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Structure–function relationships in human cytochrome *c*: The role of tyrosine 67

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ABSTRACT

Spectroscopic and functional properties of human cytochrome *c* and its Tyr67 residue mutants (i.e., Tyr67His and Tyr67Arg) have been investigated. In the case of the Tyr67His mutant, we have observed only a very limited structural alteration of the heme pocket and of the Ω-loop involving, among others, the residue Met80 and its bond with the heme iron. Conversely, in the Tyr67Arg mutant the Fe–Met80 bond is cleaved; consequently, a much more extensive structural alteration of the Ω-loop can be envisaged. The structural, and thus the functional modifications, of the Tyr67Arg mutant are present in both the ferric [Fe(III)] and the ferrous [Fe(II)] forms, indicating that the structural changes are independent of the heme iron oxidation state, depending instead on the type of substituting residue. Furthermore, a significant peroxidase activity is evident for the Tyr67Arg mutant, highlighting the role of Arg as a basic, positively charged residue at pH 7.0, located in the heme distal pocket, which may act as an acid to cleave the O–O bond in H_2O_2. As a whole, our results indicate that a delicate equilibrium is associated with the spatial arrangement of the Ω-loop. Clearly, Arg, but not His, is able to stabilize and polarize the negative charge on the Fe(III)–OOH complex during the formation of Compound I, with important consequences on cytochrome peroxidation activity and its role in the apoptotic process, which is somewhat different in yeast and mammals.

1. Introduction

It is now well recognized that cytochrome *c* (cyt c) plays different roles in various areas of the cell and a functional migration of cyt c may occur from the mitochondria to the cytosol, and even to the nucleus [1–3]. In mitochondria, cyt c is localized in the inter-membrane space in a loosely membrane-bound state and it is responsible for the primary function of electron transfer from complex III (coenzyme Q–cytochrome *c* reductase) to complex IV (cytochrome *c* oxidase). When cyt c acts as an electron shuttle, the heme iron is hexa-coordinated and no interaction of the heme with small ligands (such as H_2O_2, azide and CO) is possible. However, in mitochondria cyt c plays not only a physiological role as electron carrier, but has also a detoxifying function by regulating the production of ROS [4]. Approximately 15% of cyt c binds tightly to cardiolipin (CL) in the inner mitochondrial membrane [5,6], giving rise to a loosening of its tertiary structure and disruption of the Met80–Fe ligation. To date, in all the cytochrome *c* proteins that have been tested (both from yeast and from mammals) dissociation of Met80 from the sixth coordination position of the heme iron generates non-native cyt c conformers, which can give rise to functions that are different from those of the native protein [7,8]. Some of the non-native conformers have spectroscopic properties typical of the molten globule state [9]. In such a context, one can envisage that the partial unfolding of CL-bound cyt c facilitates the access of small molecules, such as H_2O_2, into the heme site of the protein, inducing significant cyt c peroxidase activity. In this state, cyt c catalyzes CL peroxidation, which triggers the onset of apoptosis by the release of cyt c from the inter-membrane space of the mitochondria into the cytoplasm [10,11]. In fact, in response to apoptotic stimuli, cells release cyt c from the mitochondria into the cytoplasm where it binds to apoptotic protease-activating factor 1 (Apaf 1). Upon binding cytochrome *c* and ATP, this protein forms an oligomeric apoptosome which binds and cleaves procaspase 9 protein, releasing its mature, activated form. Activated caspase 9

Abbreviations: Apaf 1, apoptotic protease-activating factor 1; Cyt c, cytochrome *c*; CL, cardiolipin; CD, circular dichroism; CT, charge transfer; CT1, charge transfer transition [$a_{2u}(\pi) \rightarrow e_g(d\pi)$]; YR, Tyr67Arg human cytochrome *c* mutant; YH, Tyr67His human cytochrome *c* mutant; RR, resonance Raman; 5c, 5-coordinate; 6c, 6 coordinate; HS, high spin; LS, low spin; WT, wild type.

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stimulates the subsequent caspase cascade that commits the cell to apoptosis. Although the release of cyt c into the cytosol is common for both mammalian and yeast cyt c, the lack of interaction with Apaf-1, and the consequent absence of a pro-apoptotic activity by the released cyt c in yeast, is not yet understood completely [12]. In fact, it is noted that the structural basis of the peroxidase activity of cyt c remains elusive, as underlined by the observation that the peroxidase activity of yeast cyt c decreases in the presence of CL, whereas that of horse cyt c increases significantly upon CL binding [12]. The conformational changes linked to the appearance of peroxidase activity in cyt c are modulated by ATP which, at the appropriate concentration, disrupts the cyt c–CL complex enabling the protein to refold into its native structure [13,14].

In this work, we have tested the hypothesis that the tyrosine residue at position 67, which is a highly conserved residue located within the helix 59–69 in all mitochondrial cytochromes c, modulates the structural properties of the heme cavity and the peroxidase activity of human cyt c. Indeed, H-bonds associated with Tyr67 connect two Ω loops (i.e., 40–57 and 71–85, see Fig. 1), regulating the dynamics of the Ω loop containing Met80 and helping to maintain the sulfur atom of Met80 bound to the heme iron [15].

