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Horseradish peroxidase-mediated aerobic and anaerobic oxidations of 3-alkylindoles

Ke-Qing Ling and Lawrence M. Sayre*

Department of Chemistry, Case Western Reserve University, Cleveland, OH 44106, USA

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Abstract—Little is known about the HRP-mediated oxidations of 3-alkylindoles. Whereas 3-methylindole and 3-ethylindole were found to be turned over smoothly by HRP, reactions of tryptophol and *N*-acetyltryptamine were inefficient. Oxidations of the former two indoles by HRP under aerobic conditions led to the corresponding ring-opened *o*-acylformanilides and oxindoles, whereas anaerobic oxidations resulted in oxidative dimerization. The major products of anaerobic oxidation of 3-methylindole were shown to be two hydrated dimers, while anhydrodimers were obtained in the 3-ethyl case. The proposed mechanism involves HRP-mediated one-electron oxidation to give an indole radical that either dimerizes (anaerobic conditions) or reacts with O_2 (aerobic conditions). Measured kinetics of indole oxidation by HRP compounds **I** and **II** mirrored their relative reactivities under turnover conditions. The observed comparable binding affinities for all four indole substrates investigated suggest that the low reactivity of tryptophol and *N*-acetyltryptamine reflect binding to HRP in an orientation that is disadvantageous to electron transfer oxidation of the indole ring.

1. Introduction

Horseradish peroxidase (HRP) catalyzes the oxidation of phenols, anilines, and a variety of other electron-rich compounds by H_2O_2 and alkylhydroperoxides.[1] Reaction of native HRP, PFe(III), with H_2O_2 first produces HRP compound **I**, a two electron oxidized species known to be a ferryl porphyrin radical cation $P^{+\cdot}Fe(IV)=O$. One-electron reduction of compound **I** by substrate gives rise to compound **II**, PFe(IV)=O, which is then further reduced by one electron to the resting state of the enzyme. In some cases, compound **I** can be reduced directly to PFe(III) via an overall two-electron oxidation process such as in the case of oxidation of sulfides, although this reaction appears to involve direct *oxygen transfer* from compound **I** to the sulfur atom.[2] In the presence of high concentrations of H_2O_2, compound **II** can be converted to compound **III**, which can be subsequently converted to an irreversibly inactivated form of the enzyme absorbing at 670 nm.[1]

The indole structure can be found in many biologically important compounds such as the plant hormone indole-3-acetic acid (IAA), the pineal gland hormone melatonin, the neurotransmitter serotonin, the amino acid tryptophan and many other naturally occurring products. Research on HRP mediated oxidation of indole derivatives has been mainly focused on IAA in connection with its oxidative degradation by plant peroxidase, important for regulating plant growth.[3] IAA is known to react with HRP either in the presence or absence of H_2O_2 through a complicated mechanism involving HRP compounds **I**, **II**, and **III**, and ultimately the dead enzyme P-670.[4] Under physiological conditions, IAA exists as a carboxylate, and its degradation by HRP involves *decarboxylation* of the initially generated IAA radical cation, leading to the formation of CO_2 and skatolyl radical, the latter then reacting with O_2 to form indole hydroperoxide and other secondary degradation products.[4] However, there has been only limited investigation of the chemistry of HRP-mediated oxidation of 3-alkylindoles other than IAA, where decarboxylation would not be the key reaction feature. A recent study reported the ability of tryptophan, tryptamine (and its *N*-acetyl derivative), melatonin, and serotonin to reduce HRP compounds **I** and **II**, and the rates paralleled the ease of one-electron oxidation of these indoles.[5]

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* Corresponding author. Tel.: +1 216 368 3704; fax: +1 216 368 3006; e-mail: lms3@case.edu

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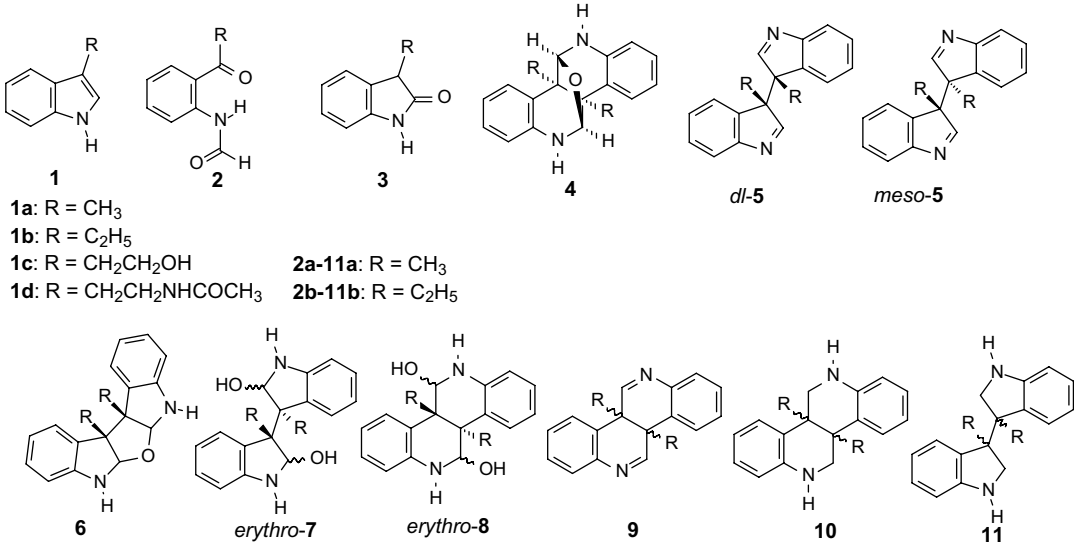
However, no turnover products were reported in this study, and turnover would be limited in most cases by the rather slow rate-limiting reduction of compound **II**. In fact, whereas HRP-mediated oxidations of melatonin and tryptophan were found in another study to generate the corresponding ring-opened 2-ketoformanilides (presumably via dioxetane intermediates), formation of these products required a high concentration of H_2O_2, was sensitive to superoxide dismutase, and was proposed to involve compound **III** rather than the normal peroxidative cycle.[6]

