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Chemical and Enzymatic Intermediates in the Peroxidation of *o*-Dianisidine by Horseradish Peroxidase. 2. Evidence for a Substrate Radical–Enzyme Complex and Its Reaction with Nucleophiles†

Al Claiborne and Irwin Fridovich*

ABSTRACT: Changes in the optical absorption spectrum of horseradish peroxidase, during the oxidation of *o*-dianisidine at pH 7.5, reveal an intermediate distinct from the previously described compounds I and II. The rate of decay of this new complex appeared to be rate limiting for the catalytic cycle, in this pH range, since imidazole, which augments the catalytic reaction, also enhanced the rate of decay of this complex. Nitrogenous compounds reportedly unable to ligate to hemes, such as 2-methylimidazole and benzimidazole, were nevertheless capable of augmenting the HRP-catalyzed rate of oxidation of *o*-dianisidine. The activity of nitrogenous compounds, in this regard, appeared to be a function of their nucleophilicity and was sensitive to steric factors but relatively free of a deuterium solvent isotope effect. The data presented in this and in the preceding paper [Claiborne, A., & Fridovich, I. (1979) *Biochemistry 18* (preceding paper in this issue)] lead to the suggestion that the nucleophile-responsive intermediate is an enzyme–dianisidine radical complex and that abstraction of the second electron from the bound radical is facilitated by binding of nitrogenous nucleophiles.

The preceding paper (Claiborne & Fridovich, 1979) presents evidence indicating that the peroxidation of *o*-dianisidine (3,3′-dimethoxybenzidine) by horseradish peroxidase does not occur via the classical univalent mechanism (George, 1952; Chance, 1952a,b), but rather involves direct two-electron oxidation of dianisidine by HRP[1] with the dianisidine quinonediimine as the first free product. Studies with stoichiometric concentrations of HRP were undertaken to further probe this conclusion. These results form the basis of this report. We have also investigated the basis of the stimulation of HRP-catalyzed dianisidine peroxidation by nitrogenous compounds (Fridovich, 1963). Our results suggest that these compounds act as nucleophilic catalysts facilitating electron

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[1] Abbreviations used: HRP, horseradish peroxidase; 2,6-DCQCI, 2,6-dichloroquinone 4-chloroimine; EPR, electron paramagnetic resonance.

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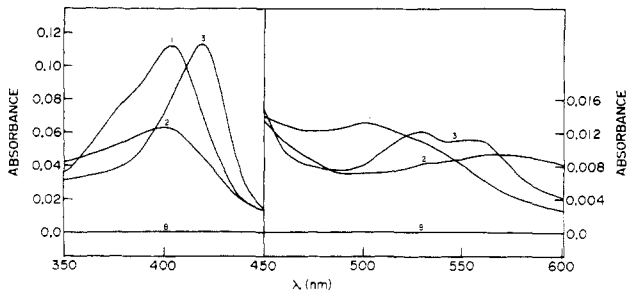

FIGURE 1: Spectra of HRP, HRP–compound I, and HRP–compound II. HRP was diluted to a final concentration of 1.1 μM in deionized water at 25 °C and scanned 2 min after mixing (scan 1). Scan 2 was obtained immediately after addition of 2.1 μM H_2O_2; scan 3 was taken 2 min after the subsequent addition of 1.5 μM sodium ascorbate. Final pH was 7.2; all spectra were recorded at a rate of 5.0 nm/s, thus yielding a complete spectrum in 50 s. Spectra in the 450–600-nm region were obtained by fivefold manual expansion of the recorded spectra at 10-nm intervals.

transfer from a substrate radical moiety in a rate-limiting step.

