Introduction

Cytoglobin (Cygb) is a relatively new member of the vertebrate hemoglobin superfamily [1,2] and displays hexacoordination (His-Fe-His) similar to neuroglobin [3–6]. Binding of exogenous ligands is therefore competitive with the endogenous histidine, giving multiphasic binding kinetics following flash photolysis; behavior that has been reported in other hexacoordinate globins such as rice non-symbiotic hemoglobin [7,8].

Previously Cygb has been shown to be an intermolecular disulfide linked homo-dimer, however recent studies have identified a monomeric form and also the presence of an internal disulfide bond [9,10]. The presence of this bond (C38–C83) has been shown to affect ligand binding kinetics and affinities [10] and other characteristics including the ability to interact with lipids in the ferric form [11]. In our previous stopped-flow studies we proposed that the formation of an intramolecular disulfide bond leads to movement of helices B and E, which results in a change in the distal histidine dissociation rate constant. Lechauve et al. [9] have previously investigated the functional properties of Cygb and have related ligand recombination rates, following flash photolysis, to conformations of the protein that depend on the absence or presence of disulfide bonds. They also conclude that the histidine dissociation rate constant is decreased in the presence of dithiothreitol, which disrupts disulfide bonds.

In this study we extend our previous work and that of Lechauve et al. [9] by undertaking further laser photolysis experiments. The use of defined monomeric and dimeric forms and mutation of specific cysteine residues (C38) has demonstrated that the intramolecular disulfide bond between C38 and C83 controls the dissociation rate constant of the intrinsic histidine (H81) from the central iron. Through this mechanism control of binding extrinsic ligands such as CO is exerted. Our conclusions are in overall qualitative agreement with Lechauve et al. [9] but we report some important quantitative differences in the values for CO binding rate constants and histidine binding and dissociation rate constants. Through time-resolved spectra we have assigned unequivocally CO binding to hexa- and penta-coordinate forms and have made direct measurement of histidine rebinding following photolysis. We present a model that summarizes our conclusions and those of earlier work, which incorporates changes in ligand affinities, ligand binding rate constants and distal histidine association and dissociation rate

* Corresponding author.
E-mail address: reedb@essex.ac.uk (B.J. Reeder).

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constants controlled by the cysteine residue redox state in the monomeric form [9,10,12].

2. Materials and methods

2.1. Cytoglobin engineering, expression and purification

Human cytoglobin was expressed and purified as previously described [11,13]. The monomeric fraction with an internal disulfide bond ($monomer_{S-S}$), dimeric fraction with intermolecular disulfide ($dimer_{S-S}$) and polymeric fraction were separated using G-75 Superdex column (600 mm × 16 mm, GE Healthcare) equilibrated with 0.1 M sodium phosphate buffer (pH 7.4) with an AKTA purification system. Fractions were collected corresponding to the absorbance peaks at 280 nm. The mutated C38R Cygb was prepared as previously described with the monomeric fractions used and is subsequently referred to as $monomer_{S-H}$ [11]. Monomeric distal histidine mutant of Cygb (H81Y), which showed penta-coordination of the heme iron, was generated by site directed mutagenesis and expressed and purified using the same protocol as the WT.

2.2. Preparation of the ferrous protein

It has been previously shown that the disulfide bond in Cygb is reduced by sodium dithionite with a half time of ~2000 s [11]. Therefore samples of ferrous Cygb, prepared by addition of a slight excess of dithionite to degassed protein solution, were used within approximately 5 min. Our previous studies have shown that there is negligible change in CO binding kinetics as a result of disulfide bond reduction over this time period [11].

2.3. Carbon monoxide titrations

A solution of CO was prepared by the equilibration of 0.1 M sodium phosphate buffer (pH 7.4) with 1 atmosphere of CO at 20 °C to give a solution of ~1 mM CO. The precise concentration of CO was calculated by a titration against ferrous myoglobin, which binds at a 1:1 stoichiometry. A sealed quartz cuvette with no headspace was filled with degassed 0.1 M sodium phosphate buffer (pH 7.4). Ferrous Cygb $monomer_{S-S}$, $monomer_{S-H}$ and $dimer_{S-S}$ were diluted to a concentration of 5 μM and titrated with the CO solution using a micrometer screw-gage AGLA syringe with 5 μL addition giving ~0.5 μM CO additions. Upon each addition changes in absorbance between 350 and 600 nm were recorded. Data was fitted, using Graphpad 6.0, to Eq. (1) below as previously reported [13], where $[P_T]$ is the total protein concentration, $[S]$ is the total ligand concentration (CO) and K is the dissociation constant.

