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Electron-balance during the oxidative self-inactivation of cytochrome *c*

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Abstract

During the oxidative self-inactivation of cytochrome *c*, four molecular events were detected and kinetically characterized; heme destruction, loss of the iron–sulfur coordination, protein multimerization, and protein inactivation. Electron transfer balance from the four molecular events account for 100% of the electrons transferred in the first minute of protein incubation with hydrogen peroxide. However, an unidentified electron source started donating electrons after the first minute and reaches 23% of the transferred electrons after 10 min incubation. Thus, the electron transfer kinetics and its balance allowed us to detect a non-considered electron transfer and free radical formation.

Keywords: Cytochrome *c*; Oxidative inactivation; Peroxidase activity; Modeling; Molecular events

1. Introduction

The industrial application of enzymes is growing as consequence of the demand for clean and low energy-requiring new processes. Peroxidasic activity has interesting potential applications in many different fields. However, present and future commercial uses have been limited, mainly, by the low stability of peroxidases in the presence of their natural substrate, hydrogen peroxide. All hemeproteins, including peroxidases, are inactivated in the presence of catalytic concentrations of hydrogen peroxide [1]. This inactivation process is especially important in the absence of reducing substrate and its mechanism has not been fully elucidated [1]. The peroxidasic activity is originated by the production of a free radical in the active site (heme). Electrons are subtracted then from the substrate in two one-electron steps for productive transfers. However, these electrons can also be subtracted by unproductive or inactivating transfers from amino acid residues or from the iron–porphyrin prosthetic group [1]. The molecular mechanism underlying the hydrogen peroxide-mediated inactivation of hemeproteins presents an extraordinary complexity arising from the fact that a multitude of reactions may occur subsequent to the hydroperoxide reaction with the heme [1].The non-enzymatic protein cytochrome *c* is able to catalyze peroxidase-like reactions in the presence of an electron acceptor, such as hydrogen peroxide or an organic hydroperoxide [2]. Site-directed mutagenesis and chemical modifications of cytochrome *c* have been performed to enhance its activity on polycyclic aromatic hydrocarbons (PAH's) [3–5], thiophenes and organosulfides to form sulfoxides [6,7], and on the recalcitrant asphaltene fraction from petroleum [8].

Cloning and expression of peroxidases has showed only limited success and protein engineering remain difficult [9,10]. Cytochrome *c* is a non glycosylated small protein with available crystallogarphic structure, easy to be expressed in *E. coli* for protein engineering, and able to perform peroxidase reactions. In this work, we have used cytochrome *c* as the simplest peroxidase model to study the molecular events occurring during the oxidative inactivation in the presence of hydrogen peroxide and in the absence of reducing substrate. The kinetic of different molecular events were determined and an electron transfer balance was obtained.

2. Experimental

Horse heart cytochrome *c*, pinacyanol chloride, β-mercaptoethanol and hydrogen peroxide were obtained from

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Sigma-Aldrich (St. Louis, MO). Buffer salts and organic solvents were purchased from J.T. Baker (Phillipsburg, NJ).

Peroxidase activity was determined as the decolorization of 5 μg ml^{-1} of pinacyanol chloride ($\varepsilon_{603\,nm}$ = 82,350 M^{-1} cm^{-1}) in 100 mM phosphate buffer pH 6.1 containing 15% acetonitrile [11]. The reaction was started by adding 1 mM hydrogen peroxide, and then the absorbance at 603 nm was monitored in a Beckman 650 spectrophotometer (Fullerton, CA). The hydrogen peroxide concentration in the stock solution was determined spectrophotometrically at 240 nm (ε_{240} = 36,000 M^{-1} cm^{-1}) [12].

Soret bleaching was evaluated spectrophotometrically at 408 nm. Horse heart cytochrome *c* (10 μM) preparation was incubated in the presence of 1 mM hydrogen peroxide in a 60 mM phosphate buffer, pH 6.1, and the absorbance data were recorded from 0 to 12 min every minute and afterwards, every 3 min up to 83 min.

Loss of the axial coordination bond between the heme iron and Met80 was evaluated as the fate of the 695 nm band using the net absorbance calculation method from the spectrophotometer options, in which the absorbance is calculated from a spectrum valley–valley baseline.

