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Identification of Intermediate and Product from Methemoglobin-Catalyzed Oxidation of *o*-Phenylenediamine in Two-Phase Aqueous–Organic System

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Abstract—Methemoglobin (metHb) was used as a mimetic enzyme for peroxidase to catalyze the oxidation reaction of *o*-phenylenediamine (OPDA) with H_2O_2 functioning as an oxidant. A reaction intermediate was obtained in two-phase aqueous–organic system and an absorption peak at 710 nm was confirmed to be that of the intermediate in relation to OPDA. The isolated product and intermediate were characterized by UV-Vis and IR spectrophotometry and HPLC-tandem mass spectrometry. The results showed that the product is 2,3-diaminophenazine, the molecular mass of the intermediate is 212 daltons, and a conceivable structure of the intermediate is suggested. Combining the catalyzed reaction mechanism of peroxidase and our experimental results, a conceivable oxidation reaction mechanism of OPDA and H_2O_2 using metHb as catalyst is proposed.

Key words: hemoglobin, *o*-phenylenediamine, intermediate, reaction mechanism

Hemoproteins are widespread in nature and have many crucial roles. Although their functions and reactivities vary widely, all they have iron protoporphyrin IX or one of its derivatives (heme) as their prosthetic group. The four main functions of hemoproteins are the transport and storage of oxygen (hemoglobin (Hb) and myoglobin (Mb), respectively), the catalysis of H_2O_2 dismutation (catalase), the transport of electrons (e.g., cytochrome *c* and cytochrome b_5), and the catalysis of the oxidation of substrates (e.g., horseradish peroxidase (HRP) and cytochrome P-450) [1].

The variety of functions of hemoproteins stems from differences in the way the apoprotein interact with the heme and with potential substrates. The mechanisms by which the apoprotein controls the intrinsic reactivity of the heme are of both theoretical and practical interest, as are the mechanisms by which the apoprotein interacts with substrates.

Horseradish peroxidase is one of the most widely studied members of the plant peroxidase superfamily [2-4]. *o*-Phenylenediamine (OPDA) is a general substrate for the enzymatic reaction catalyzed by HRP. In a study of HRP activity with H_2O_2 and OPDA as the substrate, Gallati and Brodbech [5] assumed that azobenzene was the resulting product. Furthermore, Tarcha et al. [6] reported that the product of the oxidation of OPDA by H_2O_2, uncatalyzed or catalyzed by HRP, was 2,3-diaminophenazine. There are conflicting results on the reaction products from the oxidation of OPDA.

Hemoglobin is the major heme protein of red blood cells and is responsible for the transport of oxygen to the tissues. The function of Hb depends upon the ability of ferrous iron in the heme group to bind and release oxygen. Despite its principle role as an oxygen-carrier, the Hb molecule also possesses various enzymatic activities [7]. Hemoglobin has been reported to be able to oxidize aniline [8], lipids [9], S- and N-heterocycles [10, 11], styrene [12], *p*-cresol [13], and OPDA [14]. The last cited reaction is one, which is typically catalyzed by HRP. Zhang et al. [15] reported that the catalytic effectiveness of methemoglobin (metHb) with OPDA as a substrate was the highest compared with other mimics of peroxidase such as hemin, βCD-hemin, and manganese (III) 5,10,15,20-tetrakis(4-carboxylatophenyl) porphyrin (MnTCPP). Moreover, metHb had a higher catalytic activity than HRP when their catalytic effectiveness is

Abbreviations: OPDA) *o*-phenylenediamine; Hb) hemoglobin; metHb) methemoglobin; HRP) horseradish peroxidase.

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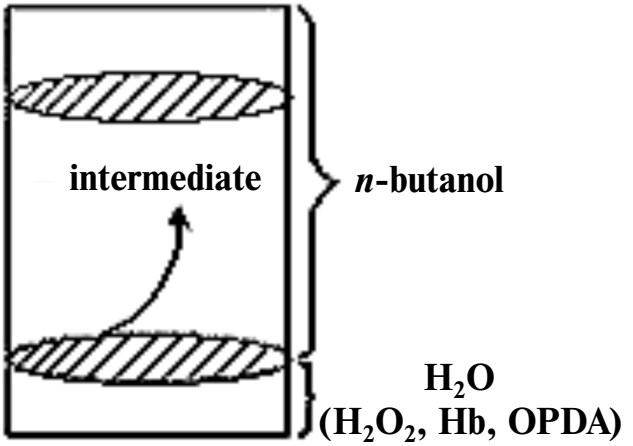
expressed on a molar basis rather than in terms of unit weight [15]. It was assumed that the product of H_2O_2-dependent oxidation of OPDA catalyzed by metHb was 2,3-diaminophenazine [15]. But its molecular structure has not been identified and the detailed molecular mechanisms of these reactions have not been worked out yet.

