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Functional and structural roles of the *N*-terminal extension in *Methanosarcina acetivorans* protoglobin ☆

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ABSTRACT

Functional and structural properties of protoglobin from *Methanosarcina acetivorans*, whose Cys(101)E20 residue was mutated to Ser (*Ma*Pgb*), and of mutants missing either the first 20 *N*-terminal amino acids (*Ma*Pgb*-ΔN20 mutant), or the first 33 *N*-terminal amino acids [*N*-terminal loop of 20 amino acids and a 13-residue Z-helix, preceding the globin fold A-helix; (*Ma*Pgb*-ΔN20Z mutant)] have been investigated. In keeping with the *Ma*Pgb*-ΔN20 mutant crystal structure, here reported at 2.0 Å resolution, which shows an increased exposure of the haem propionates to the solvent, the analysis of ligand binding kinetics highlights high accessibility of ligands to the haem pocket in ferric *Ma*Pgb*-ΔN20. CO binding to ferrous *Ma*Pgb*-ΔN20 displays a marked biphasic behavior, with a fast binding process close to that observed in *Ma*Pgb* and a slow carbonylation process, characterized by a rate-limiting step. Conversely, removal of the first 33 residues induces a substantial perturbation of the overall *Ma*Pgb* structure, with loss of α-helical content and potential partial collapse of the protein chain. As such, ligand binding kinetics are characterized by very slow rates that are independent of ligand concentration, this being indicative of a high energy barrier for ligand access to the haem, possibly due to localized misfolding. This article is part of a Special Issue entitled: Oxygen Binding and Sensing Proteins.

1. Introduction

Protoglobins (Pgbs) are recently discovered single domain haemproteins composed of ~195 amino acids. They are structurally related to hemoglobins, myoglobins, and to the *N*-terminal domain of archaeal and bacterial globin coupled sensor proteins (GCS). GCS are indeed chimeric haem proteins, which couple a globin-like sensor domain to a transmitter domain of variable structure and function [1]. However,

Abbreviations: Pgb, protoglobin; GCS, globin coupled sensors; *Ma*Pgb, *Methanosarcina acetivorans* protoglobin; *Ma*Pgb*, *Methanosarcina acetivorans* protoglobin whose Cys(101)E20 is replaced by Ser; Hb, hemoglobin; Mb, myoglobin; *Ma*Pgb*-ΔN20, *Methanosarcina acetivorans* protoglobin whose first 20 amino acids at the *N*-terminal have been deleted; *Ma*Pgb*-ΔN20Z, *Methanosarcina acetivorans* protoglobin whose 33 *N*-terminal amino acids have been removed; BIS–TRIS propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane; CD, circular dichroism; *Ma*Pgb*-Fe(II), ferrous *Ma*Pgb*; *Ma*Pgb*-ΔN20-Fe(II), ferrous *Ma*Pgb*-ΔN20; *Ma*Pgb*-ΔN20Z-Fe(II), ferrous *Ma*Pgb*-ΔN20Z; *Ma*Pgb*-ΔN20-Fe(III), ferric *Ma*Pgb*-ΔN20; *Ma*Pgb*-Fe(III), ferric *Ma*Pgb*; *Ma*Pgb*-ΔN20Z-Fe(III), ferric *Ma*Pgb*-ΔN20Z; *Ma*Pgb*-Fe(II)-CO, carbonylated *Ma*Pgb*-Fe(II); *Ma*Pgb*-ΔN20-Fe(II)-CO, carbonylated *Ma*Pgb*-ΔN20-Fe(II); *Ma*Pgb*-ΔN20Z-Fe(II)-CO, carbonylated *Ma*Pgb*-ΔN20Z-Fe(II); *Ma*Pgb*-ΔN20-Fe(III)-cyanide, cyanide-bound *Ma*Pgb*-ΔN20-Fe(III); *Ma*Pgb*-Fe(II)-O_2, oxygenated *Ma*Pgb*-Fe(II); *Ma*Pgb*-Fe(III)-cyanide, cyanide-bound *Ma*Pgb*-Fe(III); *Ma*Pgb*-ΔN20-Fe(III)-formate, formate-bound *Ma*Pgb*-ΔN20-Fe(III)

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albeit Pgbs belong phylogenetically to the same cluster as the GCS group within the globin superfamily [2], they miss the covalently-bound transmitter domain normally present in GCSs.

