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Nitrate as a probe of cytochrome *c* surface: Crystallographic identification of crucial "hot spots" for protein–protein recognition

Matteo De March [a], Nicola Demitri [a], Rita De Zorzi [a], Angela Casini [b], Chiara Gabbiani [c], Annalisa Guerri [d], Luigi Messori [d], Silvano Geremia [a,*]

[a] Centre of Excellence in Biocrystallography, Department of Chemical and Pharmaceutical Sciences, University of Trieste, via L. Giorgeri 1, 34127 Trieste, Italy
[b] Dept. Pharmacokinetics, Toxicology and Targeting, Research Institute of Pharmacy, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands
[c] Department of Chemistry and Industrial Chemistry, University of Pisa, I-56126 Pisa, Italy
[d] Department of Chemistry, University of Florence, I-50019 Sesto Fiorentino (FI), Italy

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ABSTRACT

The electrostatic surface of cytochrome *c* and its changes with the iron oxidation state are involved in the docking and undocking processes of this protein to its biological partners in the mitochondrial respiratory pathway. To investigate the subtle mechanisms of formation of productive macromolecular complexes and of their breakage following the electron transfer process, the X-ray structures of horse heart ferri-cytochrome *c* (trigonal form) and ferro-cytochrome *c* (monoclinic form) were obtained using nitrate ions both as a crystallizing agent and an anionic probe for mapping the electrostatic surface changes. Both crystal forms contain three protein molecules in the asymmetric unit. In addition, a total of 21.5 and 18 crystallographically independent nitrate ions were identified for the trigonal and monoclinic forms, respectively. By matching all the six crystallographically independent protein molecules, 26 different anion–protein interaction sites were identified on the surfaces of cytochrome *c*, 10 of which were found in both forms, 8 present only in the oxidized and 8 only in the reduced form. The structural analysis of the electron transfer complexes, based on this new information, suggests a specific exit strategy for cytochrome *c* after formation of productive protein–protein complexes: a directional sliding mechanism for the electron shuttle on the surface of the redox partner is proposed to take place after the electron transfer process has occurred.

1. Introduction

c-Type cytochromes are ubiquitous heme proteins involved in the cyclic redox processes of photosynthesis and respiration [1–3]. The water soluble mitochondrial cytochrome *c* (cyt *c*) participates in the crucial energy transduction pathway for eukaryotic cells [3]. This essential component of the electron-transport chain is involved in a network of transient and highly efficient protein–protein complexes. The mitochondrial respiratory chain consists of four complexes: the specific role of cyt *c* is to accept an electron from complex III and to shuttle it to complex IV. The detailed molecular mechanism underlying this electron transduction process remains ambiguous and yet not fully characterized [3]. Past investigations led to the idea that the respiratory enzymatic protein complexes are randomly inserted in the mitochondrial membrane and functionally connected by smaller, freely diffusible carriers [4,5]. More recent observations based on single-particle electron microscopy suggested the existence of associations of the main protein complexes, named "supercomplexes" [6–8]. The 3D maps of two different supercomplexes—constituted by complexes III and IV—have been reported: the bovine $I_1III_2IV_1$ supercomplex, obtained at 20 Å resolution, consisting of complex I, a dimeric form of complex III and complex IV [7]; and the yeast III_2IV_2cyt *c* supercomplex obtained at 15 Å resolution, consisting of a dimeric form of complex III, two molecules of complex IV, bound to each III subunit, and cyt *c* [6]. These and other findings support the view of cyt *c* diffusing laterally along the membrane surface to assist the electron transfer process [3]. A detailed snapshot of the interactions between cyt *c* and complex III was obtained at atomic level by X-ray crystallography [9,10]. Unfortunately, no analog experimental information for complex IV is available in the case of eukaryotic systems, and only theoretical models obtained by computational docking for complexes of bacterial cyt *c* oxidase and cyt *c* [11–13], as well as the crystal structure of *Thermus thermophilus* caa3-type cyt *c* oxidase have been reported [14].