In this work we set out to confirm whether the product of H_2O_2 mediated oxidation of OPDA catalyzed by metHb is 2,3-diaminophenazine, the same as the product of OPDA catalyzed by HRP. The formation kinetics of an intermediate and the product were studied by stopped-flow techniques at different pH values. An intermediate was prepared and purified in a two-phase aqueous–organic system. The intermediate and product were characterized by different methods such as UV-Vis and IR spectrophotometry and tandem mass spectrometry. A mechanism for the oxidation reaction of OPDA and H_2O_2 catalyzed by metHb is proposed.



Reaction scheme
Scheme 1

MATERIALS AND METHODS

Apparatuses and reagents. UV-Vis measurements were performed on a UV-1601 rapid scan spectrophotometer (Shimadzu, Japan) using a 1 cm light path quartz cuvette. A 420A pH meter (Orion Research Inc., USA) was used. IR spectra were recorded using a NEXUS 470 spectrophotometer (Nicolet, USA), a KBr pellet being used as a window. A SFA-12 stopped-flow rapid-scan system (Hi-Tech, Japan) was used for kinetic studies. Tandem mass spectrometry was performed on-line in a triple quadrupole mass spectrometer (Apex, USA) connected to a HPLC (Waters Alliance 2795, USA) via a microspray interface.

Bovine methemoglobin from the Shanghai Biochemical Institute (Shanghai, China) was used without further purification. *o*-Phenylenediamine from Chinese Wulian Chemical Chemistry (Shanghai, China) was sublimed before use. Hydrogen peroxide solutions were prepared by appropriate dilution of the 30% solution with distilled deionized water (standardized by titration with $KMnO_4$). All other reagents were of the highest available grade, and all solutions were prepared in 0.1 M citrate-phosphate buffer (pH 5.0). All experiments were carried out at room temperature (25 ± 1°C) otherwise noted.

Preparation of the product. The product was prepared according the method reported by Tarcha et al. [6] with minor changes. *o*-Phenylenediamine (4.0 g, 22 mmol) was dissolved in 100 ml of distilled water in a 250-ml round-bottom flask equipped with a magnetic stirrer. The pH was adjusted to 5.0 with 3 N HCl. Then hydrogen peroxide (5.0 ml, 30%) was added. Methemoglobin (50 mg), dissolved in distilled water (1.0 ml), was added, and the reaction was allowed to continue for 24 h at room temperature. The precipitate formed was isolated by filtration and recrystallized from methanol giving the pure product (1.56 g, 39% yield).

Preparation of the intermediate. A 0.1 M phosphate-buffered saline (20 ml, pH 7.0) containing 10^{-4} M H_2O_2, $4.6 \cdot 10^{-3}$ M OPDA, and $2 \cdot 10^{-6}$ M metHb was added into a 250-ml beaker, and 180 ml *n*-butanol was slowly injected into the beaker. The contacting area between the water and *n*-butanol was 28 cm^2. The reaction system is shown in Scheme 1. Since the solubility of the intermediate of the reaction is higher in the *n*-butanol phase than in the water phase, the intermediate itself diffused from the aqueous phase to the *n*-butanol phase. The *n*-butanol was separated and the intermediate was recrystallized from *n*-butanol thrice giving blue crystals of the intermediate.

Kinetic studies. A buffer solution (0.2 ml) containing 10^{-4} M H_2O_2, $4.6 \cdot 10^{-3}$ M OPDA, and $2 \cdot 10^{-6}$ M Hb was put into a quartz cuvette, and 3.8 ml *n*-butanol was slowly added. The absorption changes of the *n*-butanol phase were monitored by UV-Vis spectrometry. The formation of intermediate linearly increased in the first 30 sec. During this time, a good linear relationship ($r = 0.999$) between the absorbance and the time was obtained. Therefore, in subsequent experiments the reaction rate was calculated using the data recorded in the first 30 sec. The increasing rate of the absorbance of intermediate in the *n*-butanol was measured for different ratios of *n*-butanol volume to the area of the phase contact.