In this paper, we chose to investigate HRP mediated oxidations of several 3-alkylindoles (**1a**–**d**) as representatives of this biologically important class of compounds. Reactions were conducted in the presence and absence of O_2, since O_2 is often integrally involved in the product outcome of reactions involving radical intermediates. The observed differences in chemistry as a function of structural differences provide insight into the interaction of biological indoles with peroxidases.

anol, which does not alter enzyme behavior, though the rates are slower.[7] Reactions of indoles **1** were thus carried out in 50% pH 7 phosphate buffered aqueous methanol under turnover reaction conditions with a molar ratio of 1:10,000 (HRP/substrate). Starting concentrations were 15–25 mM indole substrate, 1.5–2.5 μM HRP, and the concentration of H_2O_2 was maintained at <30 μM by dropwise addition of a dilute stock solution as described in Section 4. We found that, under such turnover conditions, only **1a** and **1b** were good substrates for HRP. Reactions of **1c** and **1d** were slow, aerobically and anaerobically, and only a low conversion of substrates (<10%) could be reached after 5 h. Attempts to increase conversion by adding excess H_2O_2 resulted in loss of enzyme activity, presumably through intermediacy of compound **III** formation.[8]

Thus, compounds **1a** and **1b** became the focus of detailed investigation for HRP-mediated oxidations under both aerobic and anaerobic conditions, and the results are listed in Table 1. In addition to the identifiable prod-



2. Results and discussion

2.1. HRP-mediated oxidation of 3-alkylindoles

HRP oxidations of organic substrates only sparingly soluble in water have been carried out in aqueous methucts listed, the reactions produced variable amounts of insoluble, probably polymeric material, which accounted for the bulk of the remaining starting indole not recovered. For aerobic reactions, cleavage products **2** (major) and oxindoles **3** (minor) were obtained (Table 1). In the absence of oxygen, however, the oxidation of

Table 1. Reaction conditions and product distribution of HRP-mediated oxidation of **1a** and **1b**[a]

Substrate	Atmosphere	Reaction time[b] (h)	H_2O_2 consumed[c] (mol equiv)	Conversion (%)	Products[d] (%)			
					2	**3**	**4 + 7**	**5**
1a	Air	3	0.33	85	38	21	0	0
1a	Argon	3	0.45	77	0	0	33	0
1b	Air	5	0.32	43	38	21	0	0
1b	Argon	5	0.43	37	0	0	0	32

[a] Reactions conducted in 1:1 0.1 M pH 7.0 sodium phosphate buffer/methanol at 25 °C.
[b] The time at which point no further H_2O_2 could be consumed, as verified by the guaiacol test (see Section 4).
[c] With respect to converted indole substrate.
[d] Percent of converted indole substrate as indicated by ^{1}H NMR of the CH_2Cl_2 extract of the reaction mixture. This number may underestimate the true yields due to incompletely extraction, but otherwise, the remaining material (maximally 41–68%) represents unidentified oligomeric or polymeric products.

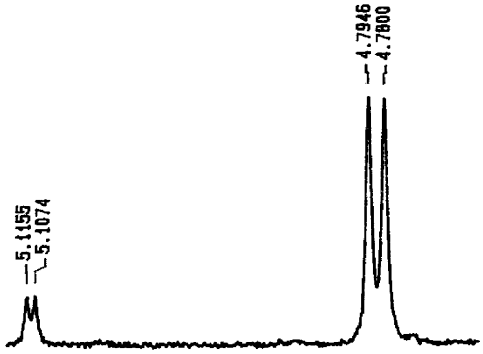
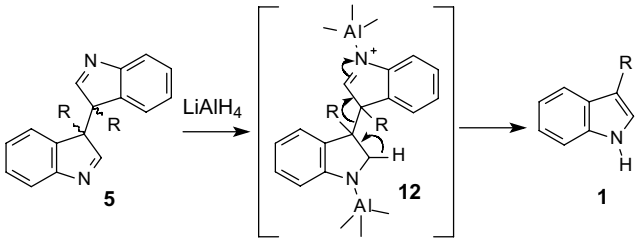


Figure 1. Crude ^{1}H NMR (300 MHz, DMSO-d_6) for the dichloromethane extract of the reaction mixture of the anaerobic oxidation of **1a**, showing the tertiary CH doublets (δ 4.79 ppm, J = 4.5 Hz, and δ 5.11 ppm, J = 2.4 Hz) of the major (**4a**) and minor (**7a**) dimers, respectively.

1a yielded two different products, both of which represent initial oxidative coupling at C3. The dimer monohydrate **4a** arising from hydration and reorganization of the initially formed dimer DL-**5a** was isolated after column chromatography.[9] The isomeric *meso*-**5a** dimer, if formed, could not be converted to a monohydrate analogous to **4a**, but could form the monohydrate **6a** or the dihydrate forms, *erythro*-**7a** or *erythro*-**8a**. The presence of one of these compounds (**6a**–**8a**) could be deduced from the crude ^{1}H NMR of the concentrated organic extract (Fig. 1), with the major doublet at 4.79 ppm being identical with that of isolated **4a** in DMSO-d_6. We were unable to isolate the minor product showing the doublet at 5.11 ppm by chromatography, probably due to its higher polarity (suggesting it is **7a** or **8a** rather than **6a**) and lower stability (see Section 4). Although **4a** is the major product as determined by TLC prior to extraction, the yield of the second product may be greater than is apparent in Figure 1 due to incomplete extraction. In sharp contrast, anaerobic reaction of **1b** gave a mixture of what ^{1}H NMR showed to be two *anhydrodimers*, whose structures were either of two possible diastereomers of **5b** and/or **9b**. No dimer hydrate was found and also no hydration reaction occurred when these anhydrodimers (**5b/9b**) were dissolved in aqueous methanol in the absence or presence of either acid (HOAc) or base (Et_3N).