To date, nine Pgbs have been identified in *Archaea* and *Bacteria* [1–4], and only two Pgbs have been characterized, from the obligate aerobic hyperthermophile *Aeropyrum pernix* and from the strictly anaerobic methanogen *Methanosarcina acetivorans* (*Ma*Pgb) [4,5]. *Methanosarcinae* are metabolically and physiologically the most versatile methanogens, being able to exploit acetate, methanol, CO_2 and CO as carbon sources for methanogenesis, through a metabolic pathway that has been proposed to be one of the first exploited by primordial microbes [6,7]. Despite the strict anaerobic nature of *M. acetivorans*, its genome hosts genes that can be related to O_2 metabolism, such as Pgb, which may play a yet undisclosed role in CO metabolism of Archaea and Bacteria. Indeed, up to date the physiological role of Pgb is totally unknown, as is the nature of its natural ligand(s). However, over the last few years several progresses have been made in the biochemical and biophysical characterizations of this unusual globin.

M. acetivorans Pgb three-dimensional structure has been unraveled by the crystallographic analysis of the Cys(101)E20→Ser mutant (hereafter termed *Ma*Pgb*), which was produced for crystallization purposes [5]. *Ma*Pgb* is folded into a 3-on-3 α-helical sandwich (helices A–H) similarly (65)G11 to hemoglobin (Hb) and myoglobin (Mb) [8], and its tertiary and quaternary homodimeric assembly is strongly related to the *N*-terminal domain of archaeal and bacterial GCSs [9,10]. Contrary to the classical globin fold [8,11], in *Ma*Pgb* an additional *N*-terminal Z-helix precedes the A-helix, and a short H′-helix (one helical turn) falls close to the C-terminus. The Z-helix is further preceded by twenty amino acids, which build up, over the protein α-helical core, an *N*-terminal loop held next to the haem propionates by several polar interactions. Thus the haem is completely buried in the Pgb protein matrix; the haem propionates are solvent inaccessible and the diffusion path of diatomic ligand to the haem distal site through the E7-gate is precluded [5].

Access of ligands to the haem cavity is granted by two orthogonal apolar tunnels reaching the haem distal site from locations at the B/G (tunnel 1) and B/E (tunnel 2) helix interfaces. Tunnel 1 (~7 Å in diameter, and ~16 Å in length) is lined by residues Ile(56)B5, Thr(59)B8, Trp(60)B9, Phe(63)B12, Phe(93)E11, Ile(97)E15, Phe(145)G7, Pro(148)G10, Ile(149)G11, Thr(152)G14, and Met(153)G15. Tunnel 2 (~5 Å in diameter, and ~10 Å in length) appears as a straight opening on the haem distal cavity, nestled between residues Tyr(61)B10, Leu(64)B13, Gly(65)B14, Leu(71)C5, Phe(74)CD1, and Leu(86)E4. All residues lining the *Ma*Pgb* tunnels are conserved in known Pgbs, suggesting functional implications for ligand diffusion to/from the haem cavity, for multi-ligand storage, and/or for Pgb (pseudo-)enzymatic actions. Moreover, the presence of the two tunnels within the protein matrix might be responsible for the slightly biphasic CO binding behavior to *Ma*Pgb [5,13]. Indeed, *Ma*Pgb exists in two distinct conformations with different affinities for CO, in an equilibrium influenced by the ligation state of the protein. This mechanism is mediated by protein dynamics and suggests that in this ancient protein an allosteric control of affinity may be relevant for a bimolecular reaction towards the yet unknown physiological substrates of the protein [13].

In consideration of the peculiar structural and functional properties displayed by *Ma*Pgb*, we focused our attention on the roles played by the *N*-terminal 33-residue region, which is not present in the classical 3-on-3 α-helical sandwich of most members of the globin superfamily [8,11]. Specifically, we designed two deletion mutants where: (*i*) the first 20 residues, devoid of a specific secondary structure, are deleted (*Ma*Pgb*-ΔN20 mutant), and (*ii*) the twenty *N*-terminal residues and the following Z-helix, thus covering residues 1–33, are omitted (*Ma*Pgb*-ΔN20Z mutant). Here the crystal structure of the *Ma*Pgb*-ΔN20 mutant, as well as the functional characterization of both *Ma*Pgb*-ΔN20 and *Ma*Pgb*-ΔN20Z mutants are reported, casting first light on the relevant structural and functional roles played by the (globin-uncommon) long *N*-terminal extension of *Ma*Pgb*.