RESULTS

UV-Vis absorption spectra. Figure 1 shows the UV-Vis absorption spectra of OPDA, H_2O_2, intermediate, and product. Both OPDA and H_2O_2 have an absorption peak between 200 and 330 nm. The product has two absorption maxima, at 259 and 430 nm, which are the same as those reported by Jiang et al. [16]. The intermediate also has

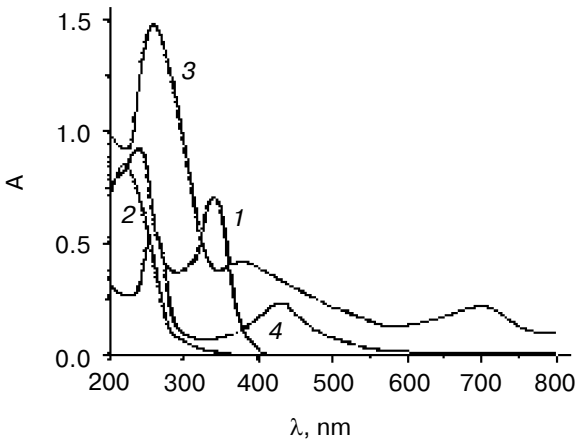


Fig. 1. UV-Vis spectra of OPDA (*1*), H_2O_2 (*2*), intermediate (*3*), and product (*4*). A 0.1 M citrate-phosphate buffer, pH 5.0, was used for OPDA, H_2O_2, and product; *n*-butanol was used for the intermediate.

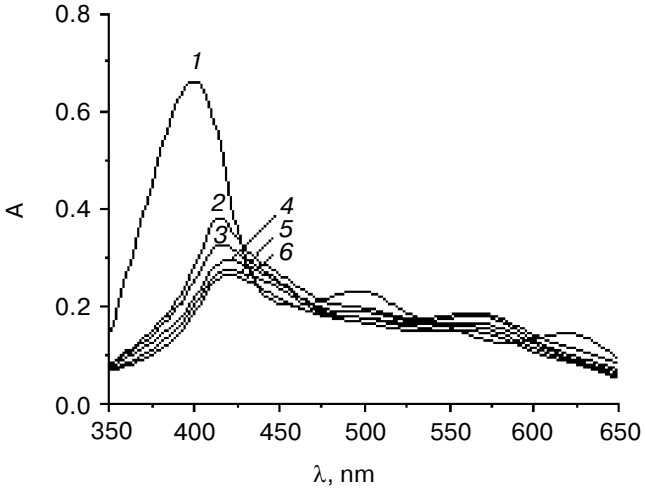


Fig. 2. UV-Vis spectra monitoring the reaction of 5 μM metHb with 25 μM H_2O_2 at 25°C in 0.1 M citrate-phosphate buffer, pH 5.0. Curves *1-6* are spectra of the reaction mixture scanned at various times (0, 1, 2, 3, 4, and 5 min, respectively).

two absorption maxima at 258 and 710 nm and a shoulder at 365 nm. The absorption at 258 nm is due to the $\pi \rightarrow \pi^*$ electronic transition of the aromatic ring, and the absorption at 710 nm due to the $n \rightarrow \pi^*$ electronic transition indicates that there is an electron-donating group in the intermediate.

Figure 2 shows the changes in absorption of metHb using the stopped-flow instrument. The observed changes in the Soret band region provide clear evidence for the formation of Hb-Compound II. Figure 2 shows the overlaid spectra of metHb during the reaction with H_2O_2 at 1 : 5 molar ratio. The metHb has absorption maxima at 400, 502, and 631 nm. Upon addition of a stoichiometric amount of H_2O_2, the Soret band shifts from 400 to 416 nm and its intensity decreases dramatically within the first minute of H_2O_2 addition. After 1 min, a second shift from 416 to 419 nm was also observed. In the visible region, within the first minute after H_2O_2 addition, the intensity of the ferric(III) bands at 502 and 631 nm decreased. New bands at 569 nm appear. These spectral bands are similar to those reported by Alayash et al. [17] in the reaction of Mb variants with H_2O_2. The Soret band at 419 nm and the other band at 569 nm involve the ferryl(IV) species–Hb(Compound II) complex [18]. The formation rate for Compound II is $7.4 \cdot 10^{-3}$ sec^{-1} at pH 5.0 and $3.9 \cdot 10^{-3}$ sec^{-1} at pH 7.0. Compound I forms very fast, with a formation rate more than ten times of that of Compound II [17, 19]. Moreover, Compound I is very unstable and easy to convert to Compound II. So it had not been detected under our experimental conditions.

Figure 3 shows the spectral changes of the *n*-butanol phase. The continuingly increasing band at 430 nm is that of the product. The bands at 365 and 710 nm increased for a while and then decreased, which demonstrates that there is a reaction intermediate during the reaction (Fig. 1). It can also be seen by visible color change that the reaction intermediate appeared first at the solution interface and then could be seen to move into the *n*-butanol phase.