Complexes III and IV are not the only redox partners for cyt *c*. Cyt *c* is known to interact with some other redox proteins involved in side pathways, such as cyt b_5 and sulfite oxidase in animals and cyt b_2 and cyt *c* peroxidase in yeast [15]. In particular, the cyt *c*–cyt *c* peroxidase electron transfer complex has been extensively characterized through X-ray crystallography [16–19], computational methods [20,21] and

* Corresponding author. Fax: +39 040 558 3903.
E-mail address: sgeremia@units.it (S. Geremia).

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NMR techniques [22–27] as a suitable model system to study the electron transfer mechanism.

Furthermore, it was shown that cyt *c* is a critical component of the apoptotic cell-death program and that electrostatic interactions between cyt *c* and the mitochondrial inner membrane are important for its apoptotic function [28–30]. In particular, cyt *c* binds to anionic phospholipids of the inner membrane, primarily cardiolipin. During the early stages of apoptosis, this bond breaks down and cyt *c* is released from mitochondria [28–30]. The precise mechanisms regulating this release process are still unknown [3,8], although it has been shown that during apoptosis NO nitrosylates the heme iron of mitochondrial cyt *c*, binding to the proximal heme coordination site of the ferro-cyt *c*/cardiolipin complex [31], which is then rapidly released into the cytoplasm. An NMR-based structure shows that the interaction between cyt *c* and the antiapoptotic protein Bclxl is also driven by electrostatic interactions involving some Lys residues located on cyt *c* surface [32]. In computational studies [33], possible interaction surfaces between beef heart oxidase and horse heart cyt *c* were calculated providing evidence for a salt bridge-stabilized complex, primarily involving two Lys residues on the electron donor molecule, and a central hydrophobic region surrounding the heme cleft. This prediction is consistent with the results from site-directed mutagenesis, steady-state and rapid kinetics using bacterial oxidases and horse heart cyt *c* [34,35]. Additionally, the presence of surface Lys residues makes it possible to use cyt *c* in superoxide biosensors [36,37].

The midpoint redox potential is approximately +260 mV for all eukaryotic cyts *c*, while it is usually about 100 mV higher in chloroplast cyts c_6; it varies from +250 to +450 mV in evolutionarily related bacterial cyts c_2 [4]. Crystallographic studies on both oxidized and reduced forms of *c*-type cyts revealed only small structural changes, mainly regarding some adjustments around the heme moiety and few side chains on the surface [38–42]. A buried water molecule located at the heme binding pocket near to the iron-bonded Met has been detected in all X-ray structures of eukaryotic *c*-type cyts. In particular, the positional change of this water molecule is the most prominent structural difference observed with the change of the iron oxidation state in the eukaryotic cyt *c*; the hydrogen-bonding network involving this water molecule is strictly related to the fine modulation of the redox potentials in *c*-type cyts [43].

The formation of productive complexes between *c*-type cyts and their redox partners is generally driven by the occurrence of favorable electrostatic interactions between the positive surface of the electron shuttle and the negative surface of the membrane complex. The docking and undocking processes need to be regulated by conformational and electrostatic changes related to the change in the oxidation state of the iron ion [44,45].

