Cryogenic absorption spectra of hydroperoxo-ferric heme oxygenase, the active intermediate of enzymatic heme oxygenation

Ilia G. Denisov[a,1], Masao Ikeda-Saito[b,c], Tadashi Yoshida[d], Stephen G. Sligar[a,*]

[a] *Departments of Biochemistry, Chemistry, and College of Medicine, University of Illinois, 116 Morrill Hall, 505 S. Goodwin Avenue, Urbana-Champaign, IL 61801, USA*
[b] *Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, OH 44106-4970, USA*
[c] *Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Katahira, Aoba, Sendai 980-8577, Japan*
[d] *Department of Biochemistry, Yamagata University School of Medicine, Yamagata 990-9585, Japan*

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Abstract Using radiolysis with ^{32}P enriched phosphate as an internal source of ionizing radiation, the formation of hydroperoxo-ferric complex from oxy-ferrous precursor with a high yield was monitored at 77 K in heme oxygenase (HO) by means of optical absorption spectroscopy. Well-resolved absorption spectra (maxima at 421 nm, 530 nm, 557 nm) of hydroperoxo-ferric intermediate of this heme enzyme were measured in 70% glycerol/buffer frozen glasses. After annealing at 210–215 K this complex converts to the product complex, α-*meso* hydroxyheme-HO. No heme degradation products were formed in control experiments with ferric HO or other heme proteins.

Key words: Absorption spectroscopy; Cryogenic reduction; Heme oxygenase; Reaction intermediates; Radiolysis; Reactive oxygen species

1. Introduction

The cytochromes P450 [1], nitric oxide synthases [2], heme oxygenases (HO) [3,4], and some other heme enzymes [5] are able to activate molecular oxygen and to form a redox active center. Other mononuclear heme enzymes (peroxidases, catalases) use instead the reduced form of oxygen, hydrogen peroxide [6,7]. All these enzymes contain ferric protoporphyrin IX, and form the same highly reactive intermediate, hydroperoxo-ferric heme complex (FeP-OOH), at the obligatory step of the catalytic cycle towards the high valent oxoferryl heme complexes [8,9]. The pivotal role of this intermediate in oxygen activation catalysis is becoming widely recognized [10,11]; however, there is still not enough information about its structure and properties because of the high reactivity and low stability of FeP-OOH enzyme complexes [12–14] as well as of porphyrin model compounds [15]. Up to now the isolation of FeP-OOH intermediates in heme enzymes was achieved mainly through radiolytic reduction of oxy-ferrous precursors at 77 K [16–26].

Heme oxygenases catalyze the degradation of hemin and may play an important role in the defensive mechanism against oxidative stress [4]. The catalytic mechanism of HO includes stepwise formation of hydroxyheme, verdoheme, and α-biliverdin with concomitant release of CO and free iron. Unlike in many other heme enzymes, the FeP-OOH complex in HO is not an intermediate on the path towards the ferryl-oxo porphyrin compound, but is itself the active complex at the first step of HO catalytic cycle [3,4]. Recently, it was prepared using cryoradiolysis, and characterized in detail by means of electronic paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) spectroscopy [23]. The primary product of heme catabolism, the α-*meso* hydroxyheme, was formed in this system when annealed above 200 K. However, the oxy-ferrous precursor and, possibly, some product species in this reaction are diamagnetic and could not be followed by EPR [23]. In this letter we use optical spectroscopy at low temperatures and ^{32}P phosphate as an internal radiation source to monitor the gradual formation of FeP-OOH complex from oxy-ferrous HO with the increase of absorbed dose, and to measure the absorption spectrum of this intermediate.

2. Materials and methods

2.1. Materials

The wild type truncated water-soluble recombinant rat HO-1 (denoted HO) was expressed, purified, and reconstituted with hemin according to published procedures [27]. ^{32}P enriched phosphate (aqueous solution, 50 mCi/ml activity, isotope half-life 14.26 days) was purchased from Amersham Pharmacia Biotech. All other chemicals (spectrophotometric grade) were obtained from Sigma-Aldrich.