For the determination of the multimerization rate, a horse heart cytochrome *c* preparation (10 μM) was incubated in 2 ml buffer in the presence of 1 mM hydrogen peroxide. Aliquots of the mixture (0.1 ml) were removed before the addition of the peroxide and afterwards, every 5 min up to 45 min and immediately denatured by boiling in one volume of 2× SDS sample buffer (125 mM Tris–HCl pH 6.8, 1% SDS, 20% glycerol, and 10% β-mercaptoethanol). All samples were simultaneously separated in a 15% PAGE gel, which was further stained using the non-ammoniacal silver staining method and dried [13]. The relative amount of the monomer protein was digitally estimated after scanning of the gel using the NIH Image software (Wayne Rasband, NIH, USA).

Kinetic equations of all molecular events were obtained by feeding the data into the EnzFitter program from Biosoft (Ferguson MO). The aim was to have a mathematical model in order to estimate the electron flow rate for each molecular event during the first minutes of the oxidative inactivation. Thus, the best fitting was used and the equation derived with respect to time to obtain the process rate at any time.

3. Results and discussion

The loss of catalytic activity of cytochrome *c*, in the presence of 1 mM hydrogen peroxide and in the absence of reducing substrate, was determined by measuring the residual peroxidase activity. Experimental data fitted into a double exponential kinetics with a good correlation coefficient (Table 1). The half-life time for horse heart cytochrome *c* was 1.45 min. This value is lower than for chloroperoxidase (115 min), horseradish peroxidase (33 min), lactoperoxidase (20 min), manganese peroxidase (8.5 min), and lignin peroxidase (6.2 min), but higher than for versatile peroxidase (1.3 min) in the same conditions. Chloroperoxidase showed the highest stability against hydrogen peroxide inactivation, however this enzyme is able to perform significant catalase activity and thus this result should be taken cautiously.

Table 1
Constant values of first order or double exponential equations of the molecular events during the oxidative inactivation of cytochrome *c*

Molecular event	A_1	k_1 (min^{-1})	A_2	k_2 (min^{-1})	r^2
Activity loss	0.339	0.0305	0.661	0.9117	0.997
Heme destruction	0.735	0.0189	0.265	0.1427	0.999
Coordination loss	1.000	0.0570	–	–	0.988
Multimerization	1.000	0.0479	–	–	0.984

Heme bleaching is another common molecular event associated with the oxidative inactivation of cytochrome *c* and peroxidases. Cytochrome heme destruction was monitored as the absorbance of Soret band at 408 nm and fitted in a double exponential decay kinetics. Despite peculiarities among different hemeproteins, a consensus mechanism includes the formation of a peroxyiron(III)porphyrin radical species (compound III) [1]. Compound III is not part of the peroxidase cycle, but is produced under excessive exposure to oxidative species and in the absence of reducing substrate. The peroxyl radical of compound III is placed very close to the porphyrin ring, thus it is reasonable to suspect that once formed, this reactive species would be potentially able to reach the tetrapyrrole structure and to oxidize the porphyrin moiety. Heme bleaching has been observed in ascorbate peroxidase, hemoglobin, myoglobin, horseradish peroxidase, microperoxidase 11, cytochrome P450, chloroperoxidase, peroxidase from *Coprinus cinereus*, as well in the non-peroxidasic hemeprotein prostaglandin H synthase [1]. Heme is particularly susceptible to oxidative attack at the meso positions to form biliverdin ring systems, an open chain tetrapyrrolic structure [14,15]. Iron release from heme degradation occurs during oxidative inactivation of cytochrome *c* [16]. Moreover, variants of yeast cytochrome constructed by site-directed mutagenesis showed no heme destruction with 1 mM hydrogen peroxide, even following loss of catalytic activity [16]. This result suggests that heme destruction and catalytic inactivation are independent processes. Disruption of a hydrogen bonding network through an internal water molecule (Wat112) seems to prevent the formation of a protein radical involved in the heme destruction (Fig. 1), but presumably not in reactions leading to the protein inactivation [16].

