Biochimica et Biophysica Acta 1837 (2014) 1717–1729

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Cytochrome c_1 exhibits two binding sites for cytochrome *c* in plants

Blas Moreno-Beltrán [a], Antonio Díaz-Quintana [a], Katiuska González-Arzola [a], Adrián Velázquez-Campoy [b,c,d], Miguel A. De la Rosa [a], Irene Díaz-Moreno [a,*]

[a] Instituto de Bioquímica Vegetal y Fotosíntesis, cicCartuja, Universidad de Sevilla - CSIC, Avda. Américo Vespucio 49, Sevilla 41092, Spain
[b] Institute of Biocomputation and Physics of Complex Systems (BIFI)-Joint Unit BIFI-IQFR (CSIC), Universidad de Zaragoza, Mariano Esquillor s/n, 50018, Zaragoza, Spain
[c] Departamento de Bioquímica y Biología Molecular y Celular, Universidad de Zaragoza, Pedro Cerbuna 12, 50009, Zaragoza, Spain
[d] Fundacion ARAID, Government of Aragon, Maria de Luna 11, 50018, Zaragoza, Spain

ARTICLE INFO

Article history:
Received 17 March 2014
Received in revised form 23 July 2014
Accepted 26 July 2014
Available online 1 August 2014

Keywords:
Arabidopsis thaliana
Cytochrome *c*
Cytochrome bc_1 complex
NMR
Respirasome
Supercomplex

ABSTRACT

In plants, channeling of cytochrome *c* molecules between complexes III and IV has been purported to shuttle electrons within the supercomplexes instead of carrying electrons by random diffusion across the intermembrane bulk phase. However, the mode plant cytochrome *c* behaves inside a supercomplex such as the respirasome, formed by complexes I, III and IV, remains obscure from a structural point of view. Here, we report *ab-initio* Brownian dynamics calculations and nuclear magnetic resonance-driven docking computations showing two binding sites for plant cytochrome *c* at the head soluble domain of plant cytochrome c_1, namely a non-productive (or *distal*) site with a long heme-to-heme distance and a functional (or *proximal*) site with the two heme groups close enough as to allow electron transfer. As inferred from isothermal titration calorimetry experiments, the two binding sites exhibit different equilibrium dissociation constants, for both reduced and oxidized species, that are all within the micromolar range, thus revealing the transient nature of such a respiratory complex. Although the docking of cytochrome *c* at the *distal* site occurs at the interface between cytochrome c_1 and the Rieske subunit, it is fully compatible with the complex III structure. In our model, the extra *distal* site in complex III could indeed facilitate the functional cytochrome *c* channeling towards complex IV by building a "floating boat bridge" of cytochrome *c* molecules (between complexes III and IV) in plant respirasome.

1. Introduction

Cytochrome *c* (C*c*) was first described as a redox carrier in the mitochondrial electron transport chain transferring electrons from cytochrome bc_1 (Cbc_1) to cytochrome *c* oxidase (CcO), which are respectively known as complexes III and IV [1–3]. Since then, several other mitochondrial C*c* partners were being reported, including recently the flavoenzyme L-galactono-1,4-lactone dehydrogenase (GALDH) in plants; GALDH is required for the correct assembly of NADH dehydrogenase (or complex I) and catalyzes the terminal step of L-ascorbate biosynthesis [4–6]. Nowadays, C*c* is capable of interacting with several protein targets not only in the mitochondria under homeostatic conditions but also in the cytoplasm and even in the nucleus under programmed cell death conditions [7–10].

Nowadays, the organization and dynamics of the respiratory complexes in the inner mitochondrial membrane are a matter of debate, for which two different models have been proposed [11]. In the random collision models, all membrane proteins and redox components catalyzing electron transport and ATP synthesis are in constant and independent diffusional motion [12]. In contrast, the supramolecular organization models of membranes are based on specific interactions between individual respiratory complexes to form stable supercomplexes. In fact, the oligomerization in supercomplexes could enhance the respiratory chain activity through spatial restriction of electron carrier diffusion [13]. In particular, the complexes I, III and IV are, in their turn, the three basic components of the so-called respirasome, which is a multisubunit respiratory supercomplex composed of dimeric complex III and single copies of complexes I and IV [14]. Such a diversity in arrangement of the respiratory complexes may modulate the capability of cells in response to diverse environmental conditions [15] as the respirasome could quickly drive electrons from NADH to dioxygen in the presence of ubiquinone and C*c* [16,17].