2. Materials and methods

2.1. Materials

*Ma*Pgb*, bearing a Cys(101)E20→Ser mutation, was heterologously expressed for crystallization purposes, as previously reported [5,13]. Analysis of the *Ma*Pgb* structure indicated that Ser(101)E20 is not involved in any specific interaction critical for structural stability, but is partly exposed to the solvent. Thus, the Cys(101)E20→Ser mutation is justifiable to prevent formation of intermolecular disulphides and safe, with no plausible harm for the correct protein folding.

The deletion mutants *Ma*Pgb*-ΔN20 and *Ma*Pgb*-ΔN20Z were obtained using the QuickChange™ site-directed mutagenesis method (Stratagene, La Jolla, CA, USA), as described previously [14]. *Ma*Pgb*-ΔN20 and *Ma*Pgb*-ΔN20Z were expressed and purified as reported for *Ma*Pgb* [5,13]. CO was purchased from Linde (Linde Gas, Rome, Italy). The CO stock solution was prepared anaerobically and stored in a sealed vessel with distilled water under CO, at $P = 760.0$ mm Hg ($T = 20$ °C). The solubility of CO in water is 1.03×10^{-3} M, at $P = 760$ mm Hg and 20 °C [15].

Gaseous NO was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and purified by flowing through a NaOH column in order to remove acidic nitrogen oxides. The NO stock solution was prepared anaerobically in a sealed vessel by keeping distilled water under purified NO, at 760 mm Hg and 20 °C. The solubility of NO in water is 2.05×10^{-3} M, at 760 mm Hg and 20 °C [15]. The NO stock solution was diluted with degassed 1.0×10^{-1} M 1,3-bis(tris(hydroxymethyl)methylamino) propane (BIS–TRIS propane) buffer (pH 9.2) to reach the desired concentration (2.0×10^{-4} M to 1.0×10^{-3} M). BIS–TRIS propane was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade and used without further purification unless stated.

2.2. Methods

2.2.1. Spectroscopic characterization of *MaPgb**, *MaPgb*-ΔN20* and *MaPgb*-ΔN20Z* derivatives

Absorption spectroscopy and circular dichroism (CD) measurements of *Ma*Pgb*, *Ma*Pgb*-ΔN20 and *Ma*Pgb*-ΔN20Z were carried out employing a Jasco V-53 UV–vis spectrophotometer (Varian Inc., Palo Alto, USA) and a Jasco J-700 spectrometer (Varian Inc., Palo Alto, USA), respectively. The spectroscopic characterizations of ferrous unligated derivatives of *Ma*Pgb*, *Ma*Pgb*-ΔN20 and *Ma*Pgb*-ΔN20Z (*Ma*Pgb*-Fe(II), *Ma*Pgb*-ΔN20-Fe(II) and *Ma*Pgb*-ΔN20Z-Fe(II), respectively) were carried out on samples kept anaerobically (under N_2 atmosphere and in the presence of 10 mg/mL dithionite) in a sealed vessel equipped with a quartz cuvette.

2.2.2. Crystallization and structure determination

Crystals of ferric *Ma*Pgb*-ΔN20 (*Ma*Pgb*-ΔN20-Fe(III)) were grown by vapor diffusion methods mixing equal volumes of the protein solution (43 mg/mL) and the precipitant solution (0.2 M magnesium formate, Crystal Screen I, condition 44, Hampton Research; Aliso Viejo, CA, USA) in the crystallization droplets, which were equilibrated against the precipitant solution at 4 °C. The crystals were transferred to the same solution supplemented with 25% (v/v) glycerol, prior to cryo-cooling and data collection. Crystals belong to the primitive monoclinic $P2_1$ space group (4 *Ma*Pgb*-ΔN20-Fe(III) molecules in the asymmetric unit) and diffracted to 2.0 Å resolution, using synchrotron radiation at -173 °C (beamline ID14-4, ESRF, Grenoble, France). All collected data were reduced and scaled using MOSFLM and SCALA [16,17].

Structure determination was achieved by molecular replacement methods with the program PHASER [18], using the *Ma*Pgb* structure (PDB accession code: 2VEB) as the search model from which the first 20 *N*-terminal residues had been deleted [5]. Crystallographic refinement was performed using the program REFMAC [19], and the program