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Topography of human cytochrome b_5/cytochrome b_5 reductase interacting domain and redox alterations upon complex formation

Alejandro K. Samhan-Arias[a,*], Rui M. Almeida[a], Susana Ramos[a], Cristina M. Cordas[a], Isabel Moura[a], Carlos Gutierrez-Merino[b], José J.G. Moura[a,*]

[a] UCIBIO, REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, NOVA, 2829-516 Caparica, Portugal
[b] Department of Biochemistry and Molecular Biology, Faculty of Sciences, Institute of Molecular Pathology Biomarkers, University of Extremadura, 06006 Badajoz, Spain

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ABSTRACT

Cytochrome b_5 is the main electron acceptor of cytochrome b_5 reductase. The interacting domain between both human proteins has been unidentified up to date and very little is known about its redox properties modulation upon complex formation. In this article, we characterized the protein/protein interacting interface by solution NMR and molecular docking. In addition, upon complex formation, we measured an increase of cytochrome b_5 reductase flavin autofluorescence that was dependent upon the presence of cytochrome b_5. Data analysis of these results allowed us to calculate a dissociation constant value between proteins of 0.5 ± 0.1 μM and a 1:1 stoichiometry for the complex formation. In addition, a 30 mV negative shift of cytochrome b_5 reductase redox potential in presence of cytochrome b_5 was also measured. These experiments suggest that the FAD group of cytochrome b_5 reductase increase its solvent exposition upon complex formation promoting an efficient electron transfer between the proteins.

1. Introduction

Cytochrome b_5 reductase (Cb_5R) is a protein with multiple functions in cells and tissues associated to its redox partner, Cytochrome b_5 (Cb_5) [1]. Cb_5R catalyzes the oxidation of NADH through its FAD group to reduce Cb_5. This is favored by the electrochemical potentials of NADH (≈ − 360 mV) [2], Cb_5R (≈ − 270 mV) associated to its flavin [3] and Cb_5 (≈ 0 mV) [4] that settle the so called "plasma membrane redox chain" [5], with other redox partners [6]. Modulation of the Cb_5R redox potential by some cofactors is known, i.e. Cb_5R purified from pig liver upon interaction with NADH alters its protein redox potential to − 160 mV [7]. This change has been correlated to a more reduced state of the enzyme revealing a thermodynamic barrier not allowing the interaction with other molecules [3]. The mechanism underlying the electron transfer from substrates to Cb_5R was studied by pulse radiolysis [8]. By this methodology, NADH consumption by Cb_5R purified from pig liver was proposed to occur through electron transfer to the flavin group generating an anionic semiquinone radical that, by dismutation, produces one fully reduced and one fully oxidized flavin molecule [8]. Nevertheless, the flavin reduction mechanism can change depending on the reducer. Reducers like dithionite induce a two electron reduction of the FAD group without formation of the flavin radical in absence of NAD^+ [3]. Therefore, formation of a stable complex with NAD^+ is determinant to allow the formation of the FAD semiquinone [7]. In addition, the NAD^+ free reduced enzyme by light irradiation is sensitive to the presence of oxygen which rapidly oxidizes it, with a first-order rate constant of $k = 2.1\ s^{-1}$ [7]. This correlates with formation of superoxide anion by pig liver Cb_5R located at the neuronal plasma membrane as a source of this radical [9–11], pointing out the different stability of Cb_5R complex with oxygen compared to other flavoproteins where this complex has been shown, i.e. for the NADPH cytochrome P450 reductase [7,12]. In addition, very little is known about the modulatory effect of Cb_5 on the redox potential of Cb_5R upon complex formation. The binding domain between proteins is still unidentified, although some residues have been suggested to participate in the interaction [13,14].

In respect to pig liver Cb_5R, the surface electrostatic potential of FAD protruding site is positive, in comparison to the negatively-charged surface of the heme binding site in Cb_5. Therefore the FAD area of Cb_5R is susceptible to interact with Cb_5 based on electrostatic properties.

Abbreviations: Cytochrome b_5, Cb_5; Cytochrome b_5 reductase, Cb_5R; Cytochrome *c*, Cyt *c*; Cytoglobin, Cyg; Ethylene diamine tetra acetic acid, EDTA; Flavin adenine dinucleotide, FAD; Heteronuclear single quantum coherence spectroscopy, HSQC; Isopropyl ß-D-1-thiogalactopyranoside, IPTG; Reduced nicotinamide adeninedinucleotide, NADH; Reduced nicotinamide adenine dinucleotide phosphate, NADPH; Neuroglobin, Ngb; Normal hydrogen electrode, NHE; Phenylmethylsulfonylfluoride, PMSF; Terrific Broth, TB; 2-Amino-2-(hydroxymethyl) propane-1,3-diol, Tris; Thermosensitive alkaline phosphatase, TSAP

* Corresponding authors at: UCIBIO, REQUIMTE, Departamento de Quimica, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal.
E-mail addresses: alejandro.samhan@fct.unl.pt (A.K. Samhan-Arias), jose.moura@fct.unl.pt (J.J.G. Moura).

