Noncovalent minimal assembly of exogenous histamine with hemin cofactor as a peroxidase-mimicking cooperative catalyst

Highlights

- Molecular-level cooperation between hemin and histamine for peroxidase-mimicking
- Histamine-driven proton attraction from H₂O₂ and stabilization of transition-state
- Fast oxidation of chromogenic substrates by hemin-histamine pair in complex environments
- Superior specific activity of hemin-histamine pair to well-known peroxidase mimics
Noncovalent minimal assembly of exogenous histamine with hemin cofactor as a peroxidase-mimicking cooperative catalyst

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SUMMARY

By mimicking the synergistic interplay of primary and secondary coordination spheres within native peroxidases, we demonstrate a scaffold-free, yet highly effective molecular-level cooperation between an iron(III)-containing hemin cofactor and exogenous histamine in accelerating a peroxidase-like reaction. Density functional theory computations predict that, among structurally similar molecules, the histamine is the most interactive partner of hemin to elicit a spontaneous peroxidation by electrostatically attracting the proton of hydrogen peroxide to its own imidazole and thermodynamically stabilizing a transition-state intermediate. Although the molecular weight of hemin-histamine pair is 763, 1.7% of the horseradish peroxidase, cooperative catalysis of two natural molecules exhibits 17.3 times greater catalytic efficiency (17.93 M\(^{-1}\)s\(^{-1}\)) and 57.8 times larger specific activity (36.45 \(\mu\)mol/min \(\cdot\) mg) than the hemin alone (1.04 M\(^{-1}\)s\(^{-1}\) and 0.63 \(\mu\)mol/min \(\cdot\) mg). Despite no scaffold or covalent linkage, the self-assembly with hemin is highly histamine-specific in complex environments, leading rapid color changes by substrate oxidation within 10 s.

INTRODUCTION

As biocatalysts, peroxidase enzymes efficiently catalyze substrate oxidation even at ambient conditions in a complex mixture (Chattopadhyay and Mazumdar, 2000), allowing them to be widely used in various bioanalytical applications, including enzyme-linked immunosorbent assay (ELISA) (Regalado et al., 2004), chromogenic immunohistochemistry (Petersen et al., 2018), and clinical diagnosis (Krainer and Glieder, 2015). Similar to catalytic reactions by other metalloenzymes, that in peroxidation arises from the synergistic interplay between an ion cofactor and an apoenzyme scaffold; as optimally coordinated with ligands of the primary sphere, a metal ion cooperatively interacts with one or more amino acid residues of the secondary sphere that provides appropriate substrate position and orientation for the progress of the oxidation reaction (de Visser, 2020; Hale and Szymczak, 2018). In particular, Horseradish peroxidase (HRP) relies on the sophisticated cooperation of two major catalytic moieties: a porphyrin redox cofactor and a histidine residue (Jones and Dunford, 2005; Samajdar et al., 2018). When hydrogen peroxide (H\(_2\)O\(_2\)) is sandwiched by the heme and the histidine, it is selectively decomposed to oxidize the central ferric iron-porphyrin complex, and the reactive ferryl iron-based radical rapidly perform the oxidation of incoming molecules. Arguably, such cooperative catalysis would be the best way to achieve high selectivity and high reactivity in chemical transformations although its activity is highly limited in the vulnerable protein scaffold (Lin et al., 2014; Pina et al., 2001).

For protein-free scaffolds, diverse peroxidase mimics have been developed over the past decades (Li et al., 2018; Lin et al., 2014; Marshall-Roth et al., 2020; Samajdar et al., 2018; Travascio et al., 1998; Wu et al., 2019), but simply resembling the active site of the native peroxidase has not guaranteed the effectiveness of catalytic cooperation in nature (Golub et al., 2011; Liu et al., 2016; Nastri et al., 2011). Along with the emergence of scaffold-free catalytic assemblies consisting of a single amino acid and ion cofactor (Makam et al., 2019, 2022), the histidine and the imidazole, a catalytically decisive amino acid and a minimal residue in peroxidation, have been theoretically predicted to elicit spontaneous enzymatic peroxidation mechanism with the iron(III)-containing hemin cofactor (Vidossich et al., 2010; Wirstam et al., 1999). However, when separated from apoenzymes, both molecules failed to revitalize the catalytic cooperation with the hemin, only resulting in negligible or slight increases in substrate oxidation rates (Stefan et al., 2012;
Figure 1. Accelerated peroxidation reaction induced by the cooperation of hemin with histamine

(A) DFT calculation of peroxidation pathway guided by the catalytic complex of hemin, histamine, and H$_2$O$_2$. In peroxidase mimicking, the reaction mechanism is predicted to include three successive steps: histamine protonation, formation of bifurcated hydrogen bond, and deprotonation. When the H$_2$O$_2$ is sandwiched by the histamine and hemin, its proton (H1) is transferred from the oxygen atom (O1) to the nitrogen atom in the imidazole ring of histamine, and then the protonated histamine interacts with both oxygen atoms of OOH-hemin complex by hydrogen bonds. Finally, the proton (H1) of the histamine is transferred to another oxygen atom (O2), resulting in the cleavage of the O1-O2 bond, and the newly produced reactive O1 oxidizes the iron (III) of hemin.
Uno et al., 1995). As nucleic acid scaffolds, highly ordered G-quadruplexes have been known to accommodate the hemin cofactors for peroxidation acceleration (Travascio et al., 1998), but imidazole-containing nucleobases (e.g., adenine and guanine) hardly accelerate the desired reaction apart from the nucleic acid folding structures (Kong et al., 2010; Stefan et al., 2012). Even though peptide fragments have been forced to be conjugated with the hemin by an iterative design process and rigorous characterization (Lombardi et al., 2001; Nastri et al., 2011), it should be noted that the optimal secondary sphere interaction for the peroxidase-like activity basically relies on the self-assembly of the ion cofactor with the catalytic residue originated from the apoenzyme (Lombardi et al., 2001; Vitale et al., 2015). Therefore, the development of scaffold-free peroxidase mimics with minimal primary and secondary sphere interactions in catalytic coordination remains an unmet need.