Since neither the structures **5** and **9**, nor the structures **6**–**8** could be readily distinguished from each other by NMR (HMQC, HMBC, and NOESY) due to the symmetric feature of these molecules, we sought chemical evidence to resolve the structural ambiguities. $LiAlH_4$ reduction was chosen because no equilibrium interchange among **4**–**9** could occur under such conditions.[9] Also, the anticipated reduction products **10** (from **4**, **8**, or **9**) and **11** (from **5**, **6**, or **7**) would no longer undergo any tautomerization, thus permitting their full characterization. Dimer **4a** (DL) was found to be reduced quantitatively to the expected *cis* isomer (DL) of amine **10a** by $LiAlH_4$ in absolute ether.[9] Surprisingly, similar $LiAlH_4$ reduction of the suspected anhydrodimers (**5b/9b**) from anaerobic oxidation of **1b** afforded only **1b** without



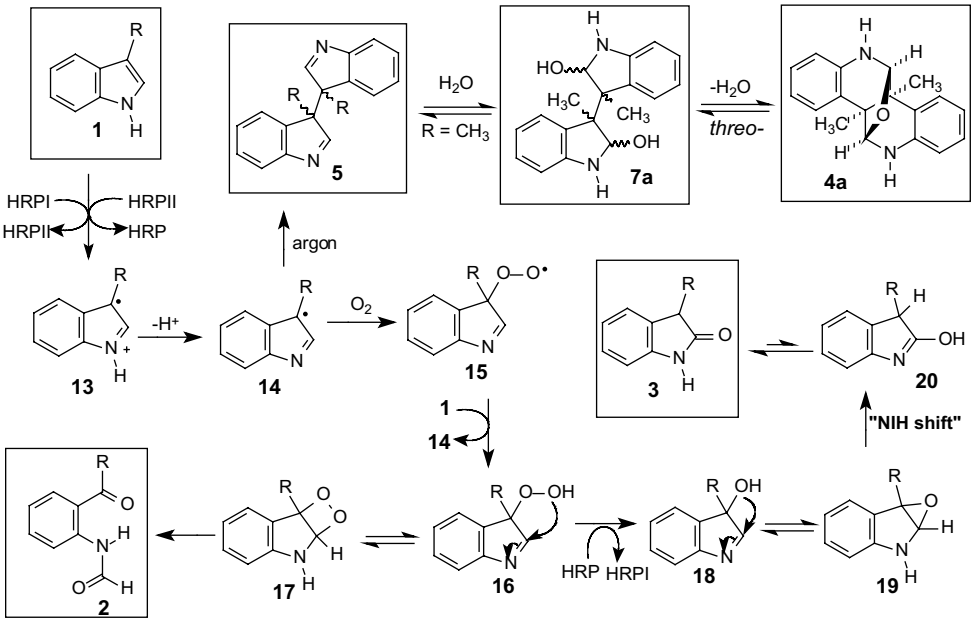
Scheme 1. $LiAlH_4$ reduction of the dimer **5**.

any products corresponding to **10b** and/or **11b**. The best explanation is that the two anhydrodimers from **1b** are *meso*- and DL-**5b**, both of which underwent facile C–C cleavage during $LiAlH_4$ reduction to give **1b** via intermediate **12b** (Scheme 1). The *aromatization* accompanying C–C cleavage in **12b** apparently provides the driving force for occurrence of this reaction to the exclusion of reduction of **12b** to **11b**. Lewis acid Al(III)-mediated C–C cleavage[10] and 1,2-alkyl shifts[11] have been observed previously in $LiAlH_4$ reductions.

The latter observation allowed us to tentatively assign the structure of the single slow-moving minor dimer hydrate (*erythro*-**7a** or *erythro*-**8a**) arising from anaerobic oxidation of **1a**. We found that $LiAlH_4$ reduction of the crude reaction mixture of anaerobic oxidation of **1a**, showing the presence of both **4a** and the minor dimer hydrate, gave only DL-**10a** along with **1a**. Since DL-**10a** arises from **4a**, the precursor to **1a** is presumably *erythro*-**7a**, which is proposed to equilibrate to its dehydrated form **5a** during the reaction with $LiAlH_4$. Reduction of *erythro*-**8a** would have been expected to give the *trans* isomer of **10a**, which was not found. The finding that the downfield CH in **7a** appears further downfield (δ 5.11 in DMSO-d_6) than for **4a** (δ 4.79 in DMSO-d_6) may reflect the fact that in the rigid structure of **4a**,[9] one of the two benzene rings is positioned to cause partial shielding of this proton.

2.2. Reaction mechanism

Control experiments showed that no reaction occurred in the absence of HRP and/or H_2O_2, indicating that *all the products are both HRP dependent and H_2O_2 dependent*. Furthermore, since catalytic amounts of HRP were used, both compounds **I** and **II** are involved in product formation (i.e., products are not being formed through a stoichiometric compound **I** pathway). The concentration of H_2O_2 was never >30 μM (at most 20:1 over HRP), and no evidence for the involvement of compound **III** was obtained. In fact, at higher [H_2O_2], where compound **III** should form, the reactions were very slow, suggesting loss of enzyme activity. TLC analysis of the reaction mixtures revealed that neither the dimers **4a** (**7a**) and **5b** were formed in aerobic reactions, nor products **2** and **3** were produced in anaerobic reactions (Table 1). This product distribution immediately leads to the conclusions that (1) products **2** and **3** are both O_2 dependent and (2) the dimeric products **4a** (**7a**) and **5b** formed under anaerobic condition are totally suppressed by O_2 under aerobic conditions.



Scheme 2. Proposed mechanism of HRP-mediated oxidation of 3-alkylindoles.

A possible mechanism for HRP mediated oxidation of 3-alkylindoles is provided in Scheme 2, with one electron oxidation of **1** by either HRP compounds **I** or **II** as the key step. The oxidation potentials of 3-alkylindoles (reduction potentials of the radical cations) have been determined in aqueous solution at pH 7 to be about 1.0–1.1 V versus NHE.[12,13] These can be compared to the redox potentials of HRP compounds **I** and **II**, both of which have been estimated to be ~0.9 V,[14] though compound **II** reactions are still typically slower than those of compound **I**.[1] This suggests that the reactions are only slightly uphill, though it has been found that HRP compound **I** oxidation of a series of ring-substituted IAAs is significantly slower than oxidation of phenols of similar potential.[4f] Such observation could signal lower binding affinities or less-productive binding modes (for electron transfer) of the indole compounds.