Materials and Methods

Horseradish peroxidase (HPOD grade, RZ = 1.0) was purchased from Worthington Biochemical Corp. and was further purified by gel filtration chromatography over a 126 × 2.7 cm column of Bio-Gel A-0.5m agarose (Bio-Rad Laboratories) in 50 mM potassium phosphate, pH 7.0. Peak fractions of A_{403}:A_{275} = 2.9–3.0 were used in these studies. Stock concentrations of peroxidase in deionized water were estimated by using an extinction coefficient $\epsilon^{403nm} = 89.5 \times 10^3$ M^{-1} cm^{-1} (Maehly, 1955). Hydrogen peroxide (30%, AR grade) was purchased from Mallinckrodt, Inc.; concentrations were determined by using an extinction coefficient $\epsilon^{240nm} = 43.6$ M^{-1} cm^{-1} (Hildebrandt & Roots, 1975). *o*-Dianisidine was a product of Sigma and of Eastman Organic Chemicals; all stocks were recrystallized as the dihydrochloride according to the procedure of Talbot et al. (1940). Concentrations of dianisidine were measured at 251 nm in 6.2 M HCl by using an extinction coefficient $\epsilon^{251nm} = 1.40 \times 10^4$ M^{-1} cm^{-1} (Møller & Ottolenghi, 1966). Sodium L-ascorbate was purchased from Sigma and was used without further purification; stock solutions were prepared fresh daily in deionized water and were protected from light to minimize autoxidation. All aqueous stock solutions were made in freshly deionized water. pH measurements were made with a Radiometer pH Meter 26 (Copenhagen).

Deuterium oxide (99.89%) was from Bio-Rad; all buffers in D_2O were corrected to final pD with the formula pD = pH + 0.4 (Lumry et al., 1951). Measurements of pH in aqueous solutions of 50% ethanol were standardized at pH 3.0 with a solution of 10^{-3} M HCl in aqueous 50% ethanol (Fridovich, 1963).

The following chemicals were purchased from Aldrich Chemical Co. at the highest available purity and were used directly: 2,6-dichloroquinone 4-chloroimine, pyridine, 4-cyanopyridine, 4-phenylpyridine, 2-benzylpyridine, 4-ethylpyridine, 4-*tert*-butylpyridine, 2-ethylpyridine, 2,6-dimethylpyridine, 3-bromopyridine, imidazole, 2-methylimidazole, and benzimidazole. Stock solutions of these compounds were, in most cases, prepared in 95% ethanol. 2,6-Diisopropylpyridine was purchased from Chemical Samples Co.; its density was estimated gravimetrically to be 0.85 g/mL. Concentrated solutions of imidazole and of 2-methylimidazole were made up in buffer whose pH was then readjusted to 7.5.

All spectral measurements and assays were made with an Aminco DW-2 UV–visible spectrophotometer, with the sample chamber and all buffers being maintained independently at 25 °C. Cuvettes were rinsed between assays with 6 M HCl:50% ethanol.

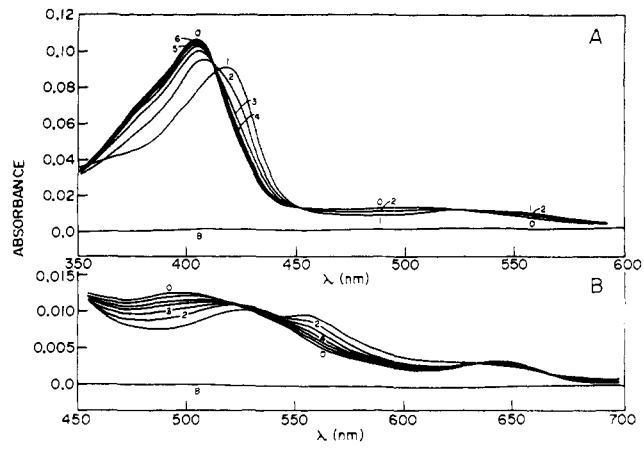

FIGURE 2: Spectral changes during the reaction of HRP with H_2O_2 and ascorbate. (A) HRP was diluted to a final concentration of 1.1 μM in 10 mM potassium phosphate (pH 7.5) and incubated for 2 min at 25 °C. Sodium ascorbate was added to a final concentration of 3.3 μM, and scan 0 was taken 1 min later. Scans 1–6 were recorded immediately following addition of 1.5 μM H_2O_2. Scan rate throughout was 5.0 nm/s, thus yielding a complete spectrum every 100 s during repetitive scans. (B) The experiment of Figure 2A was repeated in deionized water with 1.5 μM ascorbate. Scan 0 represents the HRP–ascorbate mixture after incubation for 2 min; scans 1–6 were obtained after addition of H_2O_2 at a scan rate of 5.0 nm/s. Final pH was 7.2.

