Horseradish Peroxidase Catalyzed Hydroxylations: Mechanistic Studies†

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ABSTRACT: The hydroxylation of phenol to hydroquinone and catechol in the presence of dihydroxyfumaric acid and oxygen catalyzed by horseradish peroxidase was studied under conditions where the product yield was high and the side reactions were minimal. The reaction is partially uncoupled with a molar ratio of dihydroxyfumaric acid consumed to hydroxylated products of 12:1. Hydrogen peroxide does not participate in the reaction as evidenced by the lack of effect of catalase and by the direct addition of hydrogen peroxide. Conversely, superoxide and hydroxyl radicals are involved as their scavengers are potent inhibitors. Experiments were all consistent with the involvement of compound III (oxygenated ferrous complex) of peroxidase in the reaction. Compound III is stable in the presence of phenol alone but decomposes rapidly in the presence of both phenol and dihydroxyfumaric acid with the concomitant formation of product. Therefore, phenol and dihydroxyfumaric acid must be present with compound III in order for the hydroxylation reaction to occur. A mechanism consistent with the experimental results is proposed.

Hemoproteins are ubiquitous in nature and are associated with diverse and crucial biological roles. Hemoglobin, the cytochromes P-450, and peroxidase are all proteins that contain protoporphyrin IX–heme as the prosthetic group; however, their functions and reactivities, particularly with molecular oxygen, are quite different. Hemoglobin is the oxygen-transporting protein in mammals, the cytochromes P-450 are enzymes involved in hydroxylation, and peroxidase catalyzes the peroxidative coupling of aromatic amines and phenols and is involved in lignin biosynthesis. Despite the fact that these proteins contain the same heme and, therefore, have some structural similarity, the type of chemistry carried out in each case is unique.

Hemoproteins, however, do show some common reactivities. In particular, in addition to HRP,[1] hemoglobin (Keilin & Hartree, 1935) and cytochrome P-450 (Nordblom et al., 1976) have been reported to exhibit peroxidase activity. In fact, peroxidase activity is rather common in heme-containing proteins, and in some cases, it is coupled to some other reaction such as N-demethylations by HRP (Gillette et al., 1958; Kedderis & Hollenberg, 1983) or hemoglobin (Griffin et al., 1981), peroxidative coupling of phenols by myoglobin (Keilin & Hartree, 1950), oxygen transfer by prostaglandin synthase (Marnett & Bienkowski, 1980), and hydroxylation by cytochrome P-450 (Nordblom et al., 1976).

Some time ago Mason and co-workers reported that HRP in the presence of reducing equivalents (DHFA) and O_2 catalyzed the hydroxylation of a number of aromatic compounds (Mason et al., 1957; Buhler & Mason, 1961). Although this activity of HRP, similar to that of cytochrome P-450, has received some further attention (Halliwell & Ahluwalia, 1976; Halliwell, 1977), important questions still remain unanswered regarding the differences in the chemistry of HRP-catalyzed coupling of aromatic amines and phenols vs. HRP-catalyzed hydroxylations (Whitaker, 1972). The goal of this study was to address these questions by utilizing conditions that, in contrast to those employed by others, lead to a high yield of hydroxylated products and where no appreciable side reactions with either the substrate or the product occur (Klibanov et al., 1981), thus greatly simplifying interpretation of the experimental data.

Experimental Procedures

Horseradish peroxidase (EC 1.11.1.7) type II, superoxide dismutase (EC 1.15.1.1) from bovine blood, catalase (EC 1.11.1.6) suspension from bovine liver, and dihydroxyfumaric acid were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade.

The hydroxylation of phenol by HRP was carried out in a 250-mL Erlenmeyer flask with a total volume of 25 mL. Sodium acetate (50 mM, pH 5) was used as the buffer. The temperature was controlled at 4 °C by immersing the flask in an ice–water bath. Oxygen was bubbled into the reaction vessel, and the mixture was magnetically stirred. The concentration of HRP was 0.5 mg/L (12.4 nM), and portions of DHFA (15 mg, 0.1 mmol) were added hourly. All glassware was acid-rinsed with 1 N HCl, followed by rinsing with distilled, deionized water. Product analysis was performed by HPLC. A μBondapak C_{18} reverse-phase column (Waters Assoc., Milford, MA) was used with a solvent system consisting of 10% acetonitrile in a 10 mM phosphate buffer, pH 3.0. UV detection was carried out with a Model 440 absorbance detector (Waters Assoc.) at 254 nm. DHFA was determined spectrophotometrically at 314 nm (ϵ = 3250 M^{-1} cm^{-1}).