Since electron-transfer oxidation of aryl substrates by HRP typically follows the same initial course as do other types of chemical single-electron transfer (SET) and electrochemical oxidations, it can be presumed that the first reaction of the indole substrates with HRP compounds **I** and **II** is SET to give iminium cation **13**, which has a low pK_a and can readily dissociate at pH 7 to give the neutral imine radical **14**.[15] Radical **14** is not readily oxidized further, and thus undergoes dimerization, with **5b** being a final stable products, whereas dimers **5a** are further hydrated to form **4a** and **7a**. The different behaviors of **5a** and **5b** suggest that the C=N bond in **5b** is less susceptible to water addition than **5a**, possibly due to the steric hindrance.

For the aerobic reactions, dimerization of **14** apparently cannot compete with its trapping by O_2 to afford the hydroperoxy radical **15**, which generates hydroperoxide **16** via hydrogen atom abstraction from, for example, the starting indole **1**. The hydroperoxide **16** can cyclize to dioxetane intermediate **17**, which would undergo rapid C–C cleavage, leading to the formation of product **2**, in analogy to what has been reported for peroxidase-mediated oxidation of melatonin and related indoles.[6] Although hydroperoxide **16** can theoretically also be converted to **2** by a Criegee-like rearrangement,[16] evidence for the intermediacy of the dioxetane is that the reported HRP oxidations are highly chemiluminescent.[6] Dioxetane **17** could form instead by cyclization of peroxy radical **15**, and subsequent hydrogen atom abstraction. Alternatively, **16** could be reduced to alcohol **18**, possibly by way of converting native HRP to compound **I**. Alcohol **18** should be in equilibrium with epoxide **19**, which has been shown to arise from cytochrome P450-mediated oxidation of **1a** and which is converted, by the NIH shift mechanism (labeling study) to oxindole **3**.[17] Although **19** could also form directly from compound **I**-mediated oxygenation of **1a** (at least some substrates for HRP are thought to be accessible to direct oxygenation by compound **I**), such is not the case here, because no **3** was produced in the absence of O_2.

2.3. Kinetic studies and binding experiments

The observations that oxidation of **1a** by HRP was preparatively more efficient than that of **1b** (Table 1) and that **1c** and **1d** are not turned over smoothly by HRP, suggested a unique structural dependence for the enzymatic as opposed to simple chemical oxidation, which would not be expected to discriminate among these four compounds. To gain information on the enzymologic structure–activity relationships, we investigated the kinetics and binding features of indoles **1a–d** with HRP. First, the apparent second-order rate constants k_{app} of **1a–d** with HRP compounds **I** and **II** were determined by monitoring the generation of the product species in each case, compound **II** and native HRP, respectively, under pseudo-first order reaction condi-

Table 2. Apparent second-order rate constants of HRP compound **I** and **II** with indole substrates[a]

Substrate	k_{app} or HRP compound **I** (M^{-1} s^{-1})				k_{app} or HRP compound **II** (M^{-1} s^{-1})			
	pH 7[b]	pH 8[b]	pH 9[c]	pH 10[c]	pH 7[f]	pH 8[f]	pH 9[g]	pH 10[g]
1a[d]				2.83×10^5[e]	(2.8×10^4)[h]			1.40×10^3
1b[d]				3.54×10^5[e]	(1.7×10^4)[h]			8.67×10^2
1c	1.56×10^4	1.58×10^4	1.63×10^4	1.59×10^4	4.37×10^2	4.18×10^2	92.0	32.9
1d	3.76×10^3	3.83×10^3	3.75×10^3	3.77×10^3	1.51×10^2	1.43×10^2	30.0	10.8

[a] All the experiments were conducted at 25 °C at least twice, and the average values are reported with standard deviations less than 10%.
[b] Phosphate buffer (0.1 M); [HRP] = 1.4 μM; [H_2O_2] = 14 μM and [substrate] ⩾ 14 μM.
[c] Borate buffer (0.1 M); [HRP] = 0.7 μM; [H_2O_2] = 7 μM and [substrate] ⩾ 7 μM unless otherwise stated.
[d] Rates at low pH for compounds **I** and **II** were too fast to permit their dissection.
[e] Because the reactions were fast, only a 1.75- and 2.5-fold excess of **1a** and **1b**, respectively, were used and the rate constant was obtained from the initial stage of reaction where the pseudo first-order plots were linear.
[f] Phosphate buffer (0.1 M); [HRP] = [H_2O_2] = 2.1 μM and [substrate] ⩾ 21 μM.
[g] Borate buffer (0.1 M); [HRP] = [H_2O_2] = 2.1 μM and [substrate] ⩾ 21 μM.
[h] Estimated value using a pK_E value of 8.7 for the protonated form of HRP compound **II**.[19]

tions (Table 2). We chose to study the kinetics at neutral to basic pH (7–10) because we are interested in kinetic constants near physiologic pH, and because whereas compound **I** oxidation rates are constant in this pH range, there is a group on the enzyme that titrates at pH 5.4 to give a 10-fold more weakly oxidizing compound **I** conjugate acid.[18] This would result in a compression between compound **I** and **II** rates at lower pH, making their kinetic dissection difficult. In our chosen pH range (7–10), there is an ionization of an enzyme group around pH 8.7 that converts compound **II** to an unreactive conjugate base form.[19] Thus, the already slower compound **II** rates decrease with increasing pH. The expected constancy of compound **I** rates and decrease in compound **II** rates are supported by the data obtained for substrates **1c** and **1d** (Table 2).