In this work, we demonstrated that the non-covalently assembled hemin cofactor with exogenous histamine can serve as a scaffold-free, yet highly effective peroxidase-mimetic catalyst. We assumed that the availability of the exogenous molecule, which is the most electrostatically interactive with hemin, would be the key to reproduce the secondary-sphere interactions with no scaffold by effectively sandwiching \( \text{H}_2\text{O}_2 \); among structurally similar imidazole derivatives, including the conventional histidine, our density functional theory (DFT) calculations identified that the histamine could most strongly attract the proton of \( \text{H}_2\text{O}_2 \) to its imidazole ring and stabilize a transition-state during a spontaneous peroxidation pathway owing to the protonated aliphatic amine at the C4 site of imidazole. Consistent with our computational prediction of molecular-level cooperation without covalent linkage, both hemin and histamine were experimentally demanded together to effectively oxidize chromogenic dyes, leading to \( \approx 20 \)-fold higher absorbance increase compared to the hemin-imidazole complex, the simplest mimic of the peroxidase’s catalytic core. Importantly, although the total molecular weight of the hemin-histamine pair is just 763 Da, i.e., 1.7% of the heme-containing HRP, the highly specific self-assembly of two natural chemicals allowed peroxidation-dependent colorimetric changes to be detectable within less than 10 s, even in complex environments (e.g., PBS (PBS), fetal bovine serum (FBS), and human urine). The concentration dependence of exogenous molecules in peroxidation revealed the potential of our cooperative catalyst toward the real-time detection of histamine, one of the most important neurotransmitters, capable of regulating the release of other neurotransmitters and stimulating post-synaptic receptors (Kapalka, 2009).

RESULTS

Synergistic cooperation of hemin and histamine in peroxidation

By performing DFT calculations, we found that the catalytic pathway of a hemin-histamine pair is consistent with the Poulos-Kraut mechanism of the peroxidase enzyme in nature (Figure 1A) (Poulos and Kraut, 1980), implying that the cooperative pair can be indeed an effective peroxidase-mimetic catalyst. For our DFT study, we systematically constructed an initial complex model wherein \( \text{H}_2\text{O}_2 \) is sandwiched between a pair of cooperative molecules: hemin and histamine (Figure 1A, compound 0). In this construct, we recruited the histamine (\( \text{NH}_2^+ \)) because the aliphatic amine group mostly exists as a protonated form (>96%) at neutral environment (pH 7.4) (Ganellin et al., 1973). When either the iron-containing porphyrin or the imidazole derivative is absent, i.e., hemin/\( \text{H}_2\text{O}_2 \) or histamine/\( \text{H}_2\text{O}_2 \), successful complex formation was observed, but no catalytic process was elicited (Figure S1). In contrast, the presence of both components spontaneously induced a peroxidation pathway through three major steps: protonation, bifurcated hydrogen bond formation, and deprotonation. First, a proton (H1) is transferred from the oxygen atom (O1) of \( \text{H}_2\text{O}_2 \), which is initially bound to the iron (III) of hemin, to the imidazole ring of histamine, resulting in the formation of histamine-H\(^+\) and OOH-hemin (Figure 1A, compound 0\(^*\)). Second, the position of histamine-H\(^+\) is adjusted to interact with another oxygen atom (O2) of OOH-hemin while O1 is detained by the central iron atom, resulting in a bifurcated hydrogen bond among O1-O2-H (Figure 1A, compound 0\(^{**}\)). Third, because the proton of the histamine is transferred to O2, the bonding between O1 and O2 is cleaved for the reactive O1 to oxidize the iron (III) of hemin to iron (IV). As a result, a water molecule is formed and eventually displaced (Figure 1A, compound I). Collectively, the self-assembly of hemin and histamine

Figure 1. Continued
(B) Experimental cooperativity validation of hemin and histamine in peroxidation reaction. Individually, the hemin and the histamine showed negligible peroxidase-like activities, but once paired, the peroxidation reaction was dramatically accelerated to produce a large amount of oxidized ABTS substrate, green ABTS\(^+\), even visible by our naked eyes within 20 s (left). Peroxidation-triggered absorbance changes were quantitatively monitored at 418 nm every 20 s (right).
elicits this reaction process spontaneously, thereby displaying its potential to exhibit a peroxidase-like activity.

Experimentally, the self-assembled hemin-histamine pair catalyzed the peroxidation reaction with an exceptionally enhanced rate (Figure 1B). To evaluate the degree of peroxidation, we employed a 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a colorimetric substrate, which is converted to a green radical cation (ABTS⁺, 418 nm) by oxidation (Kang et al., 2019). When the ABTS substrate was mixed with only one catalytic component, i.e., either hemin/H₂O₂ or histamine/H₂O₂, no color change occurred regardless of reaction time (Figure 1B, left). However, pairing between hemin and histamine greatly increased ABTS⁺ formation, so we could observe green color with our naked eyes within less than a minute. During monitoring time-dependent changes in absorbance (Figure 1B, right), the hemin-histamine pair rapidly increased the absorbance signal; in contrast, the hemin or the histamine alone oxidized negligible amounts of substrate or failed to cause a distinct absorbance increase. Similar to the ABTS, other peroxidase substrates, such as tetramethylbenzidine (TMB) and o-phenylenediamine (OPD), could be readily used for the cooperative peroxidation of the hemin-histamine pair (Figure S2). In principle, this cooperative effect arose from the well-organized secondary-sphere interaction capable of forming a 1:1:1 assembly of histamine, H₂O₂, and hemin, rather than the direct binding between the hemin and the histamine, evidenced by two experimental results: the histamine-irrelevant positional variation of hemin’s Soret band (Figure S3) (Samajdar et al., 2018) and the 1:1 stoichiometric interaction between the histamine and H₂O₂ in the Job’s plot analysis (Figure S4) (Xu et al., 2021). From these experimental observations, we can conclude that both hemin and histamine are essentially required together for accelerating the peroxidation in a cooperative manner, which is well-predicted by our DFT calculations.