Combining the results above, we can conclude that the band at 710 nm is due to the intermediate. But is the band at 710 nm due to the intermediate complex of heme and H_2O_2 (Compound I or Compound II) or that in rela-

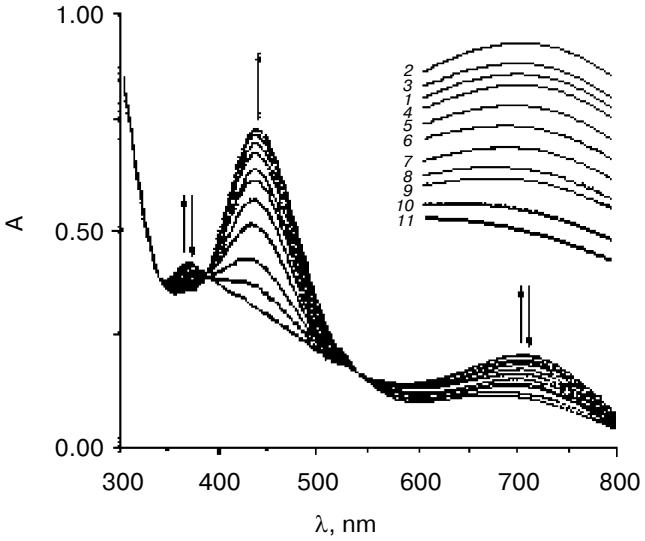


Fig. 3. UV-Vis spectra of the reaction mixture in *n*-butanol. Curves *1-11* are the spectra of the reaction mixture at various times (1, 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 min, respectively). The visible regions of curves *1-11* are also shown 4-fold expanded.

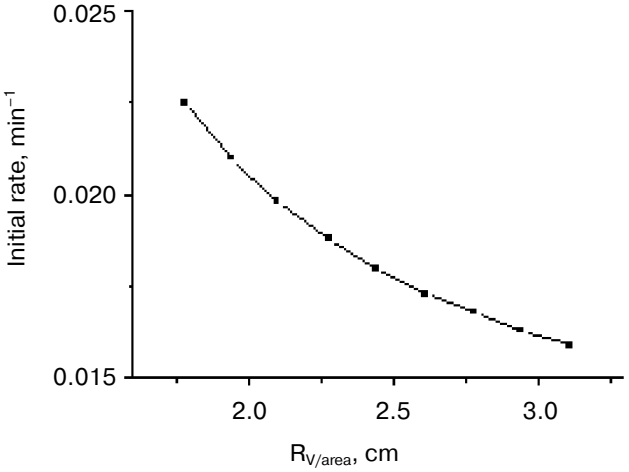


Fig. 4. Relationship between the increasing rate of the absorbance of the intermediate in *n*-butanol and the ratio of *n*-butanol volume to the area of phase contact. $R_{V/area}$ is the ratio of *n*-butanol volume to the area of phase contact. The initial rate is the absorbance increasing rate of the intermediate in *n*-butanol during the first 30 sec.

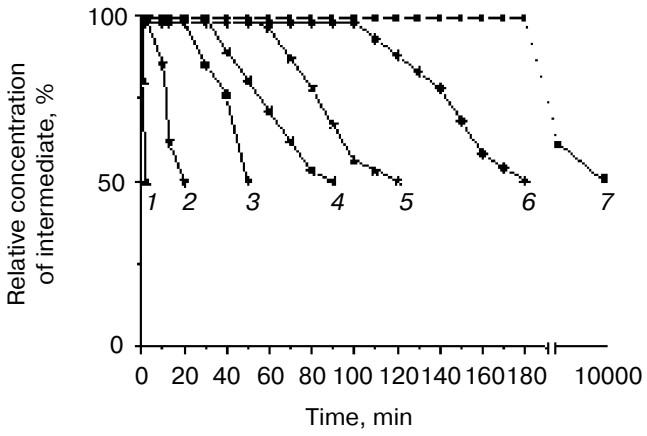


Fig. 5. Effect of temperature on the stability of the intermediate: 100 (*1*), 80 (*2*), 60 (*3*), 40 (*4*), 20 (*5*), 5 (*6*), −10°C (*7*). The solvent was *n*-butanol, and the relative concentration of the intermediate was measured by the absorption at 710 nm.

tion to OPDA? It had been reported that the reactions between Hb and H_2O_2 form the ferryl(IV) oxidation state of the protein, analogous to Compounds I and II formed in the catalytic cycle of many peroxidase enzymes [17]. These compounds have bands at 419, 539, 580, and 648, but not at 710 nm [18]. Thus, we can conclude that the 710 nm peak is due to the intermediate in relation to OPDA. The kinetic constants for the absorbance increasing rate of intermediate in the *n*-butanol at different ratios of *n*-butanol volume to the area of phase contact was tested, and the data are shown in Fig. 4. The structure of the intermediate was then studied.

Effect of temperature on the stability of the intermediate. The stability of the intermediate was studied at various temperatures in *n*-butanol. As shown in Fig. 5, the concentration of the intermediate changed with temperature, and the time required for its concentration to decrease to 50% was 2 min at 100°C, 20 min at 80°C, 50 min at 60°C, 90 min at 40°C, 120 min at 20°C, 80 min at 5°C, and 5 days at −10°C.