For the more rapidly oxidized **1a** and **1b**, compound **I** and **II** rates could be dissected only at the highest pH (10) we used, where the compound **II** rates were now slowed by two-orders of magnitude relative to compound **I** rates. Based on their pH independence, one can estimate compound **I** rates at pH 7–9 to be the same as determined at pH 10 (Table 2). Furthermore, the rate constants for compound **II** at pH 7–9 can be estimated by multiplying the rate constants at pH 10 by the Henderson–Hasselbach correction calculated using a value of 8.7[19] for the acid dissociation constant pK_E for the active (protonated) form of HRP compound **II** (the estimated value for pH 7 is listed in Table 2).

Table 2 reveals that for all four indole substrates, reaction of HRP compound **II** is rate-limiting, and that the two simple 3-alkyindoles **1a** and **1b** are much more reactive than **1c** and **1d** toward both compounds **I** and **II**. Thus, despite **1b** having a higher rate than **1a** with compound **I**, its slower rate with compound **II** explains its lower reactivity under turnover conditions. Furthermore, despite the fast reactions of **1c** and **1d** with HRP compound **I**, their rate-limiting reactions with compound **II** are two orders of magnitude slower than are the reactions with **1a** and **1b**, explaining their near inertness under turnover conditions at neutral pH.

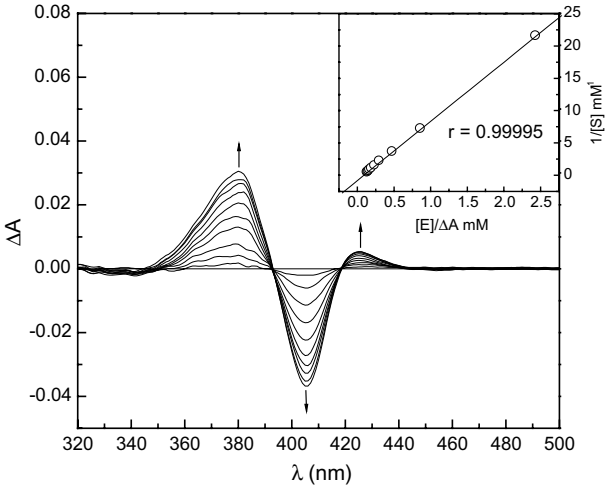


Figure 2. Difference spectra obtained by titration of HRP (10.0 μM) with 3-methylindole **1a** in 0.1 M sodium phosphate buffer (pH = 7.00) at 25 °C and the corresponding 1/[*S*] vs [*E*]/Δ*A* plots. Over the course of the titration shown, the concentration of HRP decreased from 10.0 to 9.65 μM, and the concentration of **1a** increased from 0 to 2.48 mM.

Second, the binding of **1a–d** to native HRP was examined using optical difference spectroscopy,[20] an example of which (titration of HRP with **1a**) is shown in Figure 2. From these spectra, the apparent dissociation constants K_d and the molar absorptivity differences Δε of the free and bound enzyme were obtained by correlating 1/[*S*] with [*E*]/Δ*A* (Fig. 2, Table 3, and Section 4). All four indole substrates bind to HRP and exhibit similar difference spectra with the same maxima (380 and 425 nm), minima (405 nm), and isosbestic points (393 and 417 nm). Although there are likely to be some differences between the binding of donor molecules to the activated forms of HRP (compound **I** and compound **II**) and the iron(III) resting form, it is unlikely that these differences are large, and measured binding affinities to activated HRP may not necessarily reflect productive kinetic complexes anyway.[21] Evidence that the structures of different forms of peroxidases may differ only slightly is that in the crystal structure of cytochrome

Table 3. Difference spectrum characterization and apparent dissociation constants of HRP–substrate complexes[a]

Substrate	10^3 K_d (M)	10^{-3} $\Delta\varepsilon$ (M^{-1} cm^{-1})
1a	1.24	11.4
1b	0.943	10.7
1c	1.19	6.23
1d	1.95	5.75

[a] Experimental conditions are given in Figure 2, 0.1 M sodium phosphate buffer (pH = 7.00) at 25 °C . In all four cases, the difference spectra were isosbestic and showed maxima at 380 and 425 nm and a minimum at 405 nm. Experiments were conducted at least twice and the average values are reported.

c peroxidase, compound **I** exhibits only tenths of angstrom atomic coordinate deviations from the native enzyme.[22] Most workers tend to use binding data for native HRP, obtained by optical spectroscopy[20b] or by NMR,[23] to discuss modes of binding that determine reactivity.

Studies on the redox potentials of indoles have shown little variation for substitution on the alkyl side chains (e.g., IAA vs tryptamine).[13] Thus, the divergent kinetic behavior between **1a**,**b** and **1c**,**d** is unlikely to arise from an electronic effect of the β hydroxyl and acetamido groups on the ease of one-electron oxidation of **1c**,**d**. Assuming a similarity in the redox potentials for **1a**–**d**, the slower rates for **1c**,**d** could be explained if the latter two compounds bind proportionately more weakly to compound **II**, the rate-limiting enzyme step. However, the data in Table 3 shows that there is surprisingly very little difference in binding *affinity* among the four indoles. Admittedly, the nearly twofold weaker binding for **1d** compared to **1c** could contribute to the slower rates for the former compound, but there must be an alternative explanation for the much more rapid oxidation of **1a** and **1b** relative to **1c** and **1d**. A clue to this enigma is that the molar absorptivity differences ($\Delta\varepsilon$ values) for **1c** and **1d** are lower than for **1a** and **1b**, which is consistent with the possibility that despite similar affinities, the *orientation mode of binding* of **1c** and **1d** differs from that of **1a** and **1b** in a manner that affects the kinetics of electron-transfer oxidation. Since the rates for **1c** and **1d** are retarded for both compounds **I** and **II**, it appears that the more extended and functionalized 3-alkyl side-chain impairs optimal positioning of the indole ring for SET to both oxidants, namely the heme edge in the case of compound **I** and the iron center in the case of compound **II**.[1]

Once one recognizes the possibility that rates may be affected by binding orientation independent of binding affinity, there are subtle proportionality differences in the kinetic data (Table 2) that indicate that such binding orientation may differentially affect compound **I** and compound **II** rates. For example, although the slightly higher reactivity of 3-ethyl (**1b**) vs 3-methyl (**1a**) indole toward compound **I** may reflect more favorable binding or electronic factors, the much slower reactivity of **1b** than **1a** with compound **II** probably reflects a greater steric binding requirement of compound **II**.

