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Heterolytic O—O bond cleavage: Functional role of Glu113 during *bis*-Fe(IV) formation in MauG

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ABSTRACT

The diheme enzyme MauG utilizes H_2O_2 to perform oxidative posttranslational modification on a protein substrate. A *bis*-Fe(IV) species of MauG was previously identified as a key intermediate in this reaction. Heterolytic cleavage of the O—O bond of H_2O_2 drives the formation of the *bis*-Fe(IV) intermediate. In this work, we tested a hypothesis that a glutamate residue, Glu113 in the distal pocket of the pentacoordinate heme of MauG, facilitates heterolytic O—O bond cleavage, thereby leading to *bis*-Fe(IV) formation. This hypothesis was proposed based on sequence alignment and structural comparison with other H_2O_2-utilizing hemoenzymes, especially those from the diheme enzyme superfamily that MauG belongs to. Electron paramagnetic resonance (EPR) characterization of the reaction between MauG and H_2O_2 revealed that mutation of Glu113 inhibited heterolytic O—O bond cleavage, in agreement with our hypothesis. This result was further confirmed by the HPLC study in which an analog of H_2O_2, cumene hydroperoxide, was used to probe the pattern of O—O bond cleavage. Together, our data suggest that Glu113 functions as an acid-base catalyst to assist heterolytic O—O bond cleavage during the early stage of the catalytic reaction. This work advances our mechanistic understanding of the H_2O_2-activation process during *bis*-Fe(IV) formation in MauG.

1. Introduction

Hydrogen peroxide (H_2O_2) is produced in a rich array of biological processes, including respiration and peroxisomal β-oxidation [1]. It serves as a transmitter of cellular redox signals [2], while being a biological reactive oxygen species [3]. Although H_2O_2 is a strong oxidant, it is unreactive with most biological molecules because of the high activation energy barrier. Hemoenzymes play an important role in utilizing and detoxifying cellular H_2O_2. There are three major types of H_2O_2-utilizing hemoenzymes: peroxidases, peroxygenases, and catalases (Fig. 1). Notably, the Fe(III) state is the catalytically active form of these enzymes. Unlike most Fe(II)-dependent hemoenzymes, such as cytochrome P450s, which require external electron sources to regenerate the Fe(II) heme from the Fe(III) state at the beginning of each reaction cycle, these H_2O_2-utilizing hemoenzymes work independently and return to the resting Fe(III) state after each turnover [4,5]. As a result, the two oxidizing equivalents from H_2O_2 are both utilized to oxidize the substrate. Although H_2O_2-utilizing hemoenzymes catalyze a wide variety of chemical transformations, a common high-valence heme intermediate, namely compound I, which is an Fe(IV)-oxo species coupled with a porphyrin cation radical, is involved in these enzymatic reactions (Fig. 1) [4,5]. Compound I is generated via heterolytic O—O bond cleavage, which transfers both oxidizing equivalents from H_2O_2 to the protein-bound heme moiety.

In H_2O_2-utilizing hemoenzymes, it is frequently reported that a polar amino acid residue located in the distal heme pocket facilitates the binding and activation of H_2O_2. This residue functions as an acid-base catalyst to promote heterolytic O—O bond cleavage for compound I production (Fig. 1) [6]. Our survey of the literature shows that the most common polar amino acid used in this case is histidine, which is found in the distal heme pocket of catalase [7], peroxidase [8–11], catalase-peroxidase [12], and prostaglandin synthase [13] (Table 1). Another commonly used amino acid is glutamate. The examples include fungal peroxygenase [14,15], chloroperoxidase [16], and bacterial diheme cytochrome *c* peroxidase (bCcP) [17] (Table 1). Interestingly, such polar residues are usually placed in the optimal position for catalysis via an H-bonding interaction with another polar residue or through

Abbreviations: bCcP, bacterial diheme cytochrome *c* peroxidase; EPR, electron paramagnetic resonance; MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone.

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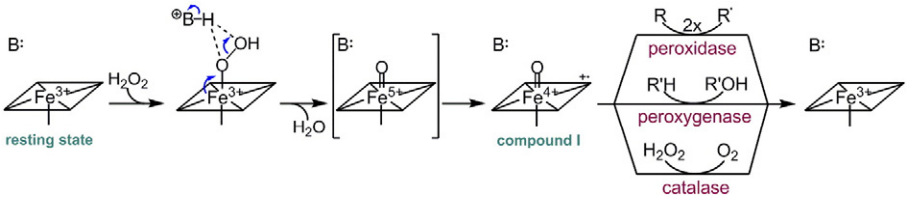

Fig. 1. General reaction mechanism of H_2O_2-utilizing hemoenzymes. "B:" represents the deprotonated state of the acid-base catalytic residue in the distal heme pocket.

a salt bridge with an adjacent charged residue (Table 1). For instance, in *Pseudomonas aeruginosa* bCcP, an asparagine residue forms an H-bonding interaction with the distal glutamate residue [17].