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Based on this, a model for the electron transfer between both proteins has been proposed [13]. Cb_5R structural dynamics upon interaction with NADH becomes altered, increasing the surface area of H49 and FAD. Involvement of K162 and K163 in the interaction between both proteins has been proposed [15]. These residues are forming part of suggested pairs to interact with Cb_5R in experiments performed where carboxylic acids of Cb_5 were modified: K41/E52 or E60 and K125/E47 and/or E48 (of Cb_5R/Cb_5 respectively) [14]. In experiments performed with K41E, K125E, K162E or K163E [15] and K41A, K125A, K163A Cb_5R mutants [16], the kinetic analysis of Cb_5 reduction also supported a role for these Cb_5R residues in the interaction with Cb_5. Mutation of these lysine residues has very little effect when artificial acceptors like ferricyanide are used to measure Cb_5R activity. Nevertheless, in presence of Cb_5 as electron acceptor, an increase of the K_m and decrease of k_{cat} was found, corroborating previous results showing a role of these residues on charge pairing [15].

By modification of carboxylic acids of Cb_5 and analysis of the interaction with Cb_5R using purified proteins from bovine liver, implication of E47, E48 and E52 residues of Cb_5 has been determined [14]. In addition, Cb_5 mutants E47A, E48A, E52A, E60A and D64A (group A) showed almost the same catalytic activities than the wild type, revealing that these residues are not involved in the electrostatic interaction with Cb_5R. Moreover, Cb_5 mutants on E41A, E42A, D57A and E63A (group B) showed an increase of the K_m values for Cb_5 with no difference on the k_{cat} values compared to the wild type, supporting a role of acidic residues from the group A in Cb_5R binding domain of Cb_5, in experiments performed with human erythrocyte Cb_5R and Cb_5 [17].

A very recent study on the crystallographic structure of porcine Cb_5R has shown the electron valence map and the hydrogen binding network of the FAD group to the peptide portion of the soluble domain recombinant pig liver Cb_5R [18]. This study shows the importance of the N5 atom of FAD hydrogen bonding to Y65 and amide group of T66 as stabilizers of the interaction of FAD with Cb_5R peptide chain. An important role has been given to H49 as part of the Cb_5 binding domain of Cb_5R, via non-classical hydrogen bonds of $C_\alpha H$ and O of Y65 and $C_\beta H$ of H49 to the N5 atom of FAD. Therefore H49 and T66 were suggested to modulate Cb_5R activity upon interaction with its electron acceptor (Cb_5), since a shorter electron transfer pathway than the classical described path was suggested to be driven through H49 [18]. The correlative residues of human Cb_5R used in this study are Y93, T94, and H77. Nevertheless due to the close proximity of the heme to the FAD group upon complexing, alteration of this network is susceptible to change. For some proteins, FAD exposure to the solvent has been elicited upon interaction with ligands [19,20]. Consequently, this is a useful susceptible tool to measure interactions.

Cb_5 interaction domain with other redox partners is known [1]. For the case of bovine erythrocyte methemoglobin as an electron partner, E43, E44, E48 and E60 of erythrocyte Cb_5 have been implicated in the interaction [21]. Some of these residues are common with glutamic and aspartic residues found in the interaction between rat hepatic Cb_5 with horse heart cytochrome *c* (Cyt *c*) (E43, E44, E48), and some of them also previously shown for the interaction between Cb_5 (E47, E48, E52) with Cb_5R from human erythrocyte and bovine liver sources [14,15,22]. Therefore these results point out the importance of these acidic residues of Cb_5 in the interaction with several of its redox partners. In this paper, we characterized the complete Cb_5R/Cb_5 interacting domain, using recombinant human erythrocyte proteins, by solution NMR and a docking model for the complex formation using BiGGER algorithm. Previous reports with Cb_5R mutants have reported a higher flavin autofluorescence with mutated amino acids of a hydrophobic domain [23]. Some of these amino acids were found in the human Cb_5R interacting domain with Cb_5. Our fluorescence experiments support that Cb_5 interacts with this hydrophobic domain of Cb_5R. Cb_5 interaction with its reductase induces a conformational change on Cb_5R that promotes an enhanced FAD exposition to the solvent that modulates Cb_5R redox potential. A negative shift in Cb_5R formal potential was observed by cyclic voltammetry and associated to a higher tendency of the protein to be oxidized in the presence of its partner, correlating with a higher amount of Cb_5 reduced upon complexing.

