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Superoxide-mediated O_2 activation drives radical cyclization in ergot alkaloid biosynthesis

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ABSTRACT

Conventional heme enzymes utilize iron–oxygen intermediates to activate substrates and drive reactions. Recently, Chen et al. discovered a novel NADPH-independent superoxide mechanism of heme catalase EasC, which facilitates an O_2-dependent radical oxidative cyclization reaction during ergot alkaloid biosynthesis. This enzyme coordinates superoxide-mediated catalysis by connecting spatially distinct NADPH-binding pocket and heme pocket via a slender tunnel, offering a novel perspective on the catalytic mechanisms of heme enzymes in nature.

Ergot alkaloids constitute a category of fungal-derived natural products renowned for their broad-spectrum of pharmacological activities [1,2]. These compounds are widely used to treat various diseases such as migraines, Parkinson's disease, cancers, and postpartum hemorrhage, which underscores their significant clinical values [3]. The tetracyclic ergoline skeleton of ergot alkaloids functions as the core pharmacophore, sharing structural resemblances with neurotransmitters and possessing the capacity to modulate neurotransmitter receptors [4]. Thus, ergot alkaloids have captivated the attention of biochemists for decades, having inspired extensive researches on their complex biosynthesis and remarkable pharmacological properties [5]. In 2019, the Gao laboratory discovered that the heme catalase chanoclavine synthase ($EasC_{Af}$) from *Aspergillus fumigatus* uniquely employs O_2 to convert pre-chanoclavine (PCC) into chanoclavine (CC), constructing the C-ring skeleton [6]. However, the molecular basis and catalytic mechanism of EasC have remained an unresolved mystery (Fig. 1a). In a groundbreaking study recently published in *Nature*, the Gao and Guo laboratories identified a novel O_2 activation pathway in $EasC_{Cf}$ from *Claviceps fusiformis* that generates superoxide, thereby mediating a complex radical oxidative cyclization reaction to convert PCC into CC during ergot alkaloid biosynthesis [7]. This mechanistic insight was elucidated through comprehensive structural analyses and *in vitro* biochemical assays.

In nearly all determined heme enzyme structures, substrates bind and undergo transformation above the porphyrin plane in the heme pocket (*e.g.*, cytochrome P450 monooxygenases, heme-containing dioxygenases) [8–11]. Unlike other heme-containing enzymes, typical heme catalases possess an additional nicotinamide adenine dinucleotide phosphate (NADPH)-binding pocket, which tightly binds to NADPH preventing the substrate H_2O_2 from converting the enzyme into an inactive state [11] (Fig. 1b). Despite extensive efforts, Chen and colleagues could not obtain the structure of $EasC_{Cf}$ in complex with NADPH. In contrast, they observed an unexpected binding mode in their high-resolution cryo-electron microscopy (cryo-EM) structure of $EasC_{Cf}$ in complex with its substrate (2.33 Å, PDB ID: 9JDC): the PCC was found to occupy the NADPH-binding pocket of $EasC_{Cf}$ rather than the canonical heme pocket. The two pockets in $EasC_{Cf}$ are connected by a slender tunnel, and the carboxyl carbon of the substrate PCC is positioned 20.6 Å from the heme centre, which represents an unusually long distance for enzymatic catalysis. Furthermore, unlike typical tetrameric catalases, $EasC_{Cf}$ exhibits a unique homodimeric architecture in both substrate-free (2.64 Å, PDB ID: 9JDB) and substrate-bound forms, as well as in solution. These unusual structural features (Fig. 1c) challenge the conventional understanding of heme enzyme-substrate interactions, suggesting a novel role for the NADPH-binding site in both substrate recognition and inter-pocket communication.

Under the guidance of these structural insights, the authors delved into the catalytic mechanism of $EasC_{Cf}$. An analysis of the heme pocket revealed a long and narrow main channel with a minimum width of 1.42 Å, which is unlikely to accommodate the entry of the sterically larger substrate PCC. A structural comparison between $EasC_{Cf}$-PCC and human catalase HEC bound to NADPH highlighted two loops in $EasC_{Cf}$ that extend into the NADPH-binding pocket [11], effectively blocking NADPH access. Isothermal titration calorimetry (ITC) experiments con-