Spectral experiments were carried out in a double-beam spectrophotometer with repetitive scans (Perkin-Elmer Model 553). The cuvettes were cooled to 4 °C by circulating ice-chilled water, and air (also cooled to 4 °C) was passed through the cuvette chamber. All solutions were equilibrated to 4 °C. The buffer used throughout the spectral studies was sodium acetate (50 mM, pH 5.0), and all enzymes and reagents were dissolved in this buffer unless otherwise noted. The rate of decay of compound III in the presence of varying concentrations of DHFA was carried out in split-compartment cuvettes. The sample cuvette contained equal volumes of HRP (4 mg/mL, 100 μM) on one side and DHFA (2–8 mM) on the

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[1] Abbreviations: HRP, horseradish peroxidase; DHFA, dihydroxyfumaric acid; SOD, superoxide dismutase; HQ, hydroquinone; HPLC, high-performance liquid chromatography.

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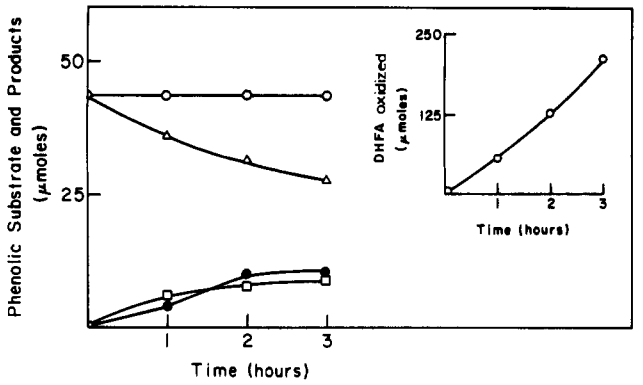


FIGURE 1: Time course and stoichiometry of HRP-catalyzed hydroxylation reaction. Reaction conditions were as described under Experimental Procedures. (○) Loss of phenol in the absence of the enzyme; (△) loss of phenol in the presence of the enzyme; (●) accumulation of hydroquinone; (□) accumulation of catechol. (Inset) (○) DHFA oxidized.

other. The reference cuvette contained HRP in one side and buffer in the other. The cuvette contents were mixed to initiate the reaction. Depending on the experiment, the course of the reaction was followed by the change in A_{417} or by repetitive scanning from 400 to 600 nm (see figure legends).

Catechol was determined colorimetrically by a modification of the Arnow method (Waite & Tanzer, 1981). The sample, 0.5 mL, was added to 0.3 mL of 0.5 N HCl, followed by addition of 0.3 mL of the molybdate nitrite solution and 0.3 mL of 1 N NaOH. The absorbance was measured at 500 nm. HRP was removed prior to catechol determinations by ultracentrifugation with Centricon-10 ultracentrifugation tubes (Amicon Corp.).

RESULTS

Phenol Hydroxylation. Phenol was chosen as a model substrate to study HRP-catalyzed hydroxylations of aromatic compounds. The time course of phenol hydroxylation is shown in Figure 1. In the presence of HRP (12.4 nM) and DHFA (0.1 mmol added hourly), nearly 40% of the phenol had reacted in 3 h. In the absence of HRP, DHFA, or O_2, no reaction took place. After 3 h, only catechol and hydroquinone were observed as products, accounting for 100% of the reacted phenol. In the same 3-h period, nearly 70% of the added DHFA was oxidized as measured by the decrease in absorbance at 314 nm. Therefore, the reaction is not highly coupled as indicated by the molar ratio of DHFA oxidized to phenol hydroxylated of about 12.

Active Oxygen Intermediates. Superoxide radicals, hydrogen peroxide, and hydroxyl radicals are reduced oxygen species that have the potential to participate, directly or indirectly, in the hydroxylation reaction. Under the assumption that one or more of these reduced oxygen species were involved in hydroxylations either as a precursor to or as the actual hydroxylating species, a series of experiments was undertaken to determine the roles of superoxide, hydrogen peroxide, and hydroxyl radicals in HRP-catalyzed hydroxylations.