$$Y = \frac{([P_T] + [S] + K) - \sqrt{([P_T] + [S] + K)^2 - 4([P_T][S])}}{2[P_T]} \quad (1)$$

2.4. Carbon monoxide flash photolysis

An Applied Photophysics LKS80 laser flash photolysis spectrometer equipped with a monochrometer and photomultiplier was used to measure changes in absorbance following laser dissociation of CO from ferrous $monomer_{S-S}$, $monomer_{S-H}$ and $dimer_{S-S}$ Cygb (5 μM) in the presence of known concentrations of CO (0–500 μM). Averages of 6 flashes were recorded and observed rate constants calculated by using the ProKinetist software. Difference spectra for each phase were calculated using changes in amplitudes at multiple wavelengths.

3. Results

3.1. Carbon monoxide titrations of ferrous $monomer_{S-S}$, $dimer_{S-S}$ and $monomer_{S-H}$ cytoglobin

Addition of saturating concentrations of CO to the 3 forms of ferro-Cygb ($monomer_{S-S}$, $monomer_{S-H}$ or $dimer_{S-S}$) led to the formation of a carbon monoxide complex, the spectral properties of which were independent of the Cygb form, i.e. Soret band at 423 nm, α and β peaks at 570 and 542 nm respectively, consistent with earlier observation [6,11] (Fig. 1 inset). Fig. 1 shows the results of CO titrations of each form in which small aliquots of a solution of CO were added to the ferrous protein. It is apparent from fitting these data (Eq. (1)) that the stoichiometry of binding is 1:1 (protein:CO) for each form. The affinities of the $monomer_{S-S}$ and $monomer_{S-H}$ are high ($K_D < 10^{-7}$ M) and could not be accurately determined from this method. The affinity of the $dimer_{S-S}$ is, however, significantly lower and we estimate from the fit a K_D of $3.3 \pm 0.9 \times 10^{-7}$ M. These results are consistent with those reported by Tsujino et al. [10].

3.2. Recombination of CO with ferrous $monomer_{S-S}$, $dimer_{S-S}$ and $monomer_{S-H}$ cytoglobin following laser photolysis

Previously reported stopped-flow studies investigating CO binding showed time courses for CO combination to $monomer_{S-S}$, $dimer_{S-S}$ and $monomer_{S-H}$ that were monophasic [11]. In contrast, following flash photolysis the time courses for CO recombination were biphasic (Fig. 2). The two phases of the $monomer_{S-S}$ CO recombination exhibited different associated spectral changes. These are shown in Fig. 2B and the optical changes of the fast phase are consistent with the binding of CO to the pentacoordinate form of the protein generated by the photolysis of the CO bond. The optical changes of the slower phase represent CO binding to the hexacoordinate form (His-Fe-His). This behavior is reminiscent of that displayed by hexacoordinate hemoglobins and is represented in Scheme 1 [8,14]. In this scheme the initial CO-complex (Scheme 1i) is photolysed to give the pentacoordinate species (ii). This penta-coordinate species may recombine in a second order process to form the initial CO-complex (i – k_{CO}). Alternatively the intrinsic distal histidine ligand binds to form a hexacoordinate

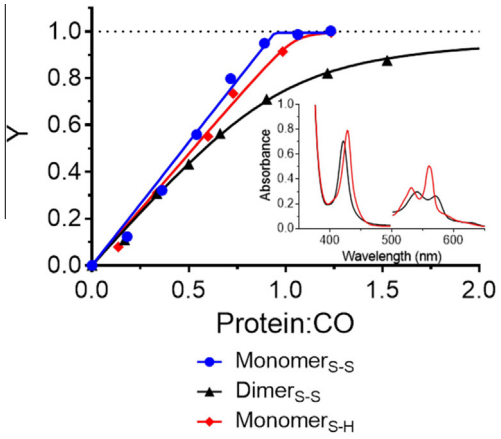

Fig. 1. Titration of ferrous cytoglobin with CO. Dithionite-reduced $monomer_{S-S}$, $dimer_{S-S}$ and $monomer_{S-H}$ Cygb were titrated with CO (436–415 nm). The continuous line represents data points fitted to Eq. (1). Inset: Spectral changes on addition of saturating concentrations of CO to ferrous Cygb. Spectra shown are the dithionite-reduced $monomer_{S-S}$ (red) and the CO-$monomer_{S-S}$ (black). Spectra of the $dimer_{S-S}$ and $monomer_{S-H}$ were identical.