Results

Enzyme Intermediates during the Peroxidation of Ascorbate. Figure 1 displays the spectrum of resting horseradish peroxidase and the spectra of HRP–compound I and HRP–compound II at neutral pH. HRP (scan 1) was diluted into deionized water at a final concentration of 1.1 μM. Compound I (scan 2) was generated by adding hydrogen peroxide to a final concentration of 2.1 μM. Without further additions, this spectrum slowly changed into a mixture of compounds I and II (not shown), due to the action of the endogenous reductant reported by Chance (1949). The use of deionized water instead of acetate or phosphate buffers in this experiment served to minimize the effect of endogenous reductant. Compound II (scan 3) was then generated by addition of 1.5 μM ascorbate to compound I at pH 7.2. These spectra are identical with those reported by Chance (1952a) at pH 7.0.

Figure 2A represents spectral changes generated during turnover of HRP with H_2O_2 and ascorbate. HRP (1.1 μM) was incubated with 3.3 μM ascorbate in 10 mM potassium phosphate at pH 7.5 (scan 0); H_2O_2 was then added to a final concentration of 1.5 μM (scans 1–6). Complete disappearance of compound I is indicated by the appearance of an isosbestic point at 412 nm for compound II and resting HRP, identical with that shown both in Figure 1 and in the report of Chance (1952a). The presence of 10 mM imidazole hydrochloride at the same pH had no qualitative effect upon the transient spectra generated and only marginally accelerated the spectral transitions observed. Figure 2B represents the results of repetitive scanning on an expanded absorbance scale during an experiment similar to that in Figure 2A. HRP (1.1 μM) was incubated with 1.5 μM ascorbate in deionized water prior to addition of 1.5 μM H_2O_2; final pH was 7.2. Complete disappearance of compound I is indicated by the appearance of an isosbestic point for compound II and HRP at 522 nm, identical with that shown in Figure 1.

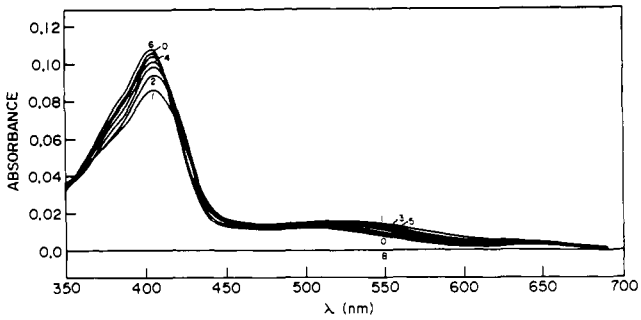

FIGURE 3: Repetitive spectra of the reaction of HRP with H_2O_2 and *o*-dianisidine. HRP (1.1 μM) was mixed with dianisidine dihydrochloride (0.3 μM) in 10 mM potassium phosphate (pH 7.5) and scanned after 1 min (scan 0). Scans 1–6 were recorded at 5.0 nm/s immediately after addition of H_2O_2 (1.5 μM).