Various anions are known to bind to the surface of cyt *c* [46–52]. Remarkably, an important study carried out by Sola and coworkers revealed that several anions, most of which of biological relevance, such as Cl^-, HPO_4^{2-}, HCO_3^-, NO_3^-, SO_4^{2-}, ClO_4^-, citrate^{3-} and oxalate^{2-}, bind specifically to the protein surface, often in a sequential manner as a result of the presence of multiple binding sites with different affinities; anion binding was shown to modulate importantly the redox potential of the iron center through a prevalent electrostatic mechanism [48]. In turn, crystallographic and NMR studies identified a few specific anion binding sites, corresponding to positively charged Lys [53]. Also, anion binding was shown to promote cyt *c* aggregation and formation of crystals. In particular, the importance of nitrate ions in the crystal growth of cyt *c* was previously stressed [54], but any structure has ever been deposited. The observed effect of the nitrate ions in facilitating cyt *c* crystallization with respect to other negative ions is probably due to their more precise localization and the possibility of being buried in relatively small sites shared by two protein molecules [54]. Neither ionic strength nor crystal-packing interactions have much influence on the overall structure of cyt *c* [45–51]. This observation suggests that crystal structures of cyt *c*, obtained in the presence of high concentrations of nitrate ions, are good models for the study of electrostatic interactions of the protein. The mapping of the cyt *c* hotspot regions involved in the electrostatic protein–protein or protein–membrane interactions may provide indications on the unknown but important mechanism of release of cyt *c* from the mitochondria during apoptosis [31]. Furthermore, they can provide new insight on the conformation variability of surface residues, for which a functional relevance can be predicted.

Based on these arguments, we present here a crystallographic study of horse heart ferri- and ferro-cyt *c* using nitrate ions as anionic probes to highlight the structural and electrostatic differences occurring between the different redox states. In this study, nitrate ions were used as a probe due to their smaller dimension with respect to other anions, allowing a more precise indication of the 'hot spots' of electrostatic charge on the surface of the protein. Furthermore, by comparing the 3D structures of the two major redox complexes of cyt *c* previously reported with the mentioned mapped surface of cyt *c*, the subtle mechanisms of formation of productive complexes and their breakage following electron transfer are analyzed and discussed.

2. Experimental

2.1. Crystallization and X-ray data collection

Horse heart cyt *c* was purchased from Sigma and used without any further purification. Crystals of cyt *c* were grown using the hanging drop technique from a 10 μL drop, containing 5 μL of protein solution (30 mg/mL) and 5 μL of reservoir solution (phosphate buffer 50 mM pH 7.4, $NaNO_3$ 3 M, saturated solution of $(NH_4)_2SO_4$ ca. 5 M). Red crystals of the oxidized form grew in 7 days. The reduction of the iron ion was performed by adding 10% dithionite in the well containing the reservoir solution before setting up the crystallization drop. Crystals of the reduced form grew in 10 days; they were paler and sharper than the oxidized ones. Crystallographic data have been collected at 100 K on

Table 1
Crystallographic data on horse heart cyt *c*. Data collection, processing and refinement statistics for the trigonal and monoclinic crystals of ferri- and ferro-cyt *c*, respectively.

	Ferri-cyt *c* (FeIII) PDB = 3O1Y	Ferro-cyt *c* (FeII) PDB = 3O20
Data collection and processing		
Space group	P 3_121	C 2
Unit cell parameters (Å)	a = b = 79.69, c = 89.25	a = 90.09, b = 52.03, c = 80.75, β = 123.07°
No of observations	261,279	58,077
No of reflections	31,828	21,667
R_{merge} (I)	0.087 (0.43)	0.116 (0.40)
Average I/σ(I)	21.5 (2.0)	9.0 (2.1)
Completeness %	100%	95.3%
Multiplicity	7.8 (4.3)	2.5 (2.0)
Refinement statistic		
Resolution range (Å)	69.01–1.75	65.09–1.90
R_factor	0.185 (0.264)	0.192 (0.228)
R_free	0.247 (0.384)	0.276 (0.303)
RMSD		
Bond lengths (Å)	0.021	0.020
Bond angles (°)	1.742	1.794
Model		
No of protein atoms	2478 (826 * 3)	2478 (826 * 3)
No of heme atoms	126 (42 * 3)	126 (42 * 3)
No of water atoms	523	365
No of atoms in the nitrate molecules	86 (4 * 21.5)	72 (4 * 18)
B factor statistics		
Main chain (Å^2)	14.4	12.7
Side chain (Å^2)	16.5	13.2
Solvent molecules (Å^2)	28.9	23.2
Nitrate ions (Å^2)	24.5	21.8

Values in parentheses are for the highest resolution shell.