The fate of axial Fe–methionine coordination during the inactivation process was followed through the measurement of 695 nm absorption band, which is characteristic of the coordination of the methionine sulfur to heme iron [17,18]. In the presence of 1 mM hydrogen peroxide, the loss of the iron–sulfur coordination in cytochrome *c* showed a first order

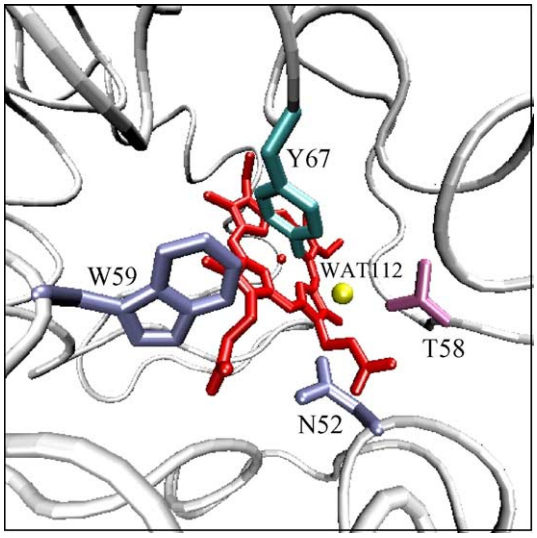

Fig. 1. Active site of horse heart cytochrome *c*.

kinetics with a half-life time of 12.2 min. Thioether methionine bond is a preferred target for free radical formation due to its low ionization potential (1.86 eV) [19]. Methionine sulfoxide cytochrome *c* was prepared by photooxidation mediated by methylene blue [20] showing significant peroxidasic activity (data not shown). Thus, the electron transfer from the methionine sulfur atom to disrupt the iron coordination bond seems to be a parallel and independent electron transfer process that does not derive into protein inactivation.

Another molecular event detected during the oxidative inactivation of horse heart cytochrome *c* is the protein polymerization. Cytochrome dimers, trimers and higher polymers were detected by gel electrophoresis. No polymerization could be found with a cytochrome variant without tyrosine residues [16], suggesting that these residues are involved in the polymerization molecular event. cytochrome *c* has four tyrosines, three of them (Tyr 48, Tyr 74 and Tyr 97) are placed on the protein surface, while Tyr 67 is embedded into the protein. A long-range electron transfer could be involved to form free radicals on the superficial tyrosines leading to the protein polymerization.

Substrate oxidation in cytochrome *c*, and heme-peroxidases as well, is a naturally imperfect process in which, to some extent, the porphyrin and the protein become alternative electron sources. Oxidative protection by the substrate comes from the favorable partition of the oxidative equivalents to the substrate, and protein disruption through oxidative damage arises as the ultimate consequence of the usage of alternative electron abstraction pathways.

Four molecular events were detected during the peroxide-mediated inactivation and kinetically characterized (Table 1). In order to analyze of the electron flows during this process, electron flow rates from hydrogen peroxide to the different molecular events were estimated as the derivative (de^-/dt) of each kinetic model:

$$\frac{de^-}{dt} = n(-k_1 A_1 e^{-k_1 t})$$

$$\frac{de^-}{dt} = n(-k_1 A_1 e^{-k_1 t} + -k_2 A_2 e^{-k_2 t})$$

where "*n*" is the number of electrons involved in the oxidation event. Five electrons ($n = 5$) are considered to be involved in the heme oxidation to biliverdin, free iron, and CO, as in the case of the heme oxygenase mediated reaction [21]. Two electrons ($n = 2$) are needed to oxidize methionone to methionine sulfoxide [22] for the loss of iron coordination. Tyrosine polymerization is carried out after tyrosyl radical formation by one-electron withdrawal, and two tyrosyl radicals form a covalent carbon–carbon bond [23]. Thus, two electrons per tyrosine–tyrosine bond are expected to be involved in the protein polymerization through superficial tyrosines. Only three from four tyrosines in cytochrome *c* are superficial and then able to react with another superficial tyrosine, so a value of $n = 6$ was considered in this molecular event. Finally, we have considered cytochrome inactivation as a two-electron process for the oxidation of one essential amino acid residue [22]. The specific electron flow rate was estimated by dividing these equations by the remaining active cytochrome *c* molecules at different times. The results are shown in Fig. 2. It is important to point out that the oxidative inactivation is a mechanism-mediated process in which only active molecules are able to transfer electrons from the different electron sources.