IR spectra of the intermediate, product, and the correlated substance. The IR absorption spectra of KBr pellets of the isolated product and intermediate are shown in Figs. 6 and 7. The spectral data are summarized in the table. The absorption at ca. 3000 and 1450-1620 cm^{-1}, which are common to OPDA and the isolated intermediate and product, are characteristics of the stretching vibration modes of the C–H and C=C bonds, respectively, of the aromatic nuclei [20, 21]. The absorption bands due to the -NH_2 stretching vibrations [20] of the amino groups of OPDA, isolated product, and intermediate were observed at 3308-3450 cm^{-1}. Two absorption peaks corresponding to the N–H stretching vibration of the amino

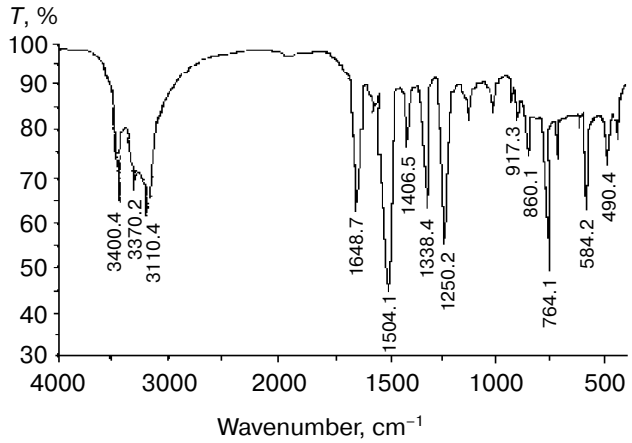


Fig. 6. IR absorption spectrum of isolated product.

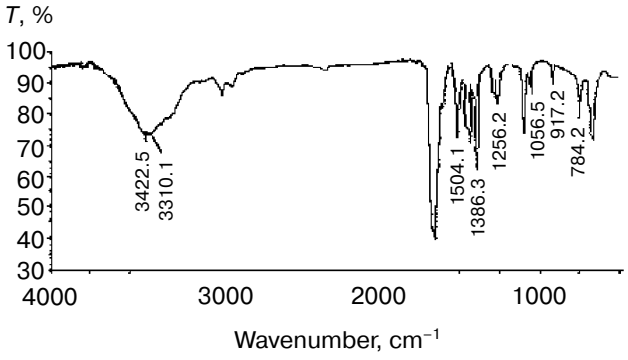


Fig. 7. IR absorption spectrum of isolated intermediate.

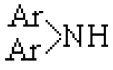
IR absorption spectra of OPDA, isolated intermediate, and product (fundamental vibrations)

Compound	Vibration mode					
	ν(C-C)[a], cm^{-1}	ν(C-H)[a], cm^{-1}	γ(C-H)[b], cm^{-1}	ν(N-H)[c], cm^{-1}	ν(C-N)[c], cm^{-1}	ν(C=N)[c], cm^{-1}
o-Phenylenediamine	1497 1590 1598	2932 3100	860	3422 3445	1255 1280	1411 1438 1499 1649
Intermediate	1504 1593	2932 3110	860 917	3422	1254	1386 1410 1439
2,3-Diaminophenazine	1504 1590 1593	2930 3110	764 860 917	3370 3400	1250 1285 1339	1439 1470 1504 1649

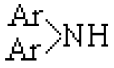
[a] Stretching vibration of the C–C and C–H bonds.
[b] Out-of-plane bending vibration of C–H bonds.
[c] Stretching vibration of the N–H, C–N, and C=N bonds.

groups, forming hydrogen bonds [20, 21], of the isolated product were observed at 3370 and 3400 cm^{-1} and one absorption peak of the isolated intermediate was observed at 3422 cm^{-1}. The absorption peaks ascribable to the stretching vibration of C–N bonds were observed at 1220-1370 cm^{-1} for all the samples [20, 21] (table). Several absorption peaks, which arise from the C–H out-of-plane bending modes [21, 22], were observed for all the samples (table). The absorption peaks of product at 1504 and 764 cm^{-1} are the characteristic absorption bands of phenazine [22]. The absorption at 1504 cm^{-1} is due to the C=C stretching vibration and the absorption peak at 764 cm^{-1} is due to the four adjacent hydrogen atoms out-of-plane bending modes of the isolated product [22].