2.2. Sample preparation and cryogenic UV-VIS spectroscopy

Oxygenated HO was prepared by bubbling of oxygen or air through the solution of ferrous HO in aqueous glycerol phosphate buffer (0.1 M, pH 7.0). Ferrous HO was obtained by reduction of ferric HO with dithionite in an anaerobic chamber and subsequent anaerobic chromatography (G-25 column) to remove excess dithionite. Absorption spectra were measured using a Cary 3 UV/Vis spectrophotometer in methacrylate semi-micro cells from Fisher used with a pathlength 4.3 mm. Cryogenic measurements were done in a home-made cryostat as described [25].

*Corresponding author. Fax: (1)-217-265 4073.
E-mail address: s-sligar@uiuc.edu (S.G. Sligar).

[1] On leave from the Institute of Macromolecular Compounds, Russian Academy of Sciences, St. Petersburg, Russia

Abbreviations: HO, heme oxygenase; FeP-OOH, hydroperoxo-ferric heme complex; EPR, electronic paramagnetic resonance; ENDOR, electron nuclear double resonance

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2.3. Cryogenic reduction

Radiolytic reduction at 77 K was achieved by γ-irradiation of the samples using the ^{60}Co source (Notre Dame Radiation Laboratory, Notre Dame University, South Bend, IN, USA) with the dose rate 11 kGy/hour for 4 h. Alternatively, the ^{32}P enriched phosphate was used as an internal source of ionizing radiation as we described in detail earlier [24]. During dose accumulation with ^{32}P, the reduction of oxy-HO and formation of the FeP-OOH complex was monitored by absorption spectroscopy at 77 K. To do this, the background absorption of the trapped electrons was photobleached prior to spectroscopic measurements by illumination with the white light, using the filter absorbing at wavelengths 450 nm and shorter, to prevent possible photolysis in the Soret region. The multiple photobleaching in the course of dose accumulation significantly improves the yield of the radiolytic reduction (see the discussion below).

3. Results and discussion

The inelastic (Compton) scattering of the high energy particles on the solvent molecules generates electrons and cation radicals. In the frozen solvent matrix at 77 K the electrons may be trapped in the potential wells created by solvent molecules [28]. The products of radiolysis are thus immobilized and accumulated in the solvent matrix, although they may later recombine with further generated radiolytic electrons, which retain mobility before they are also trapped. The latter reactions result in saturation behavior of the radiolytic yield at a higher radiation dose [29], which is usually below 40% in metalloprotein studies [30]. This limitation is due to accumulation of the positive holes with the increase of the dose absorbed by the sample and the concurrent reactions of these holes with the radiolytic electrons. The estimated yield of such holes is about 1 mM per 10 kGy of absorbed irradiation dose (as compared to 20–30 μM concentration of the heme protein); hence, at higher doses they compete for the new electrons with almost 100% efficiency, and further irradiation above the doses of 20–30 kGy does not result in formation of new product.

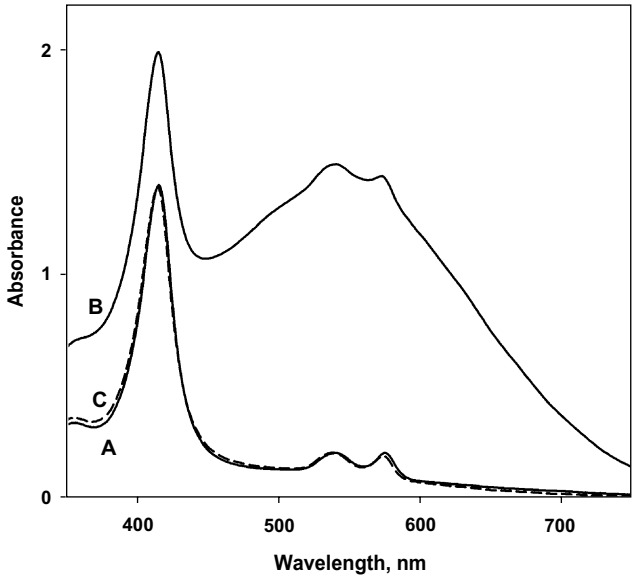

Fig. 1. Spectra of oxy-HO in the presence of ^{32}P phosphate (0.1 M phosphate, pH 7.0, 70% glycerol, 10 mCi/ml activity, $T = 80$ K). A: 30 min (50 Gy accumulated dose). B: 21 h (2.2 kGy accumulated dose). C: The same as B, after 8 min photobleaching at 77 K. See [24] for other experimental details.