By conducting steady-state kinetic assays (Harbour and Issler, 1982; Liu et al., 2016), we confirmed that the catalytic efficiency (kcat/Km) of the hemin-histamine pair is 17.3 times higher than that of the hemin cofactor alone (Table 1). Specifically, we used excessive amounts of histamine (5 mM) to make it fully assembled with the hemin (4 μM) and then measured the absorbance changes at 418 nm induced by oxidation with varying ABTS concentrations (Figure S5, left). To determine important terms of Michaelis-Menten kinetics, we obtained four kinetic parameters (kcat, KM, Vmax, and kcat/KM) of each oxidation reaction (Table 1). Compared to the hemin alone, the hemin-histamine pair yields slightly decreased Km (Michaelis constant), which is 30% lower than that of the hemin. This indicates that owing to the pairing between hemin and histamine, the binding interaction between the catalytic pair and the ABTS substrate becomes stronger. Unlike Km, the median turnover rate (kcat) dramatically increased to be 12.17-fold larger than that of the hemin. These results imply that the highly enhanced peroxidation efficiency of the hemin-histamine pair is mainly attributed to achieving a rapid turnover rate. Moreover, by varying ABTS concentrations, we further investigated kinetic parameters at different concentrations of H₂O₂ (Figure S6A and Table S1) and histamine (Figure S6B and Table S2). As a result, we found that the peroxidation reaction of the hemin-histamine pair follows the ping-pong kinetics, typical for peroxidase enzymes (Everse et al., 1990; Jian et al., 2022; Zozulia et al., 2021), and larger concentrations of histamine were revealed to induce lower Km and higher kcat, gradually increasing the catalytic efficiency. It was also observed that even with TMB substrates, the histamine-assisted peroxidation considerably increased kcat, exhibiting 14.7 times higher kcat/Km than that of the hemin (Table S3 and Figure S7).

**Table 1. Comparison of kinetic parameters for the ABTS oxidation reaction between the hemin-histamine pair and the non-paired hemin**

| Catalyst                  | Km [mM] | Vmax [10⁻⁸ M s⁻¹] | kcat [10⁻² s⁻¹] | kcat/Km [M⁻¹ s⁻¹] |
|---------------------------|---------|-------------------|-----------------|-------------------|
| Hemin-histamine pair      | 1.63 ± 0.3 | 11.70 ± 0.6       | 2.93 ± 0.2      | 17.93 ± 0.3       |
| Hemin                     | 2.33 ± 0.2 | 0.97 ± 0.2        | 0.24 ± 0.1      | 1.04 ± 0.1        |
| Histamine                 | –       | –                 | –               | –                |

**Catalytic cooperativity of imidazole derivatives with a hemin cofactor**

To identify whether other imidazole derivatives, structurally similar to histamine, can be synergistically cooperative with the hemin cofactor in catalyzing a peroxidation reaction, we chose five different histamine
Figure 2. Computational and experimental comparison of histamine and its analogs to cooperate with hemin in accelerating the peroxidation reaction

(A) Free energy change ($\Delta E_0^*$) depending on an imidazole derivative for the compound $0^*$ formation (protonation step) (left). Among six different exogenous molecules, histamine is predicted as the most efficient one to stabilize the reaction intermediate, compound $0^*$ (right).

(B) Energy diagrams relying on six imidazole derivatives during the peroxidation pathway. Compared to the other analogs, histamine has the lowest energy barrier for the conversion of compound $0^*$ to compound $I$ (red line).
analogs: histidine, 4-imidazoleacetic acid, imidazole, 4-hydroxymethyl imidazole, and 4-methylimidazole. All molecules contain an imidazole ring, essential for the peroxidation reaction, and compared to histamine, the only difference is a functional moiety at the C4 site of imidazole (–C2H5NH2 site of histamine) (Figure S8). The reason for choice for each molecule is varied (Table S4); for example, histidine is a catalytic amino acid within a peroxidase enzyme in nature, and 4-imidazoleacetic acid has a negative charge owing to its carboxyl group (–COO−), whereas histamine retains a positive charge (–NH2+) at neutral pH. We thoroughly investigated the cooperative effect of the histamine analogs by pairing them with hemin in catalyzing the peroxidation process.

Thermodynamically, histamine is the most favored partner of hemin in inducing the peroxidation pathway among imidazole derivatives (Figures 2A and 2B). By our DFT calculations, all of the chosen imidazole derivatives are supposed to ensure the Poulos-Kraut mechanism similar to the histamine (Figure S9, and see also Data S1). In general, the conversion of compound 0 to compound 0* (protonation of imidazole ring) serves as a major step to enhance the overall reaction rate, so we calculated the ability to stabilize the compound 0* intermediate state, termed a free energy ΔE0*, for each derivative (Figure 2A, left). Importantly, the order of ΔE0* is histamine > 4-hydroxymethyl imidazole > 4-imidazoleacetic acid (–COO−) > histidine > 4-methyl imidazole > imidazole (Figure 2A, right); with respect to the degree of transition-state stabilization, the histamine is predicted as the best imidazole derivative to initiate the peroxidation reaction. When we thoroughly depicted the reaction progress versus energy changes, it is further revealed that the histamine has the lowest energy barrier compared to other derivatives (Figure 2B and Table S5). After the formation of compound 0*, there exists an energy barrier, and once again, the histamine is the best to lower the energy barrier toward the final product (compound I) (Figure S10).