2. Material and methods

2.1. Cloning of human soluble Cb_5R

A commercially available construct for CYB5R3 (GenScript; CloneID:OHu12696) with the inserted sequence of NADH-cytochrome b_5 reductase 3, isoform 2 was used as template for cloning into a pET-22b. Primers were designed to incorporate a thrombin cutting site to release the His-tag when needed in our experiments. Primers (0.1 μM) (FW- 5′-CAATGCCATGGCTATGAAGCTGTTCCAGCGC-3′ and RW-5′CCCAAGCTTGCCCCGTCCGAAGACGAAGCAGCGCTC-3′) and the commercial plasmid (20 ng) were added to the buffer ($MgCl_2$ 1.5 mM, dNTPs (0.25 mM) and enzyme (NZY proof DNA polymerase kit cat#MB14601, NZYtech), and PCR was used to prepare the insert. The adjusted PCR parameters were: 30 s at 95 °C, 30 s at 60 °C, 60 s at 72 °C for denaturation, annealing and extension steps. The selected restriction enzymes for insertion into the pET-22b plasmid multicloning site were *Nco*I and *Hin*dIII. Preparation and purification of the linearized plasmid and PCR product were performed following the instructions indicated by the supplier of the commercial restriction enzymes (Invitrogen). The insert was ligated into to the cut and dephosphorylated pET-22b plasmid. Dephosphorylation was performed following the instructions from the commercial supplier with a TSAP thermosensitive alkaline phosphatase (cat#M9910, Promega) and for ligation the Rapid DNA Ligation kit (cat#11635379001, Roche) was used. Transformation was accomplished and positive colonies were picked to grow in LB Ampicillin. Purification of plasmid was achieved to obtain a stock solution of the cloned plasmid for CYB5R3 (hCYB5R3) and stored at − 80 °C.

2.2. Expression and purification of human soluble Cb_5R

Competent Rosetta 2 (DE3) cells (Novagen) were transformed with the hCYB5R3 plasmid and plated on LB agar with ampicillin and chloramphenicol. One colony was picked up and grown for 12 h at 37 °C in 50 mL Terrific broth (TB) media supplemented with riboflavin 0.1 mM. TB media (5 mL) was transferred into 1 L of supplemented TB media and growth for 24 h at 37 °C. Expression induction was performed at 25 °C for 8 h, after addition of 0.5 mM of IPTG. Cells were pelleted by centrifugation at 5,000*g* for 25 min and suspended in 150 mL of buffer: 50 mM Tris, 150 mM NaCl, 1 mM PMSF and 1 mg/mL lysozyme at pH 7.4. Lysate was incubated for 2 h on ice. Suspension was frozen and thawed 3 times at 4 °C, 50 mM $MgCl_2$ in presence of deoxyribonuclease 0.05 mg/mL (Sigma, D-5025); the mixture was incubated at 4 °C for 1 h, with gentle shaking. Soluble fraction was separated from whole cell pellet by centrifugation at 20,000*g* for 30 min [4,24] and pH was adjusted to pH 8.1 after addition of imidazole 70 mM (protein conditions to bind to Ni Sepharose were previously tested as indicated in Supp. Fig. S1A). Soluble fraction was split into two aliquots of approx. 50 mL that were sequentially loaded after Cb_5R fraction elution onto a Ni Sepharose 6 Fast Flow (cat#17351802, GE Healthcare), 2.5 × 5 cm previously equilibrated with buffer A: 20 mM Tris, 150 mM NaCl, imidazole 70 mM, PMSF 1 mM, benzamidine 1 mM pH 8.1. After loading the sample, the column was washed with 300 mL of buffer B: 20 mM Tris, 150 mM NaCl, imidazole 70 mM, PMSF 1 mM, benzamidine 1 mM pH 7.5. After this step a yellow band was visible on the column. Protein was eluted in buffer C: 20 mM Tris, 150 mM NaCl, imidazole 250 mM, PMSF 1 mM, benzamidine 1 mM pH 7.5.Afterdialysis, sample was concentrated and loaded onto a Sephadex G75 column (GE Healthcare) previously equilibrated with Tris 150 mM pH 7.5. Purified Cb_5R was eluted pure, as indicated in Supp. Fig. S1A, and concentrated with an Amicon filter 10 kDa and glycerol 30% was