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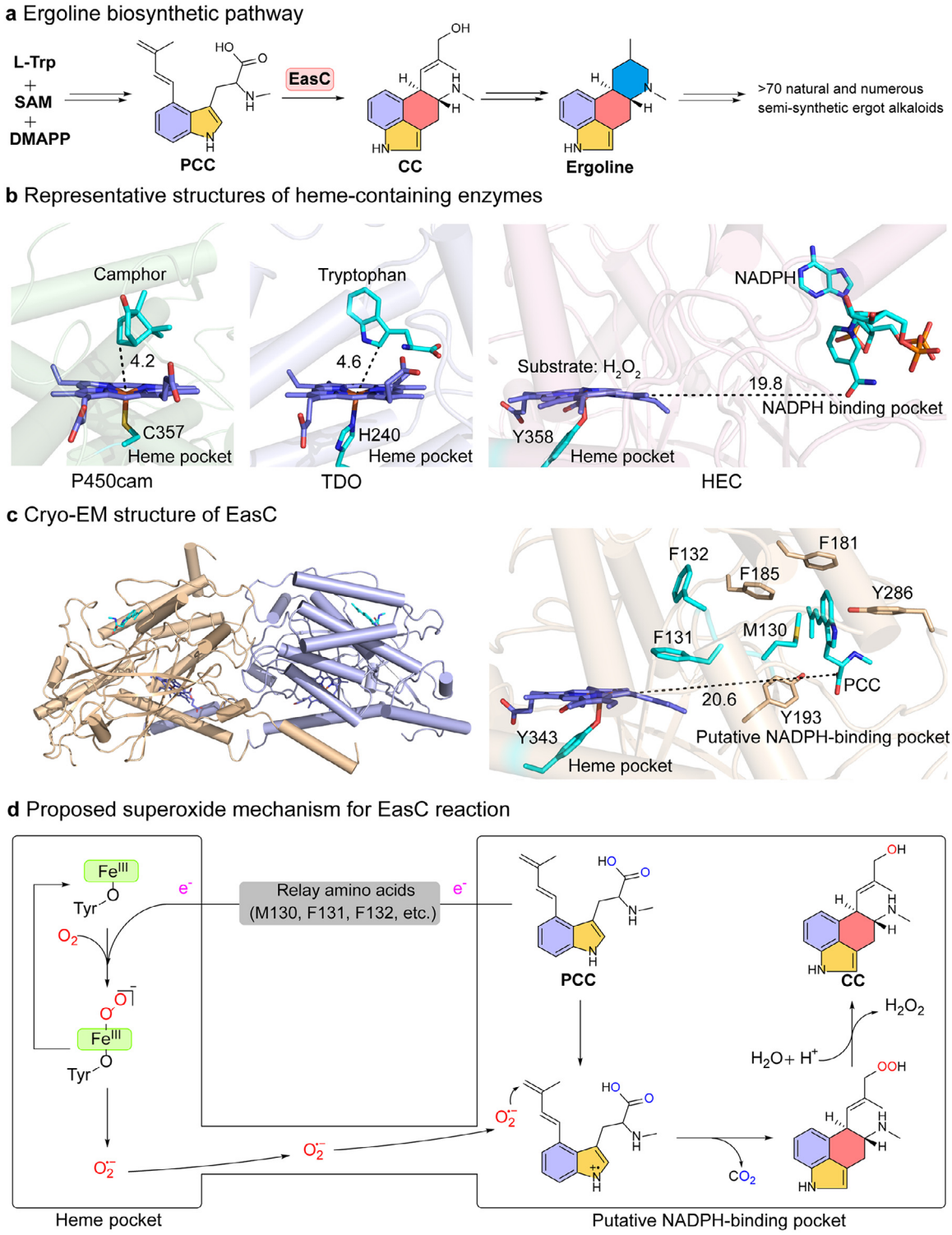

Fig. 1. NADPH-independent superoxide mechanism mediated by the heme catalase EasC.
a. Heme catalase EasC in ergot alkaloid biosynthesis. The tetracyclic ergoline (central C ring marked in red) is the pharmacophore of all clinically used ergot alkaloids. The abbreviations L-Trp, SAM, and DMAPP represent L-tryptophan, *S*-adenosylmethionine, and dimethylallyl pyrophosphate, respectively.
b. Representative structures of heme-containing enzymes. P450cam from *Pseudomonas putida* (PDB ID: 2CPP), tryptophan 2,3-dioxygenase (TDO) from *Xanthomonas campestris* (PDB ID: 2NW8), and human erythrocyte catalase (HEC, PDB ID: 1DGB).
c. Cryo-EM structure of EasC in complex with PCC (PDB ID: 9JDC). Left: overall structure of the complex of EasC with PCC; right: the zoom-in heme pocket and putative NADPH-binding pocket. The key residues in the "pseudo" NADPH-binding pocket for interacting with PCC are shown in wheat, and the key relay amino acids for electron transfer are shown in cyan.
d. Proposed superoxide mechanism for EasC reaction with superoxide-mediated cooperative catalysis of the two distant pockets.
The distances from heme centre to camphor, tryptophan, NADPH, and PCC are shown. EasC noted in figure is $EasC_{Cf}$.