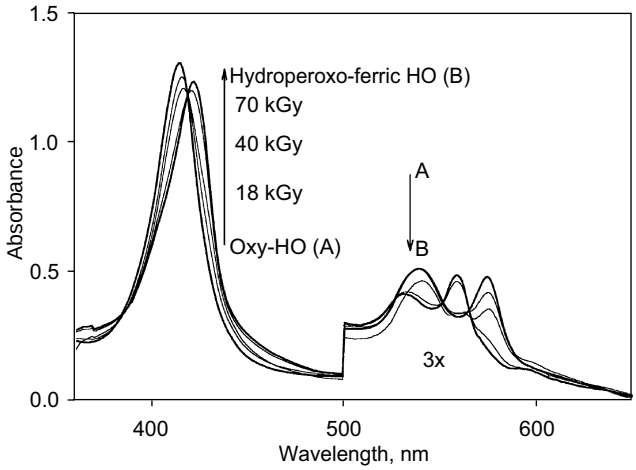

Fig. 2. Conversion of oxy-HO to hydroperoxo-HO with dose accumulation from ^{32}P phosphate (0.1 M phosphate, pH 7.0, 70% glycerol, $T = 80$ K). A: initial spectrum of oxy-HO before irradiation. B: Spectrum of hydroperoxo-HO, calculated from the experimental spectra measured at different irradiation doses.

To overcome this difficulty and to increase the yield of radiolytic reduction, we used multiple photolysis of the trapped electrons, which stimulated their recombination with positive holes. In Fig. 1 the absorption spectra of oxy-ferrous HO before and after incubation for 20 h in the presence of ^{32}P (estimated dose 2.2 kGy) are shown. The broad band corresponding to the absorption of the electron trapped in aqueous glycerol [31] is the dominating feature before photobleaching (Fig. 1B). As a result of photobleaching, most of the trapped electrons disappear through the recombination with the immobilized positive holes, as well as through the reduction of other acceptors, and the spectrum of the heme enzyme can be clearly seen (Fig. 1C). After this process is completed, the remaining concentration of positive holes is minimized, and further irradiation produces much more radiolytically reduced target species than without such treatment.

The cryoradiolysis of heme proteins using γ-rays [16–19,22–

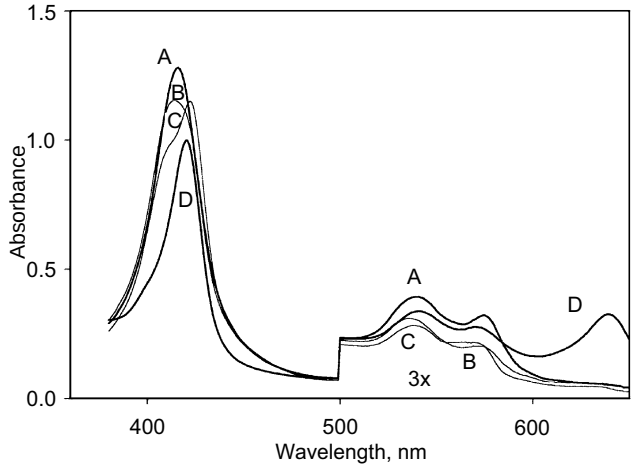

Fig. 3. Annealing of irradiated oxy-HO at 210 K (A),215 K (B), 220 K (C), and after thawing at 270 K (D). The sample was γ-irradiated using a ^{60}Co source with the total estimated dose of 44 kGy. The calculated reduction yield was 34%, the rest of the enzyme remained in oxy-ferrous form.