Hydrogen peroxide can support hydroxylations in cytochrome P-450 catalyzed reactions (Nordblom et al., 1976) and appears to be important in hemoglobin-catalyzed hydroxylations (Mieyal et al., 1976). As shown in Table I, the addition of 10 mg/L catalase (4×10^5 IU) did not affect the HRP-catalyzed hydroxylation of phenol. Although exogenous H_2O_2 was not added to the reaction mixture, the known reaction of DHFA with O_2 yields superoxide, which would then dismute leading to in situ generation of H_2O_2 (Nilsson et al., 1969). However, if this H_2O_2 were required in the hydroxylation,

Table I: Requirements for Peroxidase-Catalyzed Hydroxylation of Phenol and Effect of Reactive Oxygen Scavengers[a]

conditions	phenol hydroxylation (% maximal)
HRP + DHFA + O_2	100
−HRP	0
$-O_2$	0
$+H_2O_2$ − DHFA	0
$+H_2O_2$	100
$+H_2O_2 - O_2$	0
+catalase (10 mg/L)	100
+SOD (10 mg/L)	40
+SOD (30 mg/L)	24
+SOD (100 mg/L)	0
+SOD (dialyzed) (100 mg/L)	80
+formate (0.2 M)	0
+mannitol (0.2 M)	0
$+Me_2SO$ (0.042 M)	0
−DHFA + ascorbate (4 mM)	0
−DHFA + NADH (4 mM)	0

[a] Conditions for the hydroxylation reaction are given under Experimental Procedures, and the concentrations of the additional components are given in the table.

catalase would have inhibited the reaction. In order to determine whether H_2O_2 was produced at the active site of HRP (inaccessible to catalase) and did take part in hydroxylations, a series of experiments involving H_2O_2, DHFA, and molecular oxygen was carried out.

Direct addition of H_2O_2 (0.1 mmol) hourly did not affect the DHFA/O_2-supported hydroxylation reaction (Table I). When either DHFA or oxygen was omitted with H_2O_2 present, no hydroxylation took place. Hence, HRP is clearly distinct in this regard from cytochrome P-450, where a peroxide shunt has been shown to occur, whereby H_2O_2 can substitute for both the oxygen and electron donor and produce a heme–H_2O_2 complex that is competent for many hydroxylation reactions (Nordblom et al., 1976). Since H_2O_2 does not play a role in the hydroxylation of phenol catalyzed by peroxidase, a difference must exist between pathways of typical peroxidative oxidations and hydroxylations catalyzed by HRP.

Superoxide radical is formed from the one-electron reduction of molecular oxygen in the presence of DHFA (Nilsson et al., 1969). Yamazaki has shown that superoxide can react with the native ferric peroxidase to give oxyperoxidase, i.e., compound III (Yamazaki & Piette, 1963; Piette, 1963; Sawada & Yamazaki, 1973). To determine whether superoxide radical indeed played a role in hydroxylations catalyzed by HRP, scavenging reactions were carried out with superoxide dismutase.

Addition of SOD inhibited the hydroxylation of phenol by HRP as shown in Table I. At 100 mg/L SOD, complete inhibition of hydroxylation had taken place. Since proteins are efficient free-radical scavengers, a nonspecific scavenging might have removed superoxide or other radicals from the system, thereby inhibiting hydroxylations. To test the possibility of SOD having a unique ability to scavenge free radicals produced during the hydroxylation of phenol, inactivated enzyme was added as a control in place of active SOD. SOD (100 mg/L) was dialyzed in 1 mM EDTA at pH 3.8 for 12 h against a 50 mM sodium acetate buffer to remove the active center copper (McCord & Fridovich, 1969). The apoprotein was found to have only 20% of the dismutase activity of a nondialyzed control. As shown in Table I, as much as 80% of the initial peroxidase activity remained following addition of dialyzed SOD. Hence, most of the inhibition caused by SOD was due to specific scavenging of superoxide radicals. It is worth noting that although 100 mg/L SOD completely