The vibration peak of the C–N bond in C–NH_2 of OPDA, intermediate, and product appeared at 1250 and 1254 cm^{-1}, but only the intermediate had an additional absorption peak at 1386 cm^{-1}. The absorptions at 1380-1330 cm^{-1} are characteristics of the stretching vibration mode of the C–N bond in

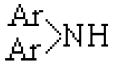


[22], and electron-donating groups of the aromatic ring tend to increase the frequency of this absorption band [22, 23]. Considering the analysis above, it is apparent that the intermediate has amino groups, which are electron-donating groups. So we can conclude the absorption at 1386 cm^{-1} of the isolated intermediate is due to the stretching vibration mode of the C–N bond in



of the intermediate.

The IR spectra of the intermediate were compared with the standard IR spectra of 5,10-dihydrophenazine. The IR spectra of the intermediate (Fig. 7) and 5,10-dihydrophenazine (Fig. 8) are the same except that 5,10-dihydrophenazine has no absorption peaks at 1254, 3422, and 860 cm^{-1}. The absorption peaks at 1254 and 3422 cm^{-1} are due to the C–N stretching vibration and the N–H stretching vibration, respectively, of the isolated intermediate [20, 21]. The absorption peak at 860 cm^{-1} is the characteristic absorption of -NH_2 out-of-plane bending mode [21, 22]. Both the intermediate (Fig. 7) and 5,10-dihydrophenazine (Fig. 8) have an absorption peak at 1386 cm^{-1}, which is assumed to be the stretching vibration mode of the C–N bond related to the carbon atom on the aromatic ring in



So we can conclude that the intermediate and 5,10-dihydrophenazine have the same structure except the intermediate has additional amino groups.

Mass spectra of the intermediate. To identify and characterize the reaction intermediate and product, mass

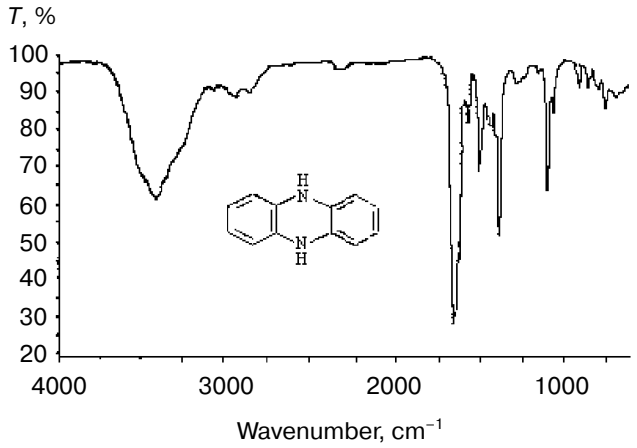


Fig. 8. The standard IR absorption spectra of 5,10-dihydrophenazine.

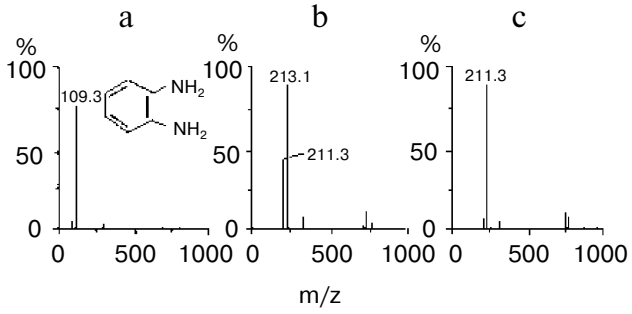
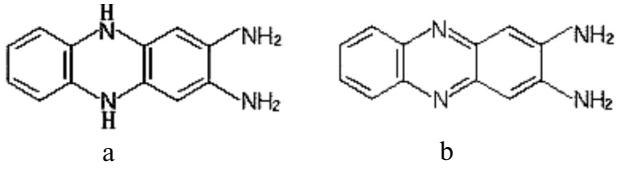


Fig. 9. Mass spectra of OPDA (a), isolated intermediate (b), and product (c).

spectra of the substrate, intermediate, and product were analyzed with positive electricity ionization mass spectrometry. The mass spectra exhibit a (M+1) signal due to the accepting of a proton under positive ionization [3]. The substrate OPDA, intermediate, and product could be seen at m/z = 109.3, 213.1, and 211.3, respectively (Fig. 9). The molecular mass of OPDA is 108.14 daltons, corresponding to the m/z = 109.3. So, we can deduce that the molecular masses of the intermediate and product are 212 and 210 daltons, respectively, from their (M+1) mass spectra signals. The appearance of m/z = 211.3 in Fig. 9b, which is corresponding to the product, indicates that some of the intermediate had converted to product during the purification and storage.

DISCUSSION

Identification of intermediate and product. Commonly, there are intermediates in every phase if an enzymatic reaction can be divided into several phases.