In this context, C*c* channeling between complexes III and IV to shuttle electrons within the supercomplexes instead of carrying them by random diffusion has recently been proposed to occur in plants [18],

Abbreviations: AIRs, ambiguous interaction restraints; AU, analytical ultracentrifugation; BD, Brownian dynamics; C*c*, cytochrome *c*; Cc_1, cytochrome c_1; Cc_2, cytochrome c_2; Cbc_1, cytochrome bc_1 complex; CcO, cytochrome *c* oxidase complex; C*f*, cytochrome *f*; CD, circular dichroism; CSP, chemical-shift perturbations; ET, electron transfer; GALDH, L-galactono-1,4-lactone dehydrogenase; HADDOCK, High Ambiguity Driven Docking approach; HSQC, heteronuclear single-quantum correlation; ITC, isothermal titration calorimetry; MD, molecular dynamics; NMR, nuclear magnetic resonance; PCA, principal component analysis; pC*c*, plant cytochrome *c*; pCc_{red}, reduced plant cytochrome *c*; pCc_{ox}, oxidized plant cytochrome *c*; pCc_1, plant Cytochrome c_1; pCc_{1ox}, oxidized plant cytochrome c_1; pCc_{1red}, reduced plant cytochrome c_1; pRieske, plant Rieske

* Corresponding author. Tel.: +34 954489513; fax: +34 954460165.
E-mail address: idiazmoreno@us.es (I. Díaz-Moreno).

http://dx.doi.org/10.1016/j.bbabio.2014.07.017
0005-2728/

in agreement with the metabolic channeling model proposed by Kholodenko and Westerhoff [19]. Within this frame, channeling would imply the affinity of C*c* molecules towards consecutive binding sites, impairing its release to the bulk phase but providing a diffusion path between its redox partners. This idea demands a detailed structural and functional analysis of the interactions between plant C*c* (pC*c*) and its respiratory partners. The structure of the mammalian supercomplexes is consistent with a single C*c* molecule bound to one of the Cc_1 subunits of complex III, as found in the yeast X-ray structure [20,21]. Unfortunately, the behavior of pC*c* in supercomplexes remains obscure from a structural point of view as not even the electron tomography studies of plant respirasomes have revealed the location of pC*c* molecules [22].

In classical redox experiments, C*c* seems to interact with either complex III or IV by forming multiple transient encounters that enable high turnover rates and efficient electron transfer (ET), even though only a few conformations of the encounter ensemble lead to a productive complex [20,23,24]. Actually, the multiphasic kinetics observed in polarographic and spectrophotometric assays for the oxidation of reduced C*c* by beef complex IV can be fitted to a model with just one catalytic site. Such a simple model includes alternative binding conformations of the transient complex, with some of them being unable to transfer electrons but affecting the ET rate at the catalytic site [25,26]. Recently, the surface residues of human C*c* contacting bovine complex IV have been mapped by nuclear magnetic resonance (NMR) [27].

Non-ET conformations within the complex between beef Cbc_1 and human C*c* were also evidenced by steady state kinetic analysis, and so a binding model with more than one molecule of C*c* per molecule of Cbc_1 was proposed to explain the observed multiphasic kinetics [28]. In addition, a second cytochrome c_2 (Cc_2) binding site was proposed for the biphasic kinetic observed by plasmon resonance in the oxidized Cbc_1–Cc_2 complex from *Rhodobacter capsulatus* [29]. However, the crystal structure of the yeast Cbc_1–C*c* complex does only show a single C*c* molecule on the native complex III dimer, mainly driven by non-polar contacts [20,30,31]. Notwithstanding, extra electrostatically charged residues seem to be also involved, as inferred from molecular dynamics (MD) calculations [32] and experimental data with chemically modified C*c* [33,34].

The Cbc_1–C*c* interaction corresponds to a short-lived complex, whose lifetime and ET mechanism are strongly dependent on ionic strength [35,36]. Rhutenium-based techniques of photooxidation have been developed to study ET in the Cbc_1–C*c* complex from *Rhodobacter sphaeroides, Paracoccus denitrificans* and yeasts [36–40]. The resulting kinetic data suggest the formation of an encounter complex guided by long-range electrostatic forces. Interestingly, ruthenium kinetics have also shown that the acidic domain of the Cbc_1 complex from *R. sphaeroides*, which is analogous to the acidic subunits in eukaryotic bc_1 complexes, does not play any significant role in ET [40].