25], X-rays [18,20,21], or ^{32}P phosphate (β-rays) [24] results in a one-electron reduction of the heme iron. We have measured the spectra of cryoreduced ferric HO at 77 K and after annealing at different temperatures in the range 80–220 K (not shown), and used them as a reference, including the direct control of possible non-specific heme degradation during radiolysis. No sign of heme degradation was detected after annealing of radiolytically reduced ferric HO. Instead, the enzyme was converted to Fe^{2+}-CO form, due to reduction by accumulated organic radicals and subsequent binding of CO, formed as the product of glycerol radiolysis [32–33]. This was also seen in our cryoradiolytic studies of cytochromes P450 [24–25] and peroxidase [26].

Fig. 2 shows the gradual disappearance of oxy-HO and formation of hydroperoxo-ferric HO at 77 K with radiation dose accumulation. The final yield of FeP-OOH intermediate is estimated as 85% ± 5% at maximum dose 70 kGy. The spectrum of this intermediate, calculated from the data in Fig. 2, is shown as Fig. 2B. The Soret band is shifted to 421 nm and has an amplitude slightly lower than that at the spectrum of oxy-HO (maximum at 416 nm). The Q bands at 530 nm and 557 nm are blue-shifted and less separated (915 cm^{-1} difference between the maxima), as compared to oxy-HO (540 nm and 575 nm, 1130 cm^{-1} difference). To our knowledge, the spectrum (Fig. 2B) is the first example of a well-resolved directly measured optical spectrum of an isolated FeP-OOH enzyme complex with histidine as a proximal ligand. The main features of this spectrum are in a good agreement with the transient absorption spectra, measured in fast mixing studies of the reaction of H_2O_2 with heme proteins [12–14], and with our recent results obtained for the same complex in horseradish peroxidase [26].

Annealing of the samples of hydroperoxo-ferric HO up to 200 K did not result in formation of new species (Fig. 3A). At 212–215 K the spectra undergo more significant changes, the primary FeP-OOH intermediate begins to disappear, and the transient species with the weaker Soret band at 406 nm is formed (Fig. 3B). This spectrum is characteristic for the ferric α-*meso* hydroxyheme, the product of heme oxygenation by HO. Once formed, it is quickly reduced by products of glycerol radiolysis and then binds CO (Fig. 3C). At higher temperatures, after thawing, the rest of the heme is also converted to the Fe^{2+}-CO form of α-*meso* hydroxyheme and further, verdoheme (Fig. 3D). The reference experiments with cryoradiolytic reduction of the ferric HO do not show any formation of these product complexes in the annealing experiments. However, after thawing and aerobic mixing of the solution of cryoreduced ferric HO, the spectra of Fe^{2+}-CO forms of α-*meso* hydroxyheme-HO and verdoheme-HO complexes were observed. The control experiments with ferric HO confirmed that the enzyme remains catalytically active after radiolytic reduction, and is still able to oxidize the bound heme using atmospheric oxygen and exogenous reductants. No heme degradation has been detected in similar experiments with other oxy-ferrous heme enzymes [24–26].

Collectively, these results show that the HO is a unique enzyme which is able to catalyze heme degradation using atmospheric oxygen and exogenous reductants through a highly stereospecific arrangement of the active FeP-OOH intermediate favorable for the heme hydroxylation at the α-*meso* position. This specific stereochemistry includes a highly bent structure of coordinated dioxygen in oxy-ferrous HO precursor complex [34–36]. We directly observed the one-electron reduction of this precursor and formation of the active intermediate (Fig. 2). As previously shown by EPR and ENDOR [23], the immediate product of this reduction, peroxo-ferric HO complex, is protonated already at 77 K due to the proton transfer from nearby water molecules, stabilized in a proper position by the side chain of Asp140 [23,36]. The FeP-OOH active intermediate transforms into a product, α-*meso* hydroxyheme, after annealing at temperatures above 210 K. In contrast, ferric HO, or oxy-ferrous complexes of other heme enzymes [24–26] do not form any such heme degradation product under identical conditions. Further studies of FeP-OOH intermediate will help to dissect the most important factors of oxygen activation by heme enzymes and bring better understanding of the chemical mechanisms of these reactions.

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