The disruption of this H-bonding network would lead to a perturbation of the tertiary structure and, consequently, also affect the chemical and biological properties of the protein. Interestingly, the Tyr67-OH group is located in the distal portion of heme pocket at a distance from the heme iron comparable to that observed for the distal histidine in peroxidases, which acts as general base/acid in classic peroxidases [16]. Mutations at the Tyr67 position have been reported previously for yeast cyt c with the intent of inducing a higher peroxidase activity [17–19]. Herein, we have successfully designed and expressed Tyr67His (YH) and Tyr67Arg (YR) variants of human cyt c, with a similar aim of introducing into the heme distal pocket basic residues to switch the protein to conformers displaying higher peroxidase activities. The spectroscopic and functional properties of the two variants have been investigated and the heme environment further probed by studying the dynamics of the heme–H_2O_2, –CO and –azide interactions. The results highlight a crucial role for Tyr67 in shaping the distal portion of the heme cavity and the Ω-loop of human cyt c. The structural and functional effects depend dramatically on the type of substituting residue, such that Arg brings about the cleavage of the Met80–heme bond and an increased peroxidase activity, while His affects the heme pocket to a much lesser extent. The present results obtained for human cyt c will be discussed and compared with those previously obtained for cyt c of different origins.

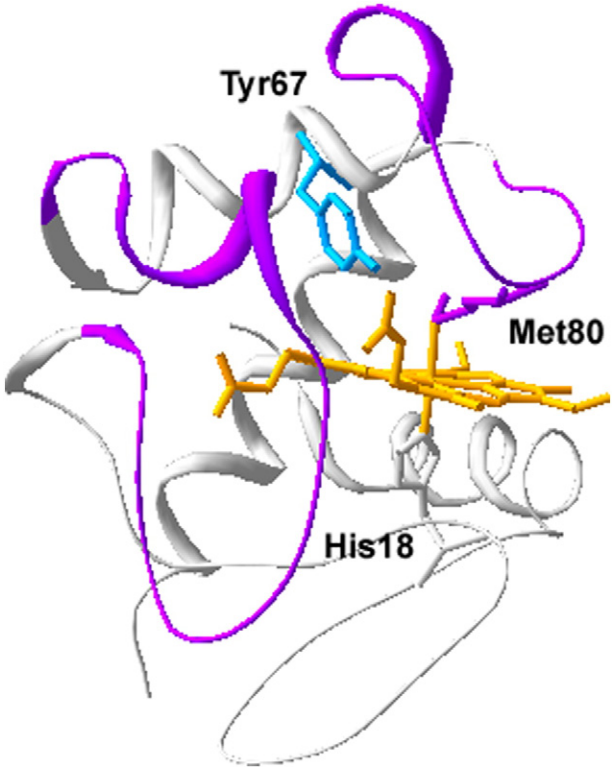

Fig. 1. Structure of human cyt c (PDB entry 3ZCF). The two Ω loops, residues 40–57 and 71–85, are shown in violet.

2. Materials and methods

2.1. Materials

Gaseous ^{12}CO and ^{13}CO were purchased from Rivoira and FluoroChem, respectively. Sodium dithionite was obtained from Fluka Biochemika. All the other chemicals were obtained from Aldrich, Steinheim, Germany. All chemicals were of analytical or reagent grade and were used without further purification.

2.2. Methods

2.2.1. Protein over-expression and purification of wild type human cyt c and mutants

The plasmid pBTR (humanCc) [20] was transformed into *Escherichia coli* strain BL21 (DE3). The human cyt c protein mutants were prepared by site-directed mutagenesis on the plasmid pBT (humanCc) using the QuikChange™ Site-directed Mutagenesis kit (Stratagene) following the manufacturers' protocol. The introduction of mutations into the nucleotide sequences was confirmed by sequence analysis. The procedures are outlined briefly in the Supplementary information.

Purification of the recombinant proteins was conducted as previously described [21]. After purification, the proteins were concentrated and stored in 0.1 M phosphate buffer at pH 7.0. The homogeneity of the wild type (WT) human cyt c and mutants (i.e., Tyr67His (YH) and Tyr67Arg (YR)) was determined by running SDS-PAGE. Recombinant WT human cyt c was used as a control in all the experiments described and reported herein as WT.

2.2.2. Sample preparation

The samples at pH 7.0 and 5.0 were obtained by 1:1 dilution of the purified proteins with 0.1 M phosphate buffer at pH 7.0 and 0.125 M citrate buffer at pH 4.3, respectively. The native protein at alkaline pH was obtained by 1:1 dilution with 0.15 M Na_2HPO_4 buffer (pH 12.1) followed by the addition of 4 μL of 1 M NaOH solution to achieve pH 12.6. Horse heart cytochrome *c* at pH 12 was obtained upon dissolution of the lyophilized protein in a 0.1 M phosphate buffer at pH 12.2.

To ensure complete oxidation, 1–3 μL of a 3 mM freshly prepared $K_4[Fe(CN)_6]$ solution were added to 40 μL protein samples.

Ferrous samples were prepared by addition of 2–3 μL of a freshly prepared sodium dithionite (10 mg/mL) solution to the ferric forms (40 μL) previously degassed with nitrogen.

The CO complexes were prepared by degassing the ferric protein solution by flushing firstly with nitrogen, then with CO or ^{13}CO and reducing the heme by addition of 2–3 μL of a freshly prepared sodium dithionite (10 mg/mL) solution.

Protein concentrations in the range 15–50 μM were used for the electronic absorption and resonance Raman (RR) samples. The protein concentration was determined on the basis of the molar absorptivity (ε) of 106 mM^{-1} cm^{-1} at 409 nm for the WT protein and the YH mutant [6,22] and of 121.7 mM^{-1} cm^{-1} at 405 nm for the YR mutant [23].

For the circular dichroism (CD) experiments, the samples (7 μM heme in the Fe(III) form both for WT and mutants) were prepared in 0.1 M phosphate buffer pH 7.0.