Experimentally, we demonstrated that histamine indeed serves as the most efficient molecule to perform a cooperative catalytic reaction of peroxidation by pairing with hemin (Figure 2C). When we monitored the ABTS+ signal relying on each imidazole derivative in the presence of hemin (13 min reaction), the order of peroxidation-enhancing efficiency was in full agreement with that of our calculation: histamine > 4-hydroxymethyl imidazole > 4-imidazoleacetic acid > histidine > 4-methylimidazole > imidazole. For instance, compared to imidazole, the simplest molecule with an imidazole ring moiety, the histamine induced a ∼20-fold higher absorbance increase, i.e., 20-fold faster ABTS oxidation. The trend was almost consistent when varying the reaction time (Figure S11); even after 1 h reaction, the histamine was always the most efficient molecule for the secondary-sphere interaction with hemin cofactor among six analyzed histamine analogs. For further comparison, we derived kinetic parameters of hemin in the presence of the histidine or the imidazole based on the ABTS oxidation rates (Table S6 and Figure S12). The histidine and the imidazole induced only a negligible increase and decrease in the catalytic efficiency of hemin compared to the histamine (Table 1), supporting our computational results again.

To clarify why the histamine, when paired with the hemin, is highly effective in accelerating the peroxidation reaction compared to the structurally similar analogs, we investigated the electric force (F_{WOT-H1}) between O1 and H1 of H2O2 before the protonation step (Figure 2D, top). The protonation of imidazole ring moiety is revealed to limit the overall peroxidation rate (Jones and Dunford, 2005), and for the protonation, H1 is demanded to be detached from O1 of H2O2 (Figure 2A); by weakening the O1-H1 bond, imidazole derivatives are supposed to accelerate the peroxidation pathway. We computationally calculated the F_{WOT-H1} at compound 0 by Coulomb’s law (Huray, 2011) (Table S7) and found that with regard to the imidazole derivatives, the order of Fe is inversely proportional to that of ΔE0* (Figure 2D, bottom): imidazole > 4-methyl imidazole > histidine > 4-imidazoleacetic acid (–COO−) > 4-hydroxymethyl imidazole > histamine. The smallest F_{WOT-H1} value of the histamine indicates the strongest attraction to the H1 of H2O2, thereby most actively supporting the production of compound 0*. 
pH dependency of exogenous molecules in cooperative peroxidation with hemin

Interestingly, pH conditions can thermodynamically influence the peroxidase-like activity of the cooperative catalyst as the charge of imidazole derivatives is another stabilization determinant of the transition state, compound 0*. Histamine and 4-imidazoleacetic acid include pH-responsive moieties, aliphatic primary amine, and carboxyl group, respectively; for example, the pKₐ value of the aliphatic amine group in the histamine is 9.40, so the imidazole derivative exists as a protonated form at physiological pH (~7.4) (Ganellin et al., 1973). To investigate the charge effect on the peroxidation, we varied the charge state of functional moieties at the C4 site of histamine and 4-imidazoleacetic acid and calculated the degree of compound 0* stabilization when paired with the hemin cofactor (Figure 3A). The calculated
stabilization energy ($\Delta E_0^*$) orders for histamine and 4-imidazoleacetic acid are histamine ($\text{H}^+$) > histamine(−NH$_2$) and 4-imidazoleacetic acid(−COO$^-$) > 4-imidazoleacetic acid(−COOH), respectively, predicting that the protonation of histamine is favorable to initiate the peroxidation unlike that of 4-imidazoleacetic acid.

The degree of substrate oxidation by histamine and 4-imidazoleacetic acid, in cooperation with hemin, indeed relied on environmental pH (Figure 3B). In three different pH buffers (7.0, 7.5, and 8.0), we experimentally measured the value of relative substrate oxidation (RSO), the ABTS$^+$ signal (absorbance at 418 nm) of the hemin-imidazole derivative complex divided by that of the hemin. Consistent with our DFT calculation, as pH increased to make histamine less protonated, the RSO value of the hemin-histamine pair decreased (top graph); at pH 7.0, it was 2.04 ± 0.04, but it decreased down to 1.67 ± 0.02 at pH 8.0. Moreover, at pH 6.0, the hemin-histamine pair would be more catalytically active, so the difference in peroxidation-enhancing efficiency between the histamine and the other imidazole derivatives became larger compared to that in the pH 7.5 condition (Figures S13 and 2C). In contrast, 4-imidazoleacetic acid preferred its deprotonated form to exhibit the peroxidase-like activity through compound 0* stabilization. Whereas the RSO value of 4-imidazoleacetic acid with hemin was 1.13 ± 0.01 only at pH 7.0, it could be elevated to 1.57 ± 0.02 at pH 8.0, which is even close to the RSO value of the hemin-histamine pair (1.67 ± 0.02) under the same pH condition. Our results indicated that along with the catalytic activity of the peroxidation reaction, its pH dependence would be controlled by the functional moiety of the imidazole derivative.