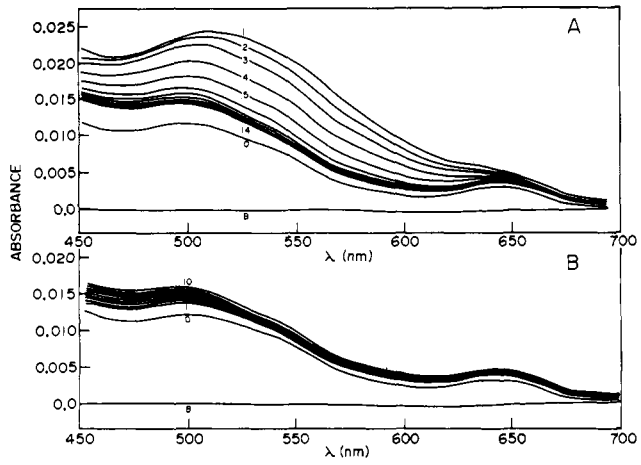

FIGURE 4: Long-wavelength spectral transitions during reaction of HRP with H_2O_2 and *o*-dianisidine. (A) The experiment presented in Figure 3 was repeated at 0.7 μM dianisidine dihydrochloride. Scan 0 represents the HRP–dianisidine mixture after 1 min; scans 1–14 were recorded after addition of H_2O_2. (B) Scan 0 represents 1.1 μM HRP in deionized water 2 min after dilution. Scans 1–10 were recorded immediately upon addition of 2,6-DCQCI to a final concentration of 0.61 mM with less than 4% overall sample dilution. Final pH was 6.9, and spectra were recorded at 5.0 nm/s. 2,6-DCQCI was added from a stock solution in 95% ethanol such that the final ethanol concentration was 3.0%.

Intermediates in the Peroxidation of o-Dianisidine by HRP. Figure 3 displays the spectral changes observed during turnover of HRP with H_2O_2 and *o*-dianisidine in 10 mM potassium phosphate at pH 7.5. HRP (1.1 μM) was incubated with 0.3 μM dianisidine (scan 0) prior to the addition of 1.5 μM H_2O_2 (scans 1–6). Compounds I and II were not observed during turnover with dianisidine. The distinct Soret band for compound II at 420 nm was absent, and the changes observed in the 450–700-nm region were completely different with dianisidine than with ascorbate.

Figure 4A expands upon the spectral changes occurring at longer wavelengths (450–700 nm) during turnover with dianisidine. This experiment, with 0.7 μM dianisidine, demonstrates a λ_{max} at 500–515 nm. Also shown in this experiment is the finite difference between the limiting spectrum (scan 14) obtained late in the reaction and the spectrum of the resting enzyme (scan 0) with dianisidine. Further addition of dianisidine alone did not alter this limiting spectrum, indicating that essentially complete reduction had indeed occurred. Furthermore, assay of the sample following this experiment showed it to be fully active (at least 90–95%). The quinonediimine product of dianisidine oxidation does absorb in this region of the visible spectrum (Claiborne & Fridovich, 1979), but was present at too small a level to contribute

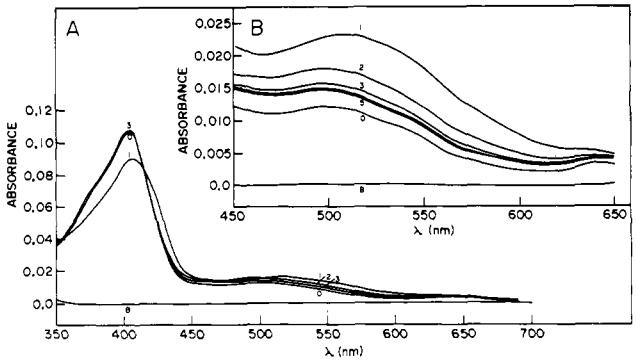

FIGURE 5: Repetitive spectra of the reaction of HRP with H_2O_2 and *o*-dianisidine in the presence of imidazole. (A) The experiment of Figure 3 was repeated in 10 mM potassium phosphate–10 mM imidazole hydrochloride (pH 7.5). Scan 0 represents the HRP–dianisidine mixture after 1 min; scans 1–3 were obtained immediately after addition of H_2O_2. (B) The experiment of Figure 4A was repeated in the phosphate–imidazole hydrochloride buffer. Scan 0 again represents the HRP–dianisidine mixture after 1 min; scans 1–5 were obtained immediately upon addition of H_2O_2. All scans were recorded at 5.0 nm/s, as in Figures 3 and 4A.