The initial electron flow rates, in mol of electrons per mol of active cytochrome per minute (e^- mol mol^{-1} min^{-1}) are: Heme destruction 0.26, coordination loss 0.11, polymerization 0.29, and protein inactivation 1.22 e^- mol mol^{-1} min^{-1}. Thus, in the presence of 1 mM hydrogen peroxide and in the absence of reducing substrate, the cytochrome molecule generate total electron transfer at a rate of 1.88 e^- mol mol^{-1} min^{-1}, from which 65% comes from the inactivating amino acid residue and only 14% comes from the iron–porphyrin group. The electron flow rate from the inactivating event decreased as the protein is incubated, while in the other three molecular events the electron flow rates increased.

As in peroxidases, the oxidative inactivation of cytochrome *c* is a mechanism-based process in which the protein should be active to promote its self-inactivation. On the other hand, the capacity of electron transfer of active cytochrome molecule may be considered constant until inactivation. From our model this capacity is 1.88 e^- mol mol^{-1} min^{-1}. The sum of all the molecular events recorded, and called "total balance" in Fig. 2, shows that in the first minute the four considered molecular events are enough to complete the electron transfer balance. However, starting from the second minute of incubation there is an unbalance. This unbalance could be due to a new electron transfer pathway from a non-considered electron source, or oxidized molecule moiety, in our model. The origin of electrons of this unknown pathway could be any low ionization potential residue. Tryptophan 59 seems to be a good candidate

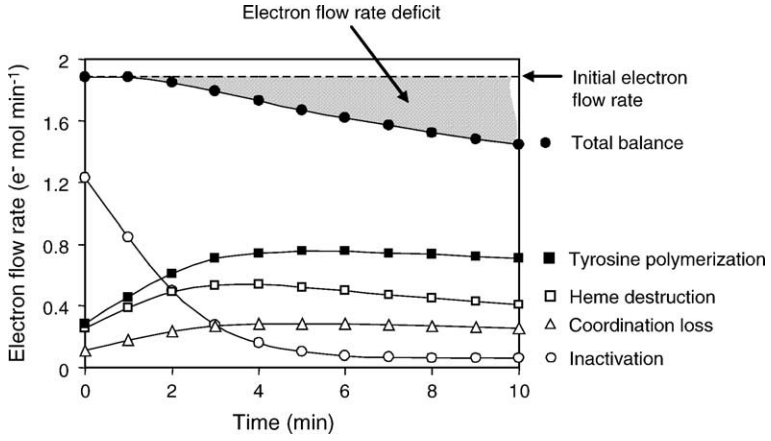

Fig. 2. Electron flow rate balance during the oxidative auto-inactivation of cytochrome *c* in the presence of 1 mM hydrogen peroxide. Four molecular events were detected and modeled; heme destruction, loss of the iron–sulfur coordination, protein multimerization, and protein inactivation. The total balance is the sum of all four kinetics.

for two reasons: The low ionization potential (IP ≈ 7.76 eV for indole [15]), and the residue position in the protein that is very close to the heme (H–O 1.76 Å or N–O 2.78 Å) forming a hydrogen bond with the heme propionate [24] (Fig. 1). The formation of a Cβ-based hydroxyl tryptophan radical has been detected in lignin peroxidase, which is auto-catalytically produced [25].

From the electron transfer balance during the oxidative self-inactivation of cytochrome *c*, we can conclude, so far, that the four molecular events account for 100% of the electrons transferred in the first minute of protein incubation with hydrogen peroxide. However, an unidentified electron source started donating electrons after the first minute and reaches 23% of the transferred electrons after 10 min incubation. Finally, the kinetics of electron transfer and its balance allowed us to detect a non-considered electron transfer forming a free radical. As far as we know, this is the first time that an electron transfer balance is calculated from experimental data during the oxidative inactivation of a protein with peroxidase activity. Elucidation of the complex electron transfers and free-radical formations, doubtless, will contribute to the stabilization of cytochrome *c* and peroxidases against hydrogen peroxide, and this might be achieved by replacing low-ionization potential residues in order to eliminate alternative free-radicals transfer pathways.

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