**Superior specific activity of hemin-histamine pair in substrate oxidation**

The hemin-histamine pair, despite the remarkably low molecular weight (763.1 in total), exhibits an excellent specific activity (SA) in substrate oxidation, which is much higher than many of heavier synthetic peroxidase mimics (Tables 2 and S8). The catalytic efficiency per quantity of catalyst can be standardized with the value of SA, which is defined as activity units per milligram of catalyst (µmol/min·mg) (Hastie et al., 2006). In oxidizing the ABTS substrate (500 µM), the SA of the hemin-histamine pair was measured to be 36.4 µmol/min·mg at room temperature for 2 min, which was 57.8 times higher than that of hemin alone (SA$_{\text{hemin}}$ 0.63 µmol/min·mg) (Table 2). Synthetic G-quadruplex DNAzymes, including PS2.M, (Travascio et al., 1998) are well-known to serve as peroxidase activity enhancers (Evans et al., 2007; Phan et al., 2004), but the most efficient PS2.M showed the SA of 7.21 µmol/min·mg, which is less than one-fifth of the SA for the histamine. Despite the lower $V_{\text{max}}$ than many peroxidase activity enhancers, our hemin-histamine pair could exhibit higher SA because its molecular weight is at least 10 times smaller than those of other catalysts. We note that the SA value of the histamine is even higher than the theoretically deduced or reported values of organic DNAzymes and inorganic nanozymes (Tables S8 and S9) (Cheng et al., 2009; Jiang et al., 2018). Although the hemin-histamine pair displayed a 2.3 times smaller SA than a native HRP enzyme, which is, however, 57.9 times larger in size, the noncovalent self-assembly catalyzed the desired reaction without the sophisticated enzymatic scaffold, only relying on a minimal molecular-level interaction in a cooperative manner.

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**Table 2. Specific activity comparison of hemin and five different peroxidation catalysts**

| Name                     | Molecular weight (MW)$^a$ | MW$_{\text{catalyst}}$ | MW$_{\text{hemin}}$ | Specific activity (SA) | SA$_{\text{catalyst}}$ | Description                |
|-------------------------|--------------------------|------------------------|---------------------|------------------------|------------------------|----------------------------|
| Hemin                   | 651.9                    | 1                      | 0.63                | 1                      | –                      |                           |
| Hemin-histamine pair    | 763.1                    | 1.17                   | 36.45               | 57.86                  | this work              |                           |
| Hemin with PS2 M        | 6359.5                   | 9.75                   | 7.21                | 11.44                  | most widely used       | G-quadruplex               |
| Hemin with c-Myc gene   | 7693.4                   | 11.80                  | 6.48                | 10.29                  | one of the most active | G-quadruplexes             |
| Hemin with G6           | 18,039.0$^b$             | 27.67                  | 0.95                | 1.51                   | Intermolecular G-quadruplex |
| HRP                     | 44,173.9                 | 67.76                  | 84.79               | 134.58                 | natural enzyme         |                           |

$^a$To calculate the MW of the hemin-histamine pair and hemin-G-quadruplex complexes, we summed the MW of hemin (651.94) and that of partner molecule (histamine: 111.15, PS2.M: 5707.7, c-Myc gene promoter: 7041.4, and G6: 4346.76).

$^b$To construct the intermolecular hemin-G-quadruplex complex, four G6s are required, so we summed the MW of hemin and that of four G6s (17,387.0).
Ultra-simple and quick histamine quantification in complex mixtures

The histamine-assisted dramatic peroxidation acceleration represents the feasibility of simple colorimetric trace analysis for the biologically important histamine. As histamine is an aminergic neurotransmitter (Nuu-tinen and Panula, 2010) and simultaneously, a mediator of allergic inflammatory response (Maintz and Novak, 2007), its quantitative detection in a clinical sample is crucial for brain study and diagnosis of histamine-related diseases (Comas-Basté et al., 2020). Only relying on catalytically cooperative hemin (4 μM) and chromogenic ABTS substrates (500 μM), we investigated histamine-dependent colorimetric changes; indeed, as the histamine concentrations increased up to 100 mM, the ABTS⁺ signal, which was detectable by naked eyes, increased together owing to the boosted peroxidase-like activity (Figure 4A). In particular, in a concentration range of histamine from 10 to 250 μM, the signal of green ABTS⁺ chromophore was measured to be linear (Figure 4A, inset), simplifying the accurate histamine quantification. Notably, the limit of detection (LOD) was determined down to 9.3 μM, and even when we used the TMB as a different substrate (Figure S14), there was no notable change in the LOD (8.2 μM), which are comparable to those of intricate histamine sensors requiring surface functionalization of histamine-recognition macromolecules, such as amine oxidase enzymes and molecularly imprinted polymers (Gagic et al., 2019; Gao et al., 2015; Gumpu et al., 2014). We note that compared to our simple and quick assay, other conventional ones, such as histamine dehydrogenase-based quantification (Sato et al., 2005) and histamine measurement using high-performance liquid chromatography (Paleologos and Kontominas, 2004), demand complicated sample preparations and multiple reaction steps, consuming more than 5-15 min.

The peroxidation-dependent colorimetric signaling was histamine-specific, and many other molecules of biogenic signaling pathways did not cause notable peroxidation activities in the presence of hemin and chromogenic substrates. In addition to the structural histamine analogs (Figure 2C), two different classes of neurotransmitters, biogenic amines, and amino acids were further scrutinized and revealed to fail in the production of the oxidized substrate, green ABTS⁺ (Figures S15 and S16). In particular, dopamine, norepinephrine, and serotonin contain an aliphatic amine group, which is essential for the histamine to cooperate with hemin in catalyzing the peroxidation reaction, and the serotonin possesses a more histamine-like structure owing to the additional five-membered ring. However, all of the neurotransmitters lack imidazole ring moieties, so there was no cooperation with hemin, leading to no boosted peroxidase-like activity (Figure S15).