firmed that $EasC_{Cf}$ displays no affinity for NADPH, which contradicts the conventional heme enzyme model that requires NADPH as an electron donor. Subsequent biochemical assays confirmed that NADPH does not act as an electron donor in $EasC_{Cf}$ catalysis but may accelerate reaction rates by stabilizing protein conformation. Using CAVER 3.0.3, the authors identified an 11.6 Å channel linking the heme and NADPH pockets, with the C-4 vinyl group of PCC positioned 5.3 Å from the channel entrance. This channel is proposed to mediate reactive oxygen species (ROS) transfer, enabling heme-generated ROS to reach the substrate pocket and drive radical reactions.

Furthermore, stopped-flow spectroscopy revealed the formation of a heme iron-oxygen transition state, Compound III (Cpd III, Fe(III)-$O_2^{\bullet-}$), which is characterized by UV–Vis absorption peaks at 416, 544, and 590 nm. EPR experiments indicated that Cpd III forms when the heme Fe(III) centre binds with O_2 while simultaneously acquiring an electron directly from PCC, bypassing the need for prior reduction to Fe(II). UV–Vis spectroscopy, mutagenesis experiments, and computational analyses supported an electron transfer mechanism similar to catalase-peroxidase KatG [12], coupling O_2 binding with electron transfer from PCC's indole nitrogen to the heme iron. Since Cpd III (Fe(III)-$O_2^{\bullet-}$) naturally decomposes into superoxide ($O_2^{\bullet-}$), the authors hypothesized that $EasC_{Cf}$ catalysis might be superoxide-mediated. ROS inhibition assays, superoxide restoration experiments, and ^{18}O-labeled superoxide competition studies confirmed that superoxide, rather than conventional iron-oxygen intermediates, is the driving force behind this catalysis.

Based on these findings, an unprecedented superoxide-mediated mechanism for $EasC_{Cf}$ is proposed (Fig. 1d), which unfolds in the following stages: 1) electron transfer and Cpd III formation: PCC bound to the NADPH pocket transfers an electron from its indole nitrogen to heme Fe(III), which simultaneously binds O_2 to form Fe(III)-$O_2^{\bullet-}$ (Cpd III); 2) dissociation and superoxide migration: Cpd III dissociates into Fe(III) and superoxide, which migrates through the ROS channel to the NADPH binding pocket; and 3) oxidative cyclization: at the NADPH binding pocket, superoxide interacts with PCC, facilitating oxidative cyclization to form the central C ring of CC. This proposed mechanism was further validated by a combination of mutagenesis experiments, H_2O_2 detection assays, and quantum chemical calculations.

Overall, this study is significant for uncovering a groundbreaking mechanism through which chanoclavine synthase $EasC_{Cf}$ employs an interesting NADPH-independent cooperative catalysis of the two distant pockets, in which heme-catalyzed O_2 activation generates superoxide to mediate a complex oxidative cyclization reaction of PCC. Moreover, the first detailed elucidation of the catalytic mechanism of $EasC_{Cf}$ offers a valuable template for protein engineering aimed at expanding the structural and bioactive diversity of the pharmacologically important ergot alkaloids. Unlike traditional heme enzymes, which rely exclusively on forming active iron-oxygen complexes (*e.g.*, Compounds I and II) for catalysis [8], $EasC_{Cf}$ enables O_2 to react via superoxide, suggesting that superoxide may play a more ubiquitous role in heme enzyme-mediated reactions than previously recognized. This discovery significantly broadens the scope of heme catalase research, extending it from H_2O_2-dependent enzymes to encompass O_2-dependent enzymes, thereby deepening our understanding of these biocatalysts. Furthermore, the ability of $EasC_{Cf}$ to generate and harness superoxide for catalysis opens new avenues for exploring the catalytic diversity of heme enzymes and their potential applications in synthetic biology and biotechnology. The NADPH-binding pocket of $EasC_{Cf}$ is, in fact, a "pseudo" NADPH-binding pocket, which further suggests an intriguing functional adaptation and evolutionary divergence of fungal heme catalases in natural product biosynthesis. This insight holds the potential to inspire further exploration and functional characterization of novel heme enzymes hidden in nature, thereby broadening the frontiers of scientific understanding in this field.

Declaration of Competing Interest

Given his role as Executive Editor, Dr. Shengying Li had no involvement in the peer review of this article, and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Dr. Jiazhang Lian.

CRediT authorship contribution statement

Yuanyuan Jiang: Writing – review & editing, Writing – original draft. **Zhong Li:** Writing – review & editing, Writing – original draft. **Shengying Li:** Writing – review & editing, Writing – original draft.

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