MauG is a diheme enzyme belonging to the bCcP diheme enzyme superfamily. The two ferric hemes in MauG are in different spin states: one is high-spin pentacoordinate with an axial histidine ligand and the other is low-spin hexacoordinate with an axial histidine-tyrosine ligand set (denoted as $Heme_{5C}$ and $Heme_{6C}$, respectively, as shown in (Fig. 2A) [22,24]. MauG catalyzes the final stage of the biosynthesis of a protein-derived redox cofactor, tryptophan tryptophylquinone (TTQ), which functions as the catalytic center of methylamine dehydrogenase (MADH) [25]. During this cofactor biogenesis process, H_2O_2 is utilized as an oxidant to modify two adjacent tryptophan residues of the precursor protein, preMADH, through a radical mechanism (Fig. 2B) [26]. This process is a three-step, six-electron oxidation reaction; each step consumes one equivalent of H_2O_2 and delivers two oxidizing equivalents to preMADH (Fig. 2B) [27,28]. A unique *bis*-Fe(IV) intermediate of MauG, in which $Heme_{5C}$ is present as Fe(IV) = O and $Heme_{6C}$ as Fe(IV) with the axial histidine-tyrosine ligand set retained, is involved in each oxidation step (Fig. 2C) [29–32]. This intermediate is electronically equivalent to compound I, with two oxidizing equivalents above the resting ferric state.

Similar to compound I, the *bis*-Fe(IV) intermediate is generated via heterolytic O—O bond cleavage of H_2O_2. The formation process of *bis*-Fe(IV) is complete within the dead-time of stopped-flow mixing of diferric MauG and H_2O_2 [33], suggesting that the heterolytic O—O bond cleavage is effectively facilitated by the protein matrix in the heme center. Notably, $Heme_{5C}$ is the reactive center that binds and activates H_2O_2 during catalysis, because of its coordination vacancy [34]. A survey of the binding pocket of $Heme_{5C}$ reveals a glutamate residue, Glu113, which is 5.6 Å from the iron ion of $Heme_{5C}$ (Fig. 2A). Previous studies showed that mutation of Glu113 resulted in no detectable TTQ biosynthesis activity from steady-state reactions, but the mutant enzyme achieved partial synthesis of TTQ from *in crystallo* reactions [35]. Herein, we took a closer look at the catalytic role of Glu113 and tested a hypothesis that Glu113 facilitates the activation of H_2O_2 by driving the heterolytic O—O bond cleavage during *bis*-Fe(IV) formation.

2. Experimental

2.1. Reagents

Sodium dithionite (85%), cumene hydroperoxide (80%), cumyl alcohol (97%), acetophenone (99%), and phenethyl alcohol (99%) were purchased from Sigma-Aldrich without further purification. H_2O_2 (30% v/v) was purchased from Fisher Scientific. The concentration of H_2O_2 was determined based on the molar absorptivity of 43.6 M^{-1} cm^{-1} at 240 nm.

2.2. Sequence alignment

Sequence alignment of the bCcP superfamily, including MauG and bCcP from different organisms, was performed using the Molecular Evolutionary Genetics Analysis (MEGA) software. The alignment output was further edited using ESPript [36] for better illustration.

2.3. Protein expression and purification

Recombinant wild-type (WT) MauG and the E113Q mutant were expressed in *Paracoccus denitrificans* and isolated in the periplasmic fraction as described previously [24,35]. The protein concentration was determined using the extinction coefficient of the heme Soret band as described previously [29,35]. All experiments on purified MauG were performed in 50 mM potassium phosphate buffer, pH 7.5.

2.4. Electron paramagnetic resonance (EPR) characterization

EPR experiments were performed using methods described previously [37,38]. Briefly, X-band EPR spectra were recorded in the perpendicular mode on a Bruker ER200D spectrometer coupled with a 4116DM

Table 1
H_2O_2-utilizing hemoenzymes and their corresponding acid-base catalytic residues for heterolytic O—O bond cleavage.

Enzyme	Heme ligand	Acid-base catalyst	Salt-bridge residue	H-bond partner	PDB ID	Ref.
Fatty acid hydroxylase	Cysteine	fatty acid carboxylate group[a]	Arginine	N/A	1IZO	[6,18]
Fungal peroxygenase	Cysteine	Glutamate	Arginine	Threonine[b]	2YP1	[14,15]
Chloroperoxidase	Cysteine	Glutamate	N/A	Histidine	2CPO	[6,16]
Catalase	Tyrosine	Histidine	N/A	Serine	1QQW	[7]
Prostaglandin synthase	Histidine	Histidine	N/A	Threonine	1DIY	[5,13]
KcatG	Histidine	Histidine	N/A	Asparagine	1MWV	[12]
Horseradish peroxidase	Histidine	Histidine	N/A	Asparagine	1ATJ	[6,9]
Cytochrome *c* peroxidase	Histidine	Histidine	N/A	Asparagine	2CYP	[8,11]
Ascorbate peroxidase	Histidine	Histidine	N/A	Asparagine	1APX	[10]
Dye-decolorizing peroxidase (B-type)	Histidine	Arginine	Heme propionate	N/A	3QNR	[19]
Dye-decolorizing peroxidase (D-type)	Histidine	Aspartate	Arginine	N/A	2D3Q	[20,21]
Bacterial di-heme cytochrome *c* peroxidases ($Heme_{5C}$)	Histidine	Glutamate	N/A	Asparagine[c]	2VHD	[17]
MauG ($Heme_{5C}$)	Histidine	Glutamate	N/A	Asparagine	3L4M	[22]
RoxA[d] ($Heme_{5C}$)	Histidine	N/A	N/A	N/A	4B2N	[23]

[a] The carboxylate group belongs to the fatty acid substrate.
[b] The H-bonding interaction between the glutamate residue and the threonine residue is weak.
[c] The asparagine residue is not strictly conserved.
[d] RoxA is an O_2-utilizing enzyme in the bCcP superfamily.