Proposed structures of the intermediate (a) and product (b)
Scheme 2

For enzymatic reactions, research into intermediates is very important for understanding the reaction mechanism and life phenomena [24]. It is necessary to obtain the stable intermediate in order to obtain information for analyzing the catalytic mechanism of the enzyme and for designing an inhibitor or medication. But it is usually difficult to investigate the properties of intermediates in reactions due to their low structural stability [25]. Research into the reaction intermediate will facilitate the understanding of the enzyme-catalytic mechanism and the exploration of the preparation methods of the mimetic enzyme. An intermediate related to OPDA of the oxidation reaction of OPDA catalyzed by Hb, especially connected with free radicals, has not yet been reported. We obtained an intermediate related to OPDA in the oxidation reaction of OPDA catalyzed by metHb in two-phase aqueous–organic system. The rate of absorbance increase of the intermediate in *n*-butanol with different ratios of *n*-butanol volume to area of phase contact is shown in Fig. 4. The absorbance increase rate of the intermediate in *n*-butanol is not linear with the ratio of *n*-butanol volume to the area of phase contact. This shows that the initial rate of the absorbance increase of the intermediate in *n*-butanol is diffusion dependent. The intermediate is very stable and can be conserved for a week at −10°C in *n*-butanol (Fig. 5). The structure of the intermediate and product were also studied by IR and mass spectroscopy. The experimental results show that the molecular masses of the intermediate and product are 212 and 210 daltons, respectively. In previous studies, the results obtained by Tarcha et al. [6] indicated that the product of the oxidation of OPDA by H_2O_2, catalyzed by HRP, was 2,3-diaminophenazine, while Gallati and Brodbeck [5] assumed that 2,2′-diaminoazobenzene was the resulting product. Considering the results of UV-Vis, IR, and MS spectroscopy, the product of the oxidation of OPDA by H_2O_2, catalyzed by metHb, is 2,3-diaminophenazine. Intermediate and product structure as shown in Scheme 2 is conceivable. The intermediate is 2,3-diamino-5,10-dihydrophenazine.

Mechanism of catalysis by MetHb. In general, the oxidation reactions catalyzed by heme proteins appear to follow two separate but related pathways. The first pathway is exemplified by peroxidase and involves an oxidation of ferric(III) enzyme to the ferryl(IV) form by H_2O_2 or an organic peroxide [26]:

$$H_2O_2 + \text{Protein-Fe(III)} \longrightarrow H_2O + \text{Protein}^{(+)}\text{-Fe(IV)=O}. \quad (1)$$

Two electrons are withdrawn from the ferric heme protein: one from the heme iron and one from elsewhere in the molecule. This is part of the mechanism of the peroxidase reaction. This ferryl(IV) intermediate is referred to as Compound I. Reduction of ferryl(IV) enzyme back to the native enzyme results from its interaction with any of a large number of organic compounds, which in turn, are oxidized. Normally, ferryl(IV)-peroxidases are reduced by accepting one electron at a time, thus converting the electron donor (substrate) into a free radical species [27-29]:

$$\text{Protein}^{(+)}\text{-Fe(IV)=O} + AH_2 \longrightarrow \text{Protein-Fe(IV)=O} + AH^{\cdot} + H^+, \quad (2)$$

$$\text{Protein-Fe(IV)=O} + AH_2 + H^+ \longrightarrow \text{Protein-Fe(III)} + AH^{\cdot} + H_2O. \quad (3)$$

In reaction (2), Compound I undergoes a one-electron reduction to form Compound II (which still contains a ferryl(IV)-iron) and in reaction (3) Compound II is further reduced by a single electron to form the native peroxidase. The free radical $AH^{\cdot}$ can turn into a non-radical product by different pathways [30]:

$$AH^{\cdot} + AH^{\cdot} \longrightarrow AH\text{–}AH, \quad (4)$$

$$AH^{\cdot} + AH^{\cdot} \longrightarrow A + AH_2, \quad (5)$$

$$AH^{\cdot} + AH^{\cdot} \longrightarrow \text{AH-polymers}. \quad (6)$$

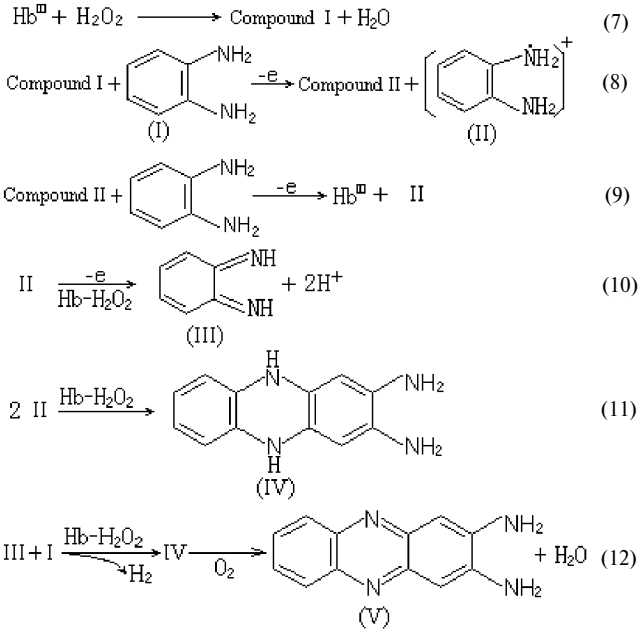
The second mechanism, which is characteristic for most oxygenases, starts with the oxy-form of the ferrous(II) heme protein and requires the presence of a reducing agent. The detail mechanism is still somewhat obscure.