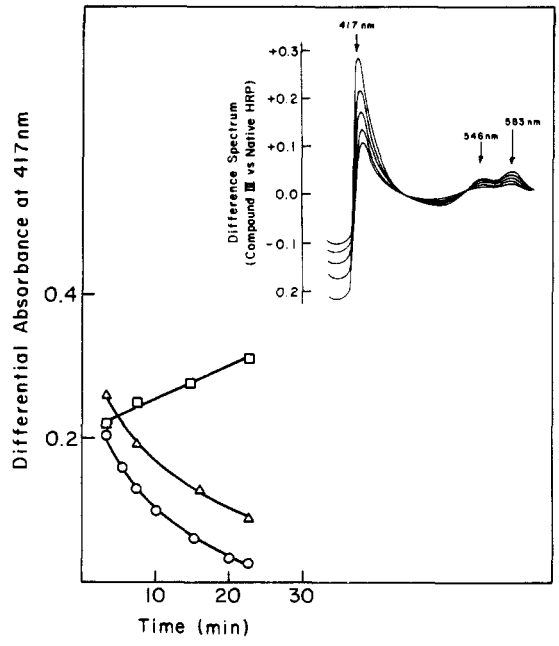


FIGURE 2: Stability of compound III vs. DHFA concentration. Reaction was carried out at 4 °C in split-compartment cuvettes. HRP (100 μM, 1 mL) was in one side of the sample and reference cuvettes and DHFA (2–8 mM, 1 mL) in the other side of the sample cuvette. Buffer (1 mL) occupied the other side of the reference cuvette. The reaction was initiated by mixing the solutions, and the difference spectrum was scanned from 400 to 600 nm as shown in the inset. The representative scan depicted in the inset had a 3 mM final concentration of DHFA. Final DHFA concentrations: (□) 1, (△) 2, and (○) 4 mM. Points represent the A_{417}.

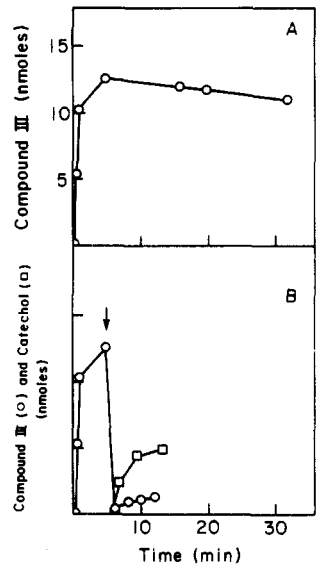


FIGURE 3: Relationship of compound III to product formation. Panel A shows the formation of compound III from HRP (140 μM) and DHFA (1 mM). Stock solutions of HRP and DHFA were chilled to 4 °C, mixed and placed on ice. At each time point, a 1:10 dilution with cold buffer was made and the difference absorbance recorded [$\Delta\epsilon$(III − HRP) = 7×10^4 M^{-1} cm^{-1}]. Panel B shows the effect of phenol addition on compound III (○) and the appearance of catechol (□). Arrow indicates the time of phenol addition. HRP and DHFA stock solutions were mixed as in (A). At 5 min, 0.1 mL was withdrawn from both the sample and reference tubes followed by addition of 0.1 mL of the buffer to the reference and 0.1 mL of buffer containing phenol to the sample. Final phenol concentration was 1 mM. Compound III concentration was 1 mM. Compound III concentration was determined as in (A) and that of catechol as reported by Waite and Tanzer (1981). Concentrations shown in the figure are corrected for the dilutions.

inhibited phenol hydroxylation, there was virtually no effect on DHFA oxidation (data not shown).

Hydroxyl radical scavengers formate, mannitol, and Me_2SO were added at the concentrations shown in Table I. No hydroxylation occurred when these scavengers were present at the given concentrations. If hydroxyl radicals form during hydroxylations catalyzed by HRP, they could hydroxylate phenol directly to catechol and hydroquinone (Norman & Radda, 1962).

Peroxidase Spectral Intermediates. Peroxidase has been shown to exist in four oxidation states (native and compounds I, II, and III) that are spectrally distinct. Compounds I and II are known to form during peroxidative oxidations (Yamazaki, 1974) and N-demethylations (Kedderis & Hollenberg, 1983) catalyzed by HRP. Compound III has been shown to form during the oxidation of DHFA, prompting speculation on a role for compound III in hydroxylations (Mason, 1957; Mason et al., 1957; Buhler & Mason, 1961). Our strategy was to preform compound III and with the addition of phenol analyze for the formation of catechol as a product of hydroxylation.

