Journal of Molecular Catalysis B: Enzymatic 85–86 (2013) 187–192

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Unusual activation during peroxidase reaction of a cytochrome c variant

S. Águila[a,*], A.M. Vidal-Limón[a], J.B. Alderete[b], M. Sosa-Torres[c], R. Vázquez-Duhalt[a]

[a] Instituto de Biotecnología, UNAM, Apartado Postal 510-3, Cuernavaca, Mor. 62210, Mexico
[b] Facultad de Ciencias Químicas, Casilla 160-C, Universidad de Concepción, Concepción, Chile
[c] Facultad de Química, UNAM, Ciudad Universitaria, Coyoacán México, DF. 04510, Mexico

ARTICLE INFO

Article history:
Received 24 April 2012
Received in revised form 11 August 2012
Accepted 12 September 2012
Available online 19 September 2012

Keywords:
Compound 0
Cytochrome c
EPR
Heme
Molecular dynamics
Peroxidase activity

ABSTRACT

Cytochrome c has been extensively used as model of peroxidase reaction. The peroxidase activity and stability of a triple mutant CYC-3 (N52I, Y67F and M80A) were studied and compared to those of wild type protein (Wt-16). The CYC-3variant showed ten-fold increased activity in styrene oxidation. An intermediary specie that resembles to Cpd 0 (Fe^{III}—OOH) of peroxidases was detected through EPR measurements during the reaction of CYC-3 with H_2O_2. Using molecular dynamics (MD) it was found that mutations in CYC-3 induce conformational changes in the M80 loop promoting the rotation of D ring propionate toward heme iron and the inclusion of transient water molecules that could explain the formation of Cpd 0 intermediate. The effects of these conformational changes on the activity increase are discussed.

1. Introduction

Cytochrome c is a heme protein involved in the electron transport chain of mitochondria [1]. Under pro-apoptotic conditions, this protein plays an important role in the modification on mitochondria inner membrane, being a structural modulator through peroxidation of phospholipids as cardiolipin [2,3]. Thus, cytochrome c could be considered as a versatile protein, showing a role as antioxidant in certain conditions, even as scavenger of superoxide radical during oxidative burst [4,5].

This protein is able to catalyze peroxidase-like reactions, as oxidative dehydrogenation, S-oxidation, epoxidation, hydroxylation, aromatic oxidation and hydrogen peroxide (H_2O_2) dismutation [6]. Peroxidase-like activity of cytochrome c have been studied in different reaction media, either in solution or immobilized, showing that the activity can be modified by the support pore-size [7,8] or by the nature of support [9–12]. On the other hand, cytochrome c has been extensively used as biocatalyst in non-conventional reaction media [13,14], showing a relative stability in organic solvents. On opposite to peroxidases, cytochrome c has the heme prosthetic group covalently bonded to cysteine residues (C14 and C17), avoiding the heme loss in increasing concentrations of solvents [15–18]. In addition, due to its conformational flexibility, the activity could be increased by mutations around heme pocket [19], or even increased in unfolded states of the protein [20].

Despite the extensive studies on this protein, its peroxidase mechanism has not been elucidated. The task is not trivial, since there is evidence from EPR measurements, that cytochrome c cleave homolytically the O—O bond of H_2O_2 [21]. In addition, tridimensional structures of cytochrome c resolved from X-ray crystallography or nuclear magnetic resonance (NMR), evidences the absence of an acid–base general catalyst in the heme vicinity that could reorganize the hydrogen peroxide protons, as in the case of peroxidases.

In our previous work, a set of multiple-point mutations was generated [22]. We selected a double mutant (N52I, Y67F) as starting point because lack of complex hydrogen bond network imposed by water 166 (W166), which in turn altered overall redox potential and increased protein flexibility [19]. In addition, we included a third mutation in axial ligand methionine 80 (M80) in order to mimic the penta-coordinated environment of peroxidases.