Even in complex environments, our histamine-specific, simple colorimetric assay enabled rapid detection of histamine within 10 s (Figures 4B and 4C). To represent physiological environments (e.g., tear, blood, and urine) where the histamine actually exists (Bruce et al., 1976), we prepared three different solutions: PBS, FBS, and human urine. For the colorimetric assay, hemin (4 μM) and TMB (500 μM) were simply mixed with three different histamine-containing complex solutions (1X PBS, 50% FBS, and 40% human urine); we note that as an oxidized ABTS is quickly decayed through disproportionation (Kong et al., 2010), the relatively stable TMB was chosen as the chromogenic substrate (Frey et al., 2000). By our naked eyes, the distinct blue color of TMB⁺ was observable within 10 s as evidence of a dramatically accelerated peroxidation reaction against the complex environments (Figure 4B). In contrast, the absence of histamine caused the oxidation of the TMB substrate to be negligible, so there were undetectable color changes. When the histamine concentrations were varied from 1 μM to 20 mM, the TMB⁺ signals (absorbance at 652 nm) monotonically increased for all different solutions (Figure 4C), confirming the histamine concentration-dependent acceleration of TMB oxidation rates. Even though abundant antioxidants in urine were supposed to undermine the desired peroxidation (Kirschbaum, 2001), it is clear that the synergistic interplay between the hemin and the histamine could achieve high levels of peroxidase-like activity even with diverse salts, small molecules, and proteins, revealing its great potential for quick and simple histamine quantification in real clinical samples.

DISCUSSION

In this study, we computationally and experimentally validated that the minimal molecular-level cooperation between hemin and histamine can accelerate spontaneous peroxidation, mainly owing to the proton attraction within H₂O₂ for reaction initiation and thermodynamic transition-state stabilization. Compared to the hemin molecule (651.9) alone, its pairing with the protonated aliphatic amine-functionalized imidazole derivative needs only a 17% increase in molecular weight (763.1), but the performance of the catalytic cooperation is dramatic, exhibiting the 17.3-fold greater catalytic efficiency and the 57.8-fold higher specific activity. Even when compared to the well-known synthetic peroxidase mimics (Tables 2, S8, and
Figure 4. Histamine-dependent peroxidation acceleration in complex mixtures enables colorimetric quantification of histamine, one of the most important neurotransmitters

(A) Histamine concentration-dependent ABTS⁺ formation (418 nm), in the presence of hemin (2 μM) (13 min reaction at room temperature). In the range of histamine concentrations from 10 to 250 μM, the absorbance intensities were linear (inset), yielding the limit of detection of 9.3 μM, based on the 3σ/slope method (σ is the SD of black signal).

(B) Naked eye observations of TMB⁺ accumulation in three different complex solutions (1X PBS, 50% FBS, and 40% human urine), in the presence or absence of the histamine (5 mM). The histamine-dependent TMB⁺ color was detectable within 10 s.

(C) Histamine concentration-dependent TMB⁺ formation (652 nm) in three different complex solutions. In this assay, the histamine concentrations were varied from 1 μM to 20 mM at the fixed hemin concentration (2 μM).
S9), the hemin-histamine pair was revealed to be more efficient or comparable in catalyzing the peroxidation. Importantly, unlike the artificial nanozymes that do not permit the controlled structural design (Wang et al., 2019), our cooperative peroxidase mimic is a fully defined minimal architecture composed of water-soluble natural molecules, allowing its accurate structural reproduction and further chemical modification.

Owing to the synergistic interplay between the primary-sphere hemin and the secondary-sphere histamine, our self-assembled peroxidase mimic can be more attractive than the native HRP, one of the most effective peroxidases. Despite the 2.3 times smaller specific activity than the HRP, the hemin-histamine pair is free from irreversible denaturation unlike the biological enzyme; the catalytically cooperative effect arises from a simple electrostatic interaction with no need for complicated structural folding. Whereas the production of the HRP exclusively relies on living organisms (Krainer and Glieder, 2015), the binary components of our cooperative catalyst can be either synthetically or biologically prepared, providing potential in vitro and in vivo applications (e.g., a cost-effective peroxidase alternative of analytical assays and an in situ signal converter of neurotransmitter effluxes). Moreover, the low-molecular-mass hemin and histamine can be readily tagged on other functional molecules, such as programmable nucleic acids (Li et al., 2021), self-assembling proteins (McMillan et al., 2005), and stimuli-responsive polymers (Moad, 2017), holding the great potential for systematically synchronizing the highly controllable peroxidation and various biophysical and cellular events, such as molecular structural alterations, ligand-receptor interactions, and membrane transports (Bongrand, 1999; Prabakaran et al., 2001; Yang and Hinner, 2015).

Limitations of the study
In this study, as an exogenous molecule that offers the optimal secondary-sphere interaction to sandwich \( \text{H}_2\text{O}_2 \) with the hemin cofactor, the histamine was screened to be the most favorable imidazole derivative among six structurally similar molecules. The only difference of other imidazole derivatives with the histamine is a functional moiety at the C4 site of imidazole ring, but the functionalization of other carbon sites would also affect the electrostatic interaction between the resulting imidazole derivatives and \( \text{H}_2\text{O}_2 \). If we could aim for deeper investigation into the effects of chemical moieties at each carbon site of imidazole, it may be possible to gain a further understanding to accelerate the peroxidation reaction.

STAR★METHODS
Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105257.