Figure 2 (inset) shows a difference spectrum indicative of the formation and decay of compound III when HRP (2 mg/mL, 50 μM) was incubated with DHFA (2 mM). There are three prominent absorbance maxima, one very intense peak at 417 nm and two smaller peaks at 546 and 583 nm, characteristic of compound III (Yamazaki & Yokota, 1965). The longer wavelength maxima are indicative of many hemoproteins in their ferrous-oxygenated states, such as oxyhemoglobin and oxymyoglobin (Waterman, 1978). With time, the decay of compound III becomes apparent (5 min between each scan), but the isosbestic points do not move, suggesting that compound III decays back to the native peroxidase without any spectrally detectable intermediates.

This decay could be due to simple dissociation of compound III yielding HRP and superoxide. However, when compound III is formed from different initial amounts of DHFA, the rate of decay is significantly affected (Figure 2). At lower DHFA concentrations, the half-life of compound III increases so that at 1 mM DHFA the formation of compound III is faster than its decay over the 25-min period that the reaction was observed. This is essentially the same result Yamazaki obtained when he reported that DHFA could not reduce compound III (Yamazaki, 1965). However, with greater amounts of DHFA, the decay of compound III is accelerated, suggesting that DHFA does participate in some way in the decomposition of compound III. The kinetics of decrease in absorbance at both 546 and 583 nm were similar to that a 417 nm (data not shown).

As further proof for the role of superoxide in the formation of compound III, we examined the formation of compound III from DHFA and oxygen in the presence of increasing concentrations of SOD. With the addition of SOD (75 mg/L), only 10% of the maximal amount of compound III formed (not shown), providing additional evidence that superoxide is required to form compound III. This is in basic agreement with the findings of Sawada & Yamazaki (1973).

The stability of compound III, when generated from 1 mM solutions of DHFA, made it possible to determine the effect of phenol on compound III without interference from DHFA. The difference in extinction coefficients between compound III and native HRP at 417 nm was found to be 7×10^4 M^{-1} cm^{-1}, which affords determination of absolute concentrations of compound III as a function of time. Upon addition of DHFA (1 mM) to HRP (140 μM), 12.5 μM compound III formed in 3 min. If no phenol was added, the decay of com-

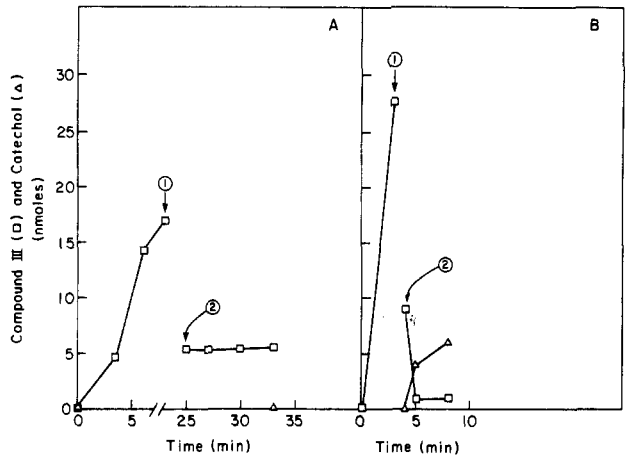


FIGURE 4: Requirement of DHFA and compound III for hydroxylation. In panel A, HRP (22 mg/mL, 0.5 mL) was mixed with DHFA (1 mM, 0.5 mL) at 4 °C. The difference absorbance against HRP and buffer was measured at 417 nm after a 1:10 dilution at the times shown. At 1 (8 min), the A_{540} was measured (isosbestic point of compound III and HRP), and 0.5 mL of the solution was applied to a Sephadex G-25 column (50–150-μm beads, 40 × 0.8 cm bed volume). The concentration of HRP in the effluent was 7.35 mg/mL (by A_{540}). At 2 (25 min), phenol (1 mM final) was added as in Figure 3. No catechol (△) formed during this incubation. HRP, which interfered with the catechol measurement, was removed with a Centricon-10 ultrafiltration tube (7000*g* for 30 min). Panel B shows nanomoles of compound III (□) and catechol (△) formed. Incubation had the same initial concentrations as in (A). At 1 (3 min), the sample was diluted with the buffer (4 °C) containing DHFA to bring the HRP concentration to 7.35 mg/mL and that of DHFA to 1 mM. At 2 (4 min), phenol was added and catechol measured as in (A).

pound III was very slow with 9.5 μM remaining after 35 min (Figure 3A). However, when phenol (1 mM final concentration) was added to the mixture, over 95% of compound III had disappeared in 0.5 min with the concomitant production of nearly 2 μM catechol (Figure 3B). The rate of this initial catechol production was 3.6 μM/min, compared to only 0.5 μM/min over the next 4.5 min. The latter rate of hydroxylation was similar to that when 1 mM DHFA and phenol are added together to initiate the reaction. Controls (data not shown) in which either DHFA or HRP was excluded from the reaction mixture produced no catechol.