The CYC-3 variant was characterized in terms of its reaction with H_2O_2 by UV/vis and EPR spectroscopic techniques. Finally, with the aid of molecular dynamic simulations, the possible conformational changes associated to the absence of M80 axial ligand are discussed.

* Corresponding author at: Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 2001, Cuernavaca, Morelos, Mexico. Tel.: +52 7773291619.
E-mail address: seaguila@ibt.unam.mx (S. Águila).

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http://dx.doi.org/10.1016/j.molcatb.2012.09.009

2. Experimental procedure

2.1. Chemicals

Hydrogen peroxide (H_2O_2), pinacyanol chloride and styrene were obtained from Sigma–Aldrich. Acetonitrile was purchased from American Bioanalytical Natick, MA, USA.

2.1.1. Site-directed mutagenesis and purification of recombinant iso-1-cytochrome c variants

The plasmid pCYC3, encoded for mutant CYC-3 (N52I, Y67F, M80A), was generated as previously described [22]. The derived plasmid was transformed into *Escherichia coli* C41strain for protein expression [23]. Mutant (CYC-3) and wild-type (Wt-16) proteins were expressed and purified according to García-Arellano et al. [24] with minor modifications. The proteins were purified trough an anion exchange chromatography using a High-S Econo-Pac cartridge (BioRad).

2.1.2. Activity assays and stability

Styrene oxidation was performed in a reaction mixture (1 mL) containing wild-type protein or iso-1-cytochrome c variant and 1 mM styrene in 60 mM phosphate buffer, pH 6.0, with 15% acetonitrile at 25 °C [7]. The reactions were started by addition of different concentrations of H_2O_2. For catalytic rate determination the reactions were stopped after 2 min by adding 1 mL of acetonitrile and the reaction extent was monitored by HPLC. All data were adjusted to Hill kinetic model.

Total turnover number (TTN) was estimated following the reactions for 30 min until no reaction was detected. The reaction progress was monitored by HPLC (Merck Hitachi) equipped with an L-6200A Intelligent pump, an UV-visible detector L-4200 model and a reverse-phase column (2.1 mm × 250 mm) RP-18 5 μm (Lichrocart). The mobile phase was acetonitrile:water 65:35 (v/v) at 0.5 mL/min. The decrease in the styrene concentration was determined by measuring the peak area at A_{254} and correlated to a standard curve. The specific activity was estimated by measuring the moles of oxidized substrate *per* mole enzyme *per* min at 25 °C. All reactions were done by triplicate, and the mean and standard deviations are reported.

Inactivation rate constants were estimated in independent assays of each cytochrome preparation. Wt-16 or CYC-3 variant (3 μM) were incubated in the presence of 1 mM H_2O_2 and the residual peroxidase activity at 0.16, 0.5, 1, 5, 10 and 20 min was determined as pinacyanol chloride oxidation ($\varepsilon_{603} = 82.35\ mM^{-1}\ cm^{-1}$) [6]. The reactions were carried out in 60 mM sodium phosphate buffer pH 6.1 at 25 °C. All measurements were carried out in a Perkin Elmer Lambda 25 UV/VIS spectrometer. Half-life time ($t_{½}$) was estimated from a first-order decay equation ($a_t = a_0\ e^{-kt}$).

2.1.3. EPR measurements

EPR spectra were recorded on a Bruker Elexys E-500 spectrometer equipped with an Oxford Instruments liquid Helium system at 9.8 GHz of potency. The protein samples (0.5 mM) were incubated in an ice-cold water bath at 276 K, prior to addition of 1 mM H_2O_2. Immediately after mixing, the samples were frozen in liquid nitrogen and stored at 77 K until recording. All EPR spectra were recorded between 4 and 20 K. The simulated spectra were obtained using standard simulation packages.