3. Conclusions

This is one of the first studies of HRP-mediated oxidation of 3-alkylindoles that delineates the different fates (aerobic vs anaerobic) of the reaction following initial electron transfer oxidation of the indole ring by HRP compounds **I** and **II**. The indole redox potentials are apparently sufficiently accessible that smooth turnover is possible, but this was observed only for the simple 3-methyl and 3-ethyl indoles **1a** and **1b**. In these cases, whereas anaerobic oxidations result in oxidative coupling products, aerobic oxidations result in oxygenated monomers. The much slower rates for **1c** and **1d**, despite similar binding affinities, suggest that the more extended and functionalized side chain of these indoles controls the orientation of the indole ring during binding in a manner that retards electron transfer oxidation by the heme edge (compound **I**) and the iron center (compound **II**). Binding of typical phenol and aniline reducing substrates for HRP is thought to reflect mainly hydrophobic interactions of the aryl rings with the heme edge of the protein. The electron-rich indole ring should be capable of interacting in a similar manner, and **1a** and **1b** fit this model. In contrast, we propose that hydrogen bonding of the β hydroxyl and acetamido groups of **1c** and **1d** with peptide residues around the heme edge preserve binding affinity despite removal of the indole ring from binding in a manner that is optimal for electron transfer, either due to increased distance and/or a sub-optimal spatial orientation. Further structure–reactivity studies on the HRP-mediated oxidations of substituted indoles should be a worthwhile objective.

4. Experimental

4.1. General procedures

In reporting NMR spectra, chemical shifts were referenced to TMS or the solvent peak. High resolution mass spectra (HRMS, EI, or FAB) were obtained on a Kratos MS-25A instrument. Solution pH was measured with a Fisher Accumet model 910 pH meter equipped with a Fisher combination glass electrode, calibrated to ±0.01 pH units using commercial standard buffers.

UV–vis spectra were obtained using a Perkin–Elmer Lambda 20 spectrophotometer equipped with a temperature-controlled multiple-cell compartment. Time-drive, spectral scan, and kinetics data were obtained using UV WINLAB software. 3-Methylindole (**1a**) and tryptophol (**1c**) were obtained from Aldrich Chemical Co. 3-Ethylindole (**1b**) was prepared by the reported method.[24] *N*-Acetyltryptamine (**1d**) was prepared by treatment of tryptamine with acetic anhydride. Horseradish peroxidase (type VI, 250–330 units/mg solid using pyrogallol) was obtained from Sigma Chemical Co. All yields reported are based on starting material converted. The enzyme concentration was determined spectrophotometrically at 403 nm using a molar absorptivity of 1.02×10^5 M^{-1} cm^{-1}.[25] Sodium phosphate monobasic and boric acid were reagent grade. All solutions

for kinetics and binding studies were prepared using water purified by a Milli-Q water purification system from Millipore. Hydrogen peroxide solutions were prepared by diluting commercially available 30% hydrogen peroxide with distilled water immediately before addition to enzyme reactions. Concentrations of diluted hydrogen peroxide solutions were determined by measuring the UV absorption at 240 nm, with $\varepsilon = 43.6\ M^{-1}\ cm^{-1}$.[26]

4.2. HRP mediated aerobic oxidation of 3-methylindole (1a)

To a vigorously stirred solution of **1a** (65 mg, 0.5 mmol) and HRP (3 mg, 0.05 μmol) in a mixture of methanol and 0.1 M pH 7.0 sodium phosphate buffer (1:1 v/v, totally 20 mL) was added H_2O_2 (34 mM) with a syringe in a manner that delivered ~10 μL drops. At the beginning of the reaction, the formation of HRP compound **I** (green) and its subsequent conversion to the corresponding HRP compound **II** (red) as well as the reproduction of native HRP (brown-yellow) from HRP compound **II** could be observed after each drop of H_2O_2 was added. As the reaction proceeded, however, such phenomenon was hard to visualize due to the formation of insoluble polymeric materials (minor oxidation products). Thus, the lack of buildup of H_2O_2 prior to more being added was verified by analyzing small aliquots of the reaction mixture for their ability to oxidize guaiacol. At the same time, TLC was conducted to monitor the consumption of starting material as well as the formation of products. Only 0.14 mmol of H_2O_2 was consumed when the reaction stopped at a conversion of 85%. The reaction proceeded smoothly only when a very low concentration of H_2O_2 in the reaction mixture was maintained throughout (if H_2O_2 was added too quickly, the reaction appeared to stop at a point reflecting low conversion of **1a**). Then methanol was removed in vacuo and the aqueous solution was extracted with chloroform (3 × 20 mL). The combined organic layer was dried (Na_2SO_4) and concentrated, and the residue was subjected to a silica gel flash chromatographic separation (a gradient of hexanes–ethyl acetate as eluent) to afford 10 mg of unreacted **1a** (85% conversion), and products **2a** (26.3 mg, 38%) and **3a** (13 mg, 21%). Compound **2a**:[27] ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 2.68 (s, 3H), 7.18 (t, 1H, J = 7.5 Hz), 7.57 (t, 1H, J = 7.5 Hz), 7.93 (d, 1H, J = 7.5 Hz), 8.50 (s, 1H), 8.75 (d, 1H, J = 7.5 Hz), 11.61 (br, 1H, NH). Compound **3a**:[28] ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 1.51 (d, 3H, J = 7.5 Hz), 3.47 (q, 1H, J = 7.5 Hz), 6.89 (d, 1H, J = 7.5 Hz), 7.03 (t, 1H, J = 7.5 Hz), 7.17–7.27 (m, 2H), 9.05 (br, 1H, NH); ^{13}C NMR (75 MHz, APT, $CDCl_3$): δ_C 15.4 (−), 41.2 (−), 109.9 (−), 122.5 (−), 123.9 (−), 128.0 (−), 131.4 (+), 141.3 (+), 181.6 (+).