Stability of Compound III. In order to determine whether DHFA was necessary for the hydroxylation of phenol with the concomitant loss of compound III, we physically separated the DHFA from compound III prior to phenol addition by passing the solution through a Sephadex G-25 column (M_r cutoff 5000). A dilution of approximately 3-fold occurred due to the peak spreading on the column. Phenol (1 μmol) was then added, and neither the decay of compound III nor the production of catechol was observed (Figure 4A). Therefore, compound III decays to result in hydroxylation only when both phenol and DHFA are present.

The concentration of HRP in this experiment was 7.35 mg/mL following the dilution due to the column. In order to confirm that DHFA is necessary for the hydroxylation reaction and to determine whether the dilution had any effect on the hydroxylation of phenol, 22 mg/mL HRP was used to form compound III, and then a 3-fold dilution was made, followed by addition of DHFA (to bring its concentration back to 1 mM) and 1 mM phenol. As shown in Figure 4B, the addition of phenol causes the decay of compound III with the concomitant hydroxylation of phenol to catechol. Thus, reducing equivalents and compound III are required for hydroxylation; dilution has no effect.

In addition to phenol, several other aromatic compounds were examined as substrates. Both catechol and (*p*-hydroxyphenyl)glycine were found to be substrates for peroxidase; catechol was oxidized to the *o*-quinone, and (*p*-hydroxyphenyl)glycine was hydroxylated to the (3,4-dihydroxyphenyl)glycine. Benzene and phenylglycine were completely unreactive. The addition of catechol or (*p*-hydroxyphenyl)glycine (both 1 mM final concentrations) to compound III caused the decay of the latter, whereas addition of either benzene or phenylglycine (also 1 mM) did not (not shown). Therefore, compounds that were oxidized also caused the decay of compound III.

Role of Electron Donor. Neither ascorbic acid nor NADH could replace DHFA as reductants in phenol hydroxylations. When ascorbic acid was used as the electron donor, compound III did not form. When compound III was preformed with DHFA, no decay was observed when 1 mM ascorbic acid was added. Since NADH is a poor one-electron donor, it was not surprising that it was unable to support hydroxylations.

The redox potential of DHFA was measured with thionine as a redox indicator dye (ZoBell, 1972). The one-electron reduction potential was 0.172 V at pH 5 and 25 °C. Under the same conditions, the redox potential for ascorbic acid was 0.127 V.

Discussion

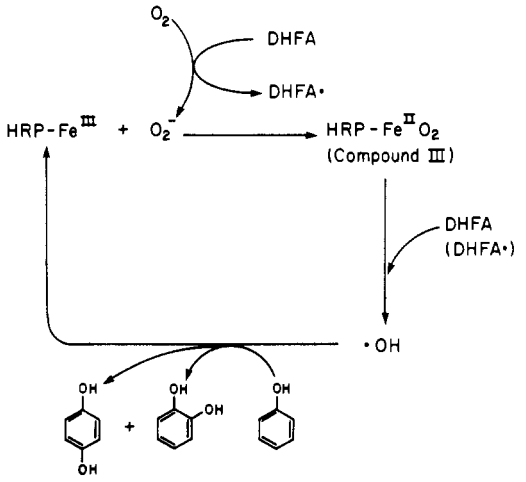
As reported previously by Klibanov and co-workers, when the HRP-catalyzed hydroxylation reaction was carried out at 4 °C, instead of at 25 °C or above, at pH 5, high yields of aromatic hydroxylation were obtained and all of the starting material could be accounted for as product (Klibanov et al., 1981); this greatly simplifies mechanistic studies and their interpretation. In this work, phenol was used as a model substrate, which yielded exclusively catechol and hydroquinone as products. Under the conditions reported here, HRP had a turnover number of 60 s^{-1}. The very fast rate of hydroxylation compared to other rather nonspecific hydroxylases prompted our consideration of the mechanism of the reaction. For example, a typical turnover number for aromatic hydroxylation catalyzed by the cytochromes P-450 is about 0.08 s^{-1} (Johnson, 1979).