2.1.4. EPR spin trapping experiments

EPR spectra were recorded using a Jeol TE300 ESR spectrometer (Tokyo, Jp) operating at 9.44 GHz with a modulation frequency of 100 kHz. All reactions were performed in 60 mM sodium phosphate solutions (pH 6.1) treated with Chelex 100 resin to inhibit possible trace-transition metal catalysis. The reactions were performed at 25 °C in a total volume of 0.25 mL with 30 mM 5,5-dimethyl-N-oxide (DMPO) and 120 μM of each variant. All the reactions were started by the addition of 5 mM hydrogen peroxide.

2.1.5. Simulation method

Molecular dynamics (MD) simulation and analysis were carried out using the NAMD and VMD programs, respectively. The CHARMM 27 force field was used with CHARMM parameters for the heme group in cytochrome c being kindly shared by Luthey–Schulten's group [25]. The simulations were performed in the presence of explicit solvent with 994 water molecules (TIP3P). For all simulations, the starting protein coordinates were taken from X-ray structure of oxidized *Saccharomyces cerevisiae* iso-1-cytochrome c (PDB code: 1YCC). The mutated proteins were constructed from the same pdb with psfgen NAMD package. Truncated sphere boundary conditions were used. An NpT ensemble was performed; constant pressure (1 atm) and temperature were applied by weak coupling to an external bath temperature coupling relaxation time. A short-range cutoff of 8 Å was used. For long-range interactions (electrostatic and van der Waals) a cutoff range of 12 Å was applied. Every 10 fs pair list was updated. Integration time of 2 fs was used for production. Analysis of trajectory coordinates and energies were written to disk every 0.1 ps. For each variant, trajectories of 50 ns were obtained at 25 °C.

3. Results

The catalytic constants for peroxidase activity of CYC-3 variant and wild-type (Wt-16) proteins were determined for styrene oxidation, which is a typical peroxidase substrate (Table 1). The CYC-3 variant is ten-times more active and shows twice catalytic efficiency than the wild-type cytochrome. The total turnover number (TTN) is also four-times higher in the variant than in the wild-type protein at 25 °C. TTN could be related to both activity rate and stability. In the CYC-3 variant the high TTN values could be attributed to the increase in activity, because the stability ($t_{½}$) is the same in both preparations (Table 1).

In order to detect differences in the nature of intermediaries during the reaction of CYC-3 with H_2O_2, EPR experiments at helium temperature were performed using fast freeze techniques. In the resting state, CYC-3 variant showed high spin signals at $g = 6.06$, 5.86 and 4.38, while low spin signals are displayed at $g = 2.05$, 20.1 and 1.97. From the data integration of each EPR signal, important differences were detected. CYC-3 contains predominantly high spin iron species (∼80%), contrarily to Wt-16 [4,26]. An amino acid based radical was also found in CYC-3 at $g = 2.0046$ (Fig. 1A), which seems to be different that those found in Wt-16 ($g = 2.0032$). The broad signal of the radical found in CYC-3 was 20.7 G, meanwhile for Wt-16 was 8.7G [27]. This amino acid radical of CYC-3 resembles to a tryptophan-based radical, since simulation of an N atom of indole group fits well to experimental data (Fig. 1C).

Immediately after mixing CYC-3 with H_2O_2, a signal was detected in the low spin region of CYC-3 (Fig. 1A). This signal at $g_x = 2.2940$; $g_y = 2.1892$; $g_z = 1.9279$ resembles to low spin Fe^{III}—OOH (Cpd 0). The spectra simulation suggests the presence of this intermediate coupled to the formation of amino acid free radical. The simulation of the intermediate Fe^{III}—OOH, fits well to experimental data (Fig. 1D), and it is similar than those from other heme proteins such as myoglobin [28] and $P450_{cam}$ [29].

To corroborate the presence of a Cpd 0 like intermediate, spin trapping experiments were carried out. Detection of hydroxyl radical could suggest the homolytic cleavage of O—O bond, thus the formation or accumulation of Cpd 0 could not be accounted. DMPO