The nature of non-productive conformations within the Cbc_1–C*c* complex and their eventual role in the respirasome assembly remain unclear. In this work, we have investigated the interaction in solution between pC*c* and the globular domain of plant cytochrome c_1 (pCc_1), which has been made soluble by truncating its membrane-anchoring hydrophobic helix located at the extreme C terminus (Fig. 1). The transient complex between the two *Arabidopsis thaliana* heme proteins has been here analyzed by using NMR, isothermal titration calorimetry (ITC) and restraint docking calculations. Surprisingly, our experimental data reveal two well-defined binding sites for pC*c* at the pCc_1 surface, namely a non-productive (or *distal*) site with a long heme-to-heme distance (>30 Å) and a functional (or *proximal*) site with the two heme groups close enough (<8 Å) as to allow ET. Though not functionally active in redox reactions, the *distal* site at the pCbc_1 adduct could play a key role in channeling pC*c* molecules within the respiratory mitochondrial supercomplexes in plants.

2. Material and methods

2.1. Design of constructs and site-directed mutagenesis

A 0.72 kb synthetic DNA fragment encoding for the soluble domain of pCc_1 subunit (amino acids 64–265, GenBank ID: *834081*) fused to a standard N-terminal periplasmic signal peptide was amplified by PCR using the oligonucleotides pC*c*1_fw (5-GCGGGATCCAGGAGGTGACCA TG-3) and pC*c*1_rv (5-GCGCTCGAGTTCCATTTCCGGTTCCGC-3), containing *BamHI* and *XhoI* restriction sites (underlined), respectively. The reaction product was inserted in the pET28a(+) expression vector. Successful cloning was confirmed by automated sequencing. Site-directed mutagenesis was performed using pET_pC*c*1 as a template and the QuikChange II method (Stratagene, http://www.stratagene.com). The primers for PCR were pC*c*1_C10A_fw (5-TGGCCTGGAAGC GCCGAACTAT-3) and pC*c*1_C10A_rv (5-ATAGTTCGGCGCTTCCAGGC CA-3).

A 0.4 kb DNA fragment encoding for the pC*c* was cloned into a pBTR1 vector [41] by adaptamer technology. The pBTR1 contains the yeast hemelyase, needed for correct heme integration in *c*-type cytochromes. The oligonucleotides used to generate the adaptamer were pC*c*_adapt_fw (5-ATATATCCATGGCGTCATTTGA-3) and pC*c*_adapt_rv (5-TCTTGGTACC TCATCACGCGGT-3). pC*c* DNA insert was amplified directly from pCytA [42]. The pET_pCc1-C10A and pBTR–pC*c* constructs were verified by automated sequencing.

Protein expression and purification protocols have been included in Supplementary Material (Materials and Methods M1).

2.2. Absorption spectroscopy

Absorption spectra were recorded in the ultraviolet–visible (UV–VIS) range at 25 °C in a V-650 spectrophotometer (Jasco, http://www.jascoinc.com). A 1 ml quartz cuvette with a path length of 10 mm was employed. Circular dichroism (CD) spectra were recorded in the UV range (190–250 nm) at 25 °C in a J-815 spectropolarimeter (Jasco, http://www.jascoinc.com), equipped with a Peltier temperature-control system, using a 1-mm quartz cuvette. Protein concentration was 3 μM in 5 mM sodium phosphate buffer (pH 6.3). Twenty scans were averaged out for each sample. Secondary structure analysis was performed by using CDPRO software [43,44]. CDSSTR was used as reference database.

2.3. Analytical ultracentrifugation

Sedimentation equilibrium experiments of pCc_1 were performed at 20 °C in an Optima XL-A Analytical Ultracentrifuge (Beckman Instruments, https://www.beckmancoulter.com) with an AN50-Ti rotor. 80 μL aliquots of a 50 μM pCc_1 solution in 5 mM sodium phosphate buffer (pH 6.3) were analyzed at three successive speeds (13,000; 15,300 and 22,500 rpm). Absorbance was measured at 523 nm after 17 h to ensure that the equilibrium condition was reached. Baseline signals were determined taking a radial scan at 13,000 rpm after running the samples for 8 h at 45,000 rpm. Conservation of mass in the cell was checked in all experiments. Sedimentation velocity experiments were performed at 45,000 rpm and 20 °C with 400 μl samples loaded into double sector cells, using the same buffer and protein concentration as in the equilibrium experiments. Radial scans at 523 nm were taken every 10 min and the sedimentation coefficient distribution was calculated by least-squares boundary modeling of the sedimentation velocity data using the program SEDFIT [45]. The experimental coefficients were converted to standard conditions. The partial specific volume of pCc_1 (0.738 g/L), calculated from the amino acid composition, as well as the buffer density and viscosity were determined with the SEDNTERP program [46].