It is a well-established fact and it has been known since the 1950s, that Hb possesses various pseudo-enzymatic activities and is able to catalyze the oxidation of a variety of compounds [31, 32]. It has considerable peroxidase activity [33], thus Hb is able to mimic the enzymatic activities of a variety of other heme enzymes. The fact that metmyoglobin (metMb) and metHb can also be oxidized to their respective ferryl(IV) forms has also been known for a long time [34, 35]. However, the exact structure of these compounds has only recently been established [36, 37].

The mechanisms of Hb-catalyzed oxidations are obscure [12]. George and Irvine demonstrated that one oxidation equivalent is retained by the hemoprotein when it reacts with hydrogen peroxide and proposed two mechanisms: (a) homolysis of hydrogen peroxide by Mb yields the hydroxyl radical and an Fe(IV)=O complex [38], or (b) that heterolysis yields an Fe(V)=O complex that is reduced so rapidly to the Fe(IV)=O species by the protein that it cannot be detected [39]. The two mechanisms proposed by George and Irvine for formation of the Hb or Mb Fe(IV)=O species predict the formation of a transient protein radical.

In our experiments, we observed (no absorbance peak at 630 nm) the characteristic spectra published by Giulivi and Davies [40] upon the addition of Na_2S to a H_2O_2-free solution of the oxidized Hb during the reaction. The ferryl(IV)–Hb obtained is the equivalent of Compound II. The formation of Compound I is so fast that it cannot be detected in our system. This is corresponding to the second (b) mechanism proposed by George and Irvine for formation of the Hb or Mb Fe(IV)=O species. Although the formation of Compound I cannot be detected in our system, there were some reports that the reactions between Hb and peroxides formed Compounds I and II analogous to those formed in the catalytic cycle of many peroxidase enzymes [17, 18, 40, 41].

According to the above mechanism and the structure of the isolated intermediate and product suggested, we proposed that the Hb-catalyzed reaction processes are as shown in Scheme 3. The metHb-catalyzed reaction involves the oxidation of metHb by H_2O_2 to give ferryl(IV) form (reaction (7)). This ferryl intermediate is referred to as Compound I analogous to that formed in the catalytic cycle of many peroxidase enzymes [17, 18, 33, 40, 41]. The ferryl(IV)–Hb can be reduced back to the ferric(III) form resulting from its interaction with OPDA, which in turn, is oxidized and converted into a free radical species (II). In reaction (8), Compound I undergoes a one-electron reduction to form Compound II (which still contains a ferryl(IV)-iron) and in reaction (9) Compound II is fur-



Mechanism of the reaction of OPDA with H_2O_2 catalyzed by metHb as a mimetic peroxidase
Scheme 3

ther reduced by a single electron to form metHb. The intermediate (IV) is formed when two molecules of (II) are catalyzed by Hb and H_2O_2 (reaction (11)). The free radicals (II) can also be converted to unstable double bond compound (III) containing two imine groups [42, 43] in the presence of metHb and H_2O_2 (reaction (10)). In our experiment, salmon pink color of the reaction solution formed first and then gradually turned to yellow. This salmon pink substance is due to (III), which is the semiquinone radical of OPDA [44]. The reaction of (III) with OPDA can also give (IV) after losing two H atoms (reaction (12)) [43]. In the end, product (V**)** is formed after the oxidation of (IV) by O_2 (reaction (12)). It was reported [43] that HRP in the last step reaction participates in the formation of product 2,3-diaminophenazine, but according to our experimental results, we think intermediate (IV) is unstable and can automatically turn into product in the presence of O_2 without the participation of HRP and H_2O_2. Six H atoms are lost in the reaction because the weight of two molecules of OPDA is 216 daltons while the molecular weight of the end product is 210. Further research about the physicochemical properties of the intermediate and the type of the free radicals generated in the oxidation reaction are in progress in our laboratory. However, this is not pertinent to the present work, which seeks identification of the intermediate and elucidation of the mechanism of metHb-catalyzed OPDA oxidation reaction.

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