The HRP-catalyzed hydroxylation of aromatic compounds is an activity that is quite different from the typical reaction catalyzed by the enzyme, namely, the peroxidative coupling of aromatic amines and phenols. The major distinction involves the inability of hydrogen peroxide to support hydroxylation. This lack of involvement of H_2O_2 was initially reported by Buhler and Mason (1961), and our findings support theirs as well as those of other laboratories studying this reaction (Halliwell, 1977). A mechanism consistent with all our results is shown in Scheme I and is different from other proposed mechanisms in that compound III is required along with substrate *and* additional reducing equivalents.

DHFA has been shown to react with oxygen in aqueous solution producing superoxide and the semiquinone of DHFA (Nilsson et al., 1969), and the reaction of superoxide with HRP leads directly to the formation of compound III (Yamazaki & Yokota, 1965; Sawada & Yamazaki, 1973). SOD, as shown in Table I, is a potent inhibitor of the hydroxylation reaction and is also equipotent in preventing the formation of compound III. Therefore, our scheme initially involves the nonenzymatic formation of O_2^- from DHFA and O_2 with the subsequent formation of compound III from HRP and O_2^-.

We also found that the stability of compound III is dependent on the concentration of DHFA. When a 1 mM solution of DHFA was used with 50 μM HRP, the resulting compound III was quite stable but decomposed rapidly when

Scheme I



higher concentrations of DHFA were used. When compound III is generated in solution by DHFA and then the DHFA is removed by gel filtration, our results show that, in order for hydroxylation of phenol to occur, phenol and additional DHFA are required. Product formation is concomitant with the quenching of the compound III chromophore. If phenol is added to compound III when the DHFA has been removed, no quenching is observed and no product is formed. Therefore, compound III is involved in the hydroxylation reaction, and the reaction requires not only substrate but also further reducing equivalents. These data allow for the completion of the minimal mechanistic scheme presented (Scheme I). These results are significant in that, for the first time, it has been shown that oxyperoxidase is directly involved in the hydroxylation reaction and that there is an absolute requirement for additional reducing equivalents in the presence of substrate. Minimally at least, one more electron is needed, and our mechanism shows that. The debate about the role of compound III, especially with regard to DHFA oxidase activity, has persisted (Yamazaki & Piette, 1963; Tamura & Yamazaki, 1972; Halliwell, 1977); however, now the species involved in HRP-catalyzed hydroxylations are clear.

We also found that compounds which were known to be substrates for HRP [catechol and (*p*-hydroxyphenyl)glycine], were competent in quenching compound III in the presence of DHFA, whereas compounds which are not substrates (benzene and phenylglycine) failed regardless of the presence of DHFA. Ascorbic acid was found by others and confirmed by us not to support hydroxylation, and we extended this to show that compound III does not form in aerobic solutions of ascorbate and HRP nor is compound III decomposed by ascorbate at any appreciable rate. The inability of ascorbate to produce compound III is definitely not related to its reduction potential because it is a stronger reductant than DHFA but instead appears to be due to its sluggish reaction with oxygen.

That catalase does not influence the reaction is mechanisticaly important. Much effort has been directed toward understanding the DHFA oxidase activity of HRP (Yamazaki & Piette, 1963). This reaction must account, at least in part, for the uncoupled stoichiometry that we observe. The DHFA oxidase reaction is somewhat complicated but does involve H_2O_2, perhaps nonenzymically (Yamazaki & Piette, 1963; Halliwell, 1977; Halliwell & DeRycker, 1978). Reports concerning various activities of HRP, e.g., aromatic amine demethylation, often can be explained by typical peroxidative chemistry. However, hydroxylation is clearly different: H_2O_2 is not involved, and therefore, compounds I and II and the chemistry associated with these structures must not be involved.