4.3. HRP mediated aerobic oxidation of 3-ethylindole (1b)

The same procedure as for **1a** was employed, though the amounts of **1b** (43.5 mg, 0.3 mmol) and HRP (2 mg, 0.03 μmol) were smaller. Aqueous H_2O_2 (34 mM) was added dropwise, using the guaiacol test to check for accumulation of H_2O_2 in the reaction mixture. The reaction stopped when 0.041 mmol of H_2O_2 was consumed. Routine workup and chromatographic separation afforded 24.8 mg of unreacted **1b** (43% conversion), and products **2b** (8.7 mg, 38%) and **3b** (4.4 mg, 21%). Compound **2b**: light brown oil, which solidified on standing;[29] ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 1.22 (t, 3H, J = 7.5 Hz), 3.08 (q, 2H, J = 7.5 Hz), 7.16 (t, 1H, J = 7.5 Hz), 7.55 (t, 1H, J = 7.5 Hz), 7.95 (d, 1H, J = 7.5 Hz), 8.49 (s, 1H), 8.74 (d, 1H, J = 7.5 Hz), 11.66 (br, 1H, NH); ^{13}C NMR (50 MHz, APT, $CDCl_3$): δ_C 8.5 (−), 33.2 (+), 121.8 (−), 121.9 (+), 123.1 (−), 130.7 (−), 134.9 (−), 139.8 (+), 159.9 (−), 205.4 (+); HRMS (FAB) calcd for $C_{10}H_{12}NO_2$ (M+1) 178.0868, found 178.0861. Compound **3b**: light brown oil;[30] ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 0.93 (t, 3H, J = 7.4Hz), 2.04 (dq, 2H, J = 7.4 and 5.6 Hz), 3.46 (t, 1H, J = 5.6 Hz), 6.89 (d, 1H, J = 7.5 Hz), 7.03 (t, 1H, J = 7.5 Hz), 7.19–7.27 (m, 2H), 8.56 (br, 1H, NH); ^{13}C NMR (50 MHz, APT, $CDCl_3$): δ_C 10.1 (−), 23.7 (+), 47.2 (−), 109.5 (−), 122.3 (−), 124.2 (−), 127.9 (−), 129.6 (+), 141.8 (+), 180.6 (+); HRMS (EI) calcd for $C_{10}H_{11}NO$ 161.0841, found 161.0847.

4.4. HRP mediated anaerobic oxidation of 3-methylindole (1a)

A solution of **1a** (65 mg, 0.5 mmol) and HRP (3 mg, 0.05 μmol) in a mixture of methanol and phosphate buffer (1:1 v/v, totally 20 mL) was placed in a well-sealed flask. The solution was stirred vigorously and bubbled continuously with argon for 30 min using inlet and outlet syringe needles. To this solution was added dropwise freshly prepared argon-purged aqueous H_2O_2 (34 mM) while argon was still kept bubbling throughout the reaction course. The reaction stopped when 0.17 mmol of H_2O_2 had been added, with a conversion of 77%. Similar workup and chromatographic separation as described above afforded 15.3 mg of unreacted **1a** (77% conversion) and dimer **4a** (15.9 mg, 30%):[9] mp 202–203 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ_H 1.18 (s, 6H), 4.78 (d, 2H, J = 4.5 Hz, becomes a singlet on adding D_2O), 6.53 (dd, 2H, J = 0.9, 7.9 Hz), 6.67 (dt, 2H, J = 1.3, 7.5 Hz), 6.90 (d, 2H, J = 4.5 Hz, exchangeable, NH), 7.03 (dt, 2H, J = 1.4, 7.5 Hz), 7.21 (d, 2H, J = 7.9 Hz); ^{13}C NMR (APT, DMSO-d_6): δ 13.1 (−), 42.5 (+), 94.8 (−), 114.0 (−), 117.8 (−), 126.2 (−), 127.5 (−), 128.0 (+), 142.6 (+); HRMS (FAB) calcd for $C_{18}H_{19}N_2O$ (M+1) 279.1497, found 279.1504.

The above oxidation was repeated with 13 mg of **1a** and 0.6 mg of HRP. After the reaction had finished, the mixture was poured into water and extracted with dichloromethane. The combined organic layer was dried (Na_2SO_4) and evaporated to dryness, and the residue was analyzed by ^{1}H NMR in different solvents. In $CDCl_3$, the apparent minor dimer product (tentatively assigned as *erythro*-**7a**) exhibited NH and CH signals appearing at 5.27 (br) and 5.31 (s),[9] whereas in DMSO-d_6 (see Fig. 1), the CH signal appeared at 5.11 ppm (d, J = 2.4 Hz).

4.5. Reduction of 4a by lithium aluminum hydride

To a suspension of $LiAlH_4$ (19 mg, 0.5 mmol) in 5 mL of absolute ether under argon was added 14 mg of **4a** (0.050 mmol) with vigorous stirring. The mixture was stirred at room temperature until **4a** had disappeared as monitored by TLC. Then the mixture was carefully added to ice, and the resultant mixture was extracted with ether. Separation, drying, and concentration of the organic layer, and subsequent chromatographic separation of the residue afforded DL-**10a**[9] (13 mg, 98%) as a colorless viscous oil that solidified on standing. Reduction of the crude anaerobic oxidation reaction mixture of **1a** containing **4a** and *erythro*-**7a** with $LiAlH_4$ was examined in an attempt to observe the reduction product of the minor dimer *erythro*-**7a**. ^{1}H NMR of the mixture after workup revealed the presence of only DL-**10a** and **1a**.