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AUTHOR CONTRIBUTIONS
S.S. Oh supervised the project. B. Kang conceived the idea and conducted experimental processes. G. park, S.H. Kim, and D. Lee carried out DFT computations. All authors discussed the results and assisted in the article preparation.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological samples** | | |
| Fetal bovine serum | Thermo Fisher Scientific | Product Code: 11573397 |
| Normal urine | Lee Biosolutions | Cat# 991-03-S |
| **Chemicals, peptides, and recombinant proteins** | | |
| Hemin (from bovine) | Sigma-Aldrich | H9039; CAS: 16009-13-5 |
| Imidazole | Sigma-Aldrich | S6750; CAS: 288-32-4 |
| Dopamine hydrochloride | Sigma-Aldrich | H8502; CAS: 62-31-7 |
| (+)-Norepinephrine (+)-bitartrate salt | Sigma-Aldrich | A9512; CAS: 108341-18-0 |
| Serine | Sigma-Aldrich | S4500; CAS: 56-45-1 |
| Glycine | Sigma-Aldrich | G7126; CAS: 56-40-6 |
| Aspartic acid | Sigma-Aldrich | A9256; CAS: 56-84-8 |
| Glutamic acid | Sigma-Aldrich | G1251; CAS: 56-86-0 |
| Sodium chloride | GA Biochem | SC1010; CAS: 7647-14-5 |
| Potassium chloride | GA Biochem | PC3013; CAS: 7447-40-7 |
| Hydrogen peroxide | GA Biochem | HP1003; CAS: 7722-84-1 |
| Triton X-100 | GA Biochem | TX1061; CAS: 9002-93-1 |
| Tris(hydroxymethyl)aminoethane | GA Biochem | TH2075; CAS: 1185-53-1 |
| Tris hydrochloride | GA Biochem | TH2075; CAS: 1185-53-1 |
| Histamine, 97% | Thermo Fisher Scientific | AC411710010; CAS: 51-45-6 |
| 4-(Hydroxymethyl)imidazole hydrochloride, 99% | Thermo Fisher Scientific | AC204870010; CAS: 32673-41-9 |
| Serotonin hydrochloride, 98% | Thermo Fisher Scientific | AC215025000; CAS: 153-98-0 |
| L-Adrenaline, 98+% | Thermo Fisher Scientific | ACO4911114; CAS: 51-43-4 |
| 3,3’,5,5’-tetratramethylbenzidine, 99+% | Thermo Fisher Scientific | AC229280010; CAS: 54827-17-7 |
| o-phenylenediamine | Thermo Fisher Scientific | 34005; CAS: 615-28-1 |
| Histamine dihydrochloride | Tokyo Chemical Industry | H0146; CAS: 56-92-8 |
| L-Histidine | Tokyo Chemical Industry | H0149; CAS: 71-00-1 |
| Imidazole-4(5)-acetic acid hydrochloride | Tokyo Chemical Industry | I0229; CAS: 3251-69-2 |
| 4-methylimidazole | Tokyo Chemical Industry | M0636; CAS: 822-36-3 |
| 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) | Roche | 10102946001; CAS: 30931-67-0 |
| Phosphate buffered saline | Tech and Innovation | BPB-9121 |
| Sodium hydroxide, bead 98% | SAMCHUN | S0611; CAS: 1310-73-2 |
| Hydrochloric acid, 35.0–37.0% | SAMCHUN | H0255; 7647-01-0 |

Deposited data

- Structures of molecules: PubChem CID: 26945, 774, 1745, 96215, 6274, 13195, 795

Software and algorithms

- Gaussian 09, Revision B.01: Frisch et al., 2016 https://gaussian.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Byunghwa Kang (kangbw93@postech.ac.kr).
Materials availability
This study did not generate new unique reagents.

Data and code availability
- This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resources table.
- This paper does not report original code, which is available for academic purposes from the lead contact upon reasonable request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

METHOD DETAILS
DFT calculations
We referred to CID: 26945, 774, 1745, 96215, 6274, 13195, and 795 as the molecular structures for DFT studies. To computationally investigate the reaction mechanism of peroxidation and free energy changes, all of the calculations in this work were performed by Gaussian 09 (G09) (Frisch et al., 2016). For more effective and faster relaxations, the heme prosthetic group without vinyl, methyl, and carboxyl groups were exploited. Catalytic complexes, consisting of three molecules (histamine or imidazole derivative, \( \text{H}_2\text{O}_2 \), and hemin), were optimized under the combination with the Lee-Yang-Parr hybrid functional (B3LYP) (Becke, 1993; Cramer, 2013; Kim and Jordan, 1994; Perdew et al., 1996; Stephens et al., 1994) and standard double-zeta potential 6-31G(d,p) basis set (Francl et al., 1982; Hariharan and Pople, 1973; Hehre et al., 1972), which is a commonly adopted combination due to its great balance between computational cost and accuracy of results (Tirado-Rives and Jorgensen, 2008). To mimic the water solution phase, the integral equation formalism variant polarizable continuum model (IEFPCM) was applied (Tomasi et al., 2005). In our calculations, the net charge of four imidazole derivatives (e.g., 4-hydroxymethyl imidazole, histidine, 4-methylimidazole, and imidazole) was set as 0, the net charge of the \( \text{H}_2\text{O}_2/\text{hemin} \) complex and histamine (\( \text{--NH}_3^+ \)) was set as +1 and the net charge of 4-imidazoleacetic acid (\( \text{--COO}^- \)) was set as -1, according to each molecule’s inherent charge at a physiological pH. Additionally, partial atomic charges of each complex are provided by Mulliken charges (Mulliken, 1955). The compound \( 0^{**} \), which is the transition state between compound \( 0^* \) and compound I, was obtained by utilizing Opt = QST2 or Opt = QST3 options.