Mechanisms that speculate on hydroxylation resulting from a compound III–substrate DHFA (or DHFA·) complex are chemically quite close to those that generate compounds I and II. Depending on the mode of oxygen–oxygen bond scission at the heme site, one can write structures that are isoelectronic with compounds I and II. The relationship of HRP hydroxylation to cytochrome P-450 is unclear at this point. While the two show some similarities, there are also mechanistic differences such as the lack of an NIH shift (Daily & Jerina, 1970), the hallmark of P-450 aromatic hydroxylations. Also, HRP-catalyzed hydroxylations are inhibited by ·OH scavengers, while most cytochrome P-450 reactions are not (Morgan et al., 1982; Ingelman-Sundberg & Johansson, 1984). HRP may be generating ·OH at the enzyme's active site, which requires reducing equivalents and the substrate to be present at that site. However, the reactivity of formate and other radical scavengers with DHFA· or a ·OH equivalent that is enzyme-bound is unknown, and these reactions cannot be ruled out as points where ·OH scavengers might influence the reaction.

In summary, by using conditions that give a high yield of products and no side reactions, we have been able to formulate a chemically sensible mechanism for this unusual reaction catalyzed by HRP. The involvement of a ferrous–oxy complex (compound III) of the enzyme has been clearly established, which is in agreement with mechanistic studies on the other well-known hemoprotein hydroxylase, cytochrome P-450 (White & Coon, 1980). The ferrous–oxy complex is stable even in the presence of the substrate phenol; however, when additional reducing equivalents are present, compound III is quenched and product is formed. Other results indicate that a hydroxy radical equivalent is involved, and one can arrive at that most simply by the addition of a single electron; however, the addition of two more electrons (three total) leads formally to a ferrous–peroxy complex that can yield ·OH by the well-known Fenton pathway.

Registry No. HRP, 9003-99-0; DHFA, 133-38-0; PhOH, 108-95-2; $O_2^{\cdot-}$, 11062-77-4; OH^-, 3352-57-6.

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Structural and Mechanistic Studies on the HeLa and Chicken Liver Proteins That Catalyze Glycinamide Ribonucleotide Synthesis and Formylation and Aminoimidazole Ribonucleotide Synthesis†

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ABSTRACT: Glycinamide ribonucleotide (GAR) transformylase from HeLa cells has been purified 200-fold to apparent homogeneity with a procedure using two affinity resins. The activities glycinamide ribonucleotide synthetase and aminoimidazole ribonucleotide synthetase were found to copurify with GAR transformylase. Glycinamide ribonucleotide synthetase and GAR transformylase were separable only after exposure to chymotrypsin. Antibodies raised to pure L1210 cell GAR transformylase were able to precipitate the glycinamide ribonucleotide transformylase and GAR synthetase activities from HeLa and L1210 cells both in their native and in their proteolytically shortened forms. The compound *N*-10-(bromoacetyl)-5,8-dideazafolate was found to inhibit formylation but to leave the ATP-requiring synthetase activities intact.

The pathway of purine de novo biosynthesis was first elucidated by Buchanan and Hartman (1959); the first five steps of the pathway are shown in Scheme I. All of the enzymes in these steps have been purified to homogeneity from avian liver due to their greater abundance in avian tissues [phosphoribosylamine (PRA)[1] amidotransferase (Hartman, 1963), GAR transformylase (Caperelli et al., 1980; Young et al., 1984), FGAM synthetase (Mizobuchi & Buchanan, 1968), and GAR synthetase and AIR synthetase (Daubner et al., 1985)]. Recent work has examined the purine synthetic pathway in neoplastic mammalian tissues, in which the levels of these enzymes are elevated in comparison to normal mammalian cells (Weber et al., 1983; Jackson et al., 1979). In particular, GAR transformylase has been purified from three murine cancer cell lines (Caperelli, 1985; Daubner & Benkovic, 1985). The recent discovery by Henikoff et al. (1985) that GAR transformylase, GAR synthetase, and AIR synthetase

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[1] Abbreviations: SDS, sodium dodecyl sulfate; HBSS, Hanks' balanced salt solution; RPMI 1640, Roswell Park Memorial Institute medium 1640; DME, Dulbecco's modified Eagle's medium; PMSF, phenylmethanesulfonyl fluoride; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; DMAC, dimethylacetamide; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; PRA, phosphoribosylamine; AIR, aminoimidazole ribonucleotide; FGAM, formylglycinamidine ribonucleotide; GAR transformylase, glycinamide ribonucleotide formyltransferase; GAR synthetase, glycinamide ribonucleotide synthetase; AIR synthetase, aminoimidazole ribonucleotide synthetase; FGAR amidotransferase, formylglycinamide ribonucleotide amidotransferase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; 10-CHO-H_4folate, (6*R*)-10-formyltetrahydrofolate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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