4.6. HRP mediated anaerobic oxidation of 3-ethylindole (1b)

To a stirred solution of **1b** (43.5 mg, 0.3 mmol) and HRP (2 mg, 0.03 μmol) under argon as described for **1a** was added dropwise aqueous H_2O_2 (34 mM). The reaction stopped when 0.048 mmol of H_2O_2 was consumed. Routine workup and chromatographic separation afforded 27.4 mg of unreacted **1b** (37% conversion) and dimer **5b** as a 1:1.4 mixture of two diastereomers (5.1 mg, 32%): mp 45–49 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 0.22 (t, 6H, J = 7.5 Hz), 0.29 (t, 6H, J = 7.5 Hz), 1.93 (dq, 2H, J = 14.6 and 7.5 Hz), 1.97 (dq, 2H, J = 14.6 and 7.5 Hz), 2.05 (dq, 2H, J = 13.4 and 7.5 Hz), 2.41 (dq, 2H, J = 13.4 and 7.5 Hz), 7.06 (d, 2H, J = 7.5 Hz), 7.14 (t, 2H, J = 7.5 Hz), 7.22–7.30 (m, 6H), 7.39 (t, 2H, J = 7.5 Hz), 7.52 (d, 2H, J = 7.5 Hz), 7.63 (d, 2H, J = 7.5 Hz), 7.97 (s, 2H), 8.11 (s, 2H); ^{13}C NMR (50 MHz, APT, $CDCl_3$): δ_C 7.83 (−), 8.05 (−), 23.2 (+), 23.7 (+), 65.8 (+), 65.9 (+), 121.4 (−), 121.6 (−), 122.4 (−), 122.9 (−), 126.1 (−), 126.4 (−), 128.4 (−), 128.7 (−), 138.6 (+), 138.8 (+), 155.7 (+), 156.2 (+), 175.1 (−), 175.5 (−); HRMS (FAB) calcd for $C_{20}H_{21}N_2$ (M+1) 289.1705, found 289.1707.

4.7. Reduction of 5b by lithium aluminum hydride

To a suspension of $LiAlH_4$ (4 mg, 0.1 mmol) in 0.5 mL of absolute ether under argon was added **5b** (3 mg, 0.01 mmol) with vigorous stirring. The mixture was stirred at room temperature until all **5b** had disappeared as monitored by TLC. Then the mixture was worked up as above to afford **1b** (2.5 mg, 0.017 mmol, 85%), whose spectral data (^{1}H and ^{13}C NMR) were identical to those of the authentic sample.

4.8. Kinetics of substrate oxidations by HRP compounds I and II

HRP compound **I** was freshly prepared by mixing 3 mL of the HRP stock solution (in buffer) with a 10-fold excess of hydrogen peroxide (2.1 mM) in a 3 mL quartz cuvette, to which was added an excess of substrate, and formation of compound **II** was monitored at 419 nm (pH = 7 and 8) or 420 nm (pH = 9 and 10). Since there is an excess of substrate, and compound **II** is also reduced by substrate, albeit more slowly, the formation of compound **II** over time underestimates that which is generated by reduction of compound **I**. The excess of H_2O_2 is used to regenerate compound **I** instantaneously when compound **II** is reduced. The cycling described is equivalent to establishment of an equilibrium between compounds **I** and **II**, where k_{II} represents the rate of compound **II** reduction. According to this equilibrium, if k_{II} is less than one tenth that of k_I, the data at early stages of the reaction, where k_{II} (compound **II**) is negligible, can be analyzed by pseudo first-order kinetics.

$$\text{compound I} \underset{k_{II}(\text{slow})}{\overset{k_I(\text{fast})}{\rightleftharpoons}} \text{compound II}$$

A fresh solution of HRP compound **II** was prepared[21] by addition of 1 mol equiv of H_2O_2 to the native HRP solution (3 mL, 2.1 μM in buffer) and then waiting 45 min before initiating reaction with substrate, at which point less than 5% of residual compound **I** was present, as determined spectrophotometrically. After addition of at least 10-fold excess of substrate to the cuvette containing the HRP compound **II**, the reaction was followed by monitoring the appearance of native HRP at 403 nm. The initial portion of the reaction, corresponding to reduction of residual compound **I**, was ignored in the processing of the data. Control experiments showed that the substrates were all stable in buffer in the absence or presence of the same concentrations of H_2O_2 used above. Blank cuvettes in these experiments contained only the appropriate buffer. In all cases, the recorded time-dependent absorption (A) curves were analyzed according to the equation, $\ln[(A_\infty - A_0)/(A_\infty - A_t)] = k_{obsd}t$, to yield pseudo-first order rate constants (k_{obsd}), which were then converted to the apparent second-order rate constants listed (k_{app}, Table 2) by dividing by the indole substrate concentration ([S], 1.2–30 μM). The second-order dependence was verified by observing a linear dependence of k_{obsd} on substrate concentration in selected cases (**1c** with compound **I** and **II** at pH 8.0), which obeyed the simple equation $k_{obsd} = k_{app}[S]$. Each experiment was repeated at least two times, and the average values were reported.

4.9. HRP binding constants from difference spectrum measurements

Difference optical spectra (enzyme–substrate complex vs enzyme) were recorded. Initially, both the sample and reference cuvettes were filled with 3 mL of the HRP solution (10 μM in 0.1 M pH 7.0 sodium phosphate buffer) and an initial baseline was recorded to compensate for slight differences between the two cuvettes. Then small volumes of the substrate solution were successively added to the sample cuvette, with concomitant addition of the same volume of buffer to the reference cuvette. After each addition, both cuvettes were well mixed, and the difference spectra were recorded. Absorption spectra of the substrates at all concentrations achieved

in the successive additions were also recorded using the same procedure but in the absence of HRP. All the different spectra were then corrected by deduction of both the initial baseline and the corresponding substrate absorption spectra using ORIGIN 6.0 software. The apparent dissociation constants K_d and the molar absorptivity differences $\Delta\varepsilon$ were obtained from equation $1/[S] = [E]\Delta\varepsilon/(K_d\Delta A) - 1/K_d$.[20b]

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Supplementary data

NMR spectra for compounds **2a**,**b**, **3a**,**b**, **4a**, **5b**, and DL-**10a**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.02.013.

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