The free energy changes (\( \Delta E_{0^*}, \Delta E_{0^{**}}, \text{and } \Delta E_{l} \)) were defined as the difference of complex energy between the next transition state and its previous state:
\[
\Delta E_{\text{int}} = E_{\text{next state}} - E_{\text{previous state}}
\]

For example, the interaction energy from compound 0 to compound \( 0^* \) (\( \Delta E_{0^*} \)) can be described as:
\[
\Delta E_{0^*} = E_{\text{compound } 0^*} - E_{\text{compound 0}}
\]

Likewise, \( \Delta E_{0^{**}} \) and \( \Delta E_{l} \) are calculated by the same method (Table S5).

Measurements of the degree of peroxidation reaction
To identify the degree of peroxidation depending on the presence of hemin and histamine, ABTS substrate and histamine were incubated together in an appropriate buffer for 5 min at room temperature. Thereafter, the mixture was transferred to a flat-bottom transparent 96-well microplate (Greiner; Kremsmünster, Austria), and then hemin solution was subsequently added to initiate the peroxidation reaction. The overall reaction condition included 500 \( \mu \text{M} \) ABTS, 5 mM histamine, 4 \( \mu \text{M} \) hemin, 10 mM KCl, 10 mM NaCl, 5 mM \( \text{H}_2\text{O}_2 \), 0.002% (v/v) Triton X-100, and 20 mM Tris-HCl (pH 7.5) (final volume: 100 \( \mu \text{L} \)). The time-dependent increase in absorbance at 418 nm was measured by the Spark 10M microplate reader, and ABTS\(^+\)-driven color change was recorded by the digital camera of a Samsung Galaxy S9+ smartphone. All peroxidation reactions were conducted at room temperature and performed in triplicate or greater.

For the steady-state kinetic assays, varying concentrations of ABTS substrate (0.1–0.8 mM) were challenged with histamine (5 mM) and hemin (4 \( \mu \text{M} \)). After 2 min, absorbance changes at 418 nm were measured. To calculate the four kinetic constants (\( K_m, k_{cat}, V_{max}, \text{and } k_{cat}/K_m \)), the extinction coefficient of ABTS\(^+\) was set as 36,000 M\(^{-1}\)cm\(^{-1}\) (Scott et al., 1993), and the Lineweaver-Burk plot was exploited.
(1/v = \(K_m/(V_{max}[S]) + 1/V_{max}\), where \(v\) is the initial velocity and \([S]\) is the initial concentration of ABTS) (Harbour and Issler, 1982; Liu et al., 2016). When we used the TMB substrate for investigating kinetic parameters, the extinction coefficient of TMB\(^+\) was set as 39,000 M\(^{-1}\)cm\(^{-1}\) (Liu et al., 2014).

To identify chosen imidazole derivatives could be cooperative with the hemin cofactor in catalyzing a peroxidation reaction, six different imidazole derivatives (1 mM) were challenged with ABTS (500 \(\mu\)M) and hemin (2 \(\mu\)M) in the identical reaction condition. Likewise, in the neurotransmitter specificity assays (biogenic amine and amino acid neurotransmitters), we used 1 mM analytes. In these two experiments, the reaction was conducted for 13 min at room temperature.

**Specific activity investigation**

To obtain the specific activity of hemin (SA\(_{hemin}\)), in the appropriate reaction condition (500 \(\mu\)M ABTS, 10 mM KCl, 10 mM NaCl, 5 mM \(H_2O_2\), 0.002\% (v/v) Triton X-100, and 20 mM Tris-HCl pH 7.5), hemin solution (4 \(\mu\)M) was introduced. Thereafter, absorbance at 418 nm was measured for 2 min. In the case of SA\(_{hemin-histamine}\), excess amounts of histamine (5 mM) were pre-mixed with 4 \(\mu\)M hemin to ensure that the concentration of the histamine-hemin pair was 4 \(\mu\)M. Likewise, compared to hemin (1 \(\mu\)M), excess amounts of G-quadruplex DNAzymes (10 \(\mu\)M) were employed to obtain the SA\(_{hemin}\) with G-quadruplex. As only low concentrations of the HRP were sufficient to elicit detectable absorbance changes, we used 10 nM HRP in this assay.

**Colorimetric histamine detection**

A mixed solution (500 \(\mu\)M ABTS, 10 mM KCl, 10 mM NaCl, 5 mM \(H_2O_2\), 0.002\% (v/v) Triton X-100, and 20 mM Tris-HCl pH 7.5) was challenged with varying concentrations of histamine (0–100 \(\mu\)M). After transferring all histamine-included solutions to the flat-bottom transparent microplate, we simultaneously added 2 \(\mu\)M of hemin (final reaction volume: 50 \(\mu\)L). Peroxidation reaction was performed for 13 min at room temperature, and then the absorbance signal (at 418 nm) of each solution was determined by the Spark 10M microplate reader, with experiments conducted in quadruplicate.

In preparing complex solutions, 100\% FBS and human urine were diluted with distilled water. Additionally, due to the anti-oxidizing environment of human urine that may prevent the oxidation of the substrate (Kirschbaum, 2001), we added excess amounts of \(H_2O_2\) to 40\% human urine, and then heat-treated (95°C for 5 min). Three prepared solutions (final concentration: 1X PBS, 50\% FBS, and 40\% human urine) were mixed with 500 \(\mu\)M TMB and 5 mM histamine, then incubated at 25°C for 5 min. Next experimental steps were performed as described above, but we measured absorbance changes at 652 nm to measure TMB\(^+\) signals, with experiments conducted in triplicate.