significantly. This product was shown in the preceding paper to be capable of pairing with appropriate electron donors in charge-transfer interactions. The possibility of similar interactions with the enzyme was therefore considered. We have shown 2,6-dichloroquinone 4-chloroimine (Claiborne & Fridovich, 1979) to be chemically similar to the dianisidine quinonediimine in terms of its reaction with butylated hydroxyanisole. We therefore tried to mimic the limiting spectrum of Figure 4A by addition of 2,6-DCQCI to a solution of resting HRP. The result of this experiment is shown in Figure 4B. There was a time-dependent positive spectral perturbation in the 450–700-nm region which was quantitatively reversible by dialysis and which led to no change in enzymatic activity measured with or without 10 mM imidazole hydrochloride at pH 7.5. Controls with ethanol, which was the solvent for 2,6-DCQCI, showed no effect on the spectrum. We conclude that the finite difference between the limiting and initial HRP spectra of Figure 4A is due to reversible binding of the dianisidine quinonediimine to HRP. This binding does not alter the catalytic activity of samples subsequently assayed at 600-fold dilution.

Imidazole and the HRP–Dianisidine Complex. Figures 5A and 5B demonstrate the effect of 10 mM imidazole hydrochloride upon the spectra of HRP during turnover with dianisidine and H_2O_2 at pH 7.5. There do not appear to be any qualitative changes in the intermediate spectra so generated; however, it is clear that the rate of turnover of the limiting enzyme–substrate complex is faster in the presence of imidazole than in its absence (Figures 3 and 4A). The kinetic aspects of the effect of imidazole are better demonstrated in Figures 6A and 6B, where enzyme turnover in the presence of dianisidine and H_2O_2 is monitored at 411 and 510 nm, respectively, each in the absence and in the presence of 10 mM imidazole at pH 7.5. The effect of imidazole upon this presumed enzyme–substrate complex provides us with a means of identifying this spectral species as a catalytically important complex and affords us the opportunity of developing a mechanistic understanding of the phenomenon of boosting of HRP-catalyzed dianisidine peroxidation by nitrogenous compounds (Fridovich, 1963).

Mechanism of Augmentation of Dianisidine Peroxidation by Nitrogenous Compounds. The work of Manning et al. (1969) showed that the anodic oxidation of 9,10-diphenylanthracene in tetraethylammonium perchlorate–acetonitrile

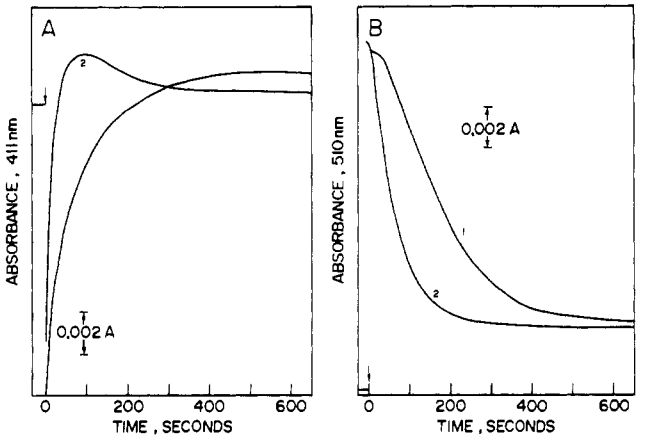

FIGURE 6: Rates of HRP turnover with H_2O_2 and dianisidine and the effect of imidazole. (A) HRP (1.1 μM) was mixed with 0.3 μM dianisidine dihydrochloride in 10 mM potassium phosphate (pH 7.5), and the base line was recorded 2 min later at 411 nm. Curve 1 represents the reaction monitored at 411 nm immediately upon addition of 1.5 μM H_2O_2, recorded at 100 s/in. Curve 2 represents the identical reaction carried out in 10 mM potassium phosphate–10 mM imidazole hydrochloride (pH 7.5). (B) Analogous experiments as in A were monitored at 510 nm, with both reactions being carried out in 0.8 μM dianisidine dihydrochloride. Curve 1 again represents the reaction in 10 mM phosphate, while curve 2 records the rate of the reaction in the phosphate–imidazole buffer. Arrows indicate base lines prior to addition of H_2O_2.

proceeded via distinct univalent steps. Oxidation of the corresponding aromatic cation radical was susceptible to stimulation by pyridine nucleophiles. Manning et al. were able to demonstrate that this stimulation was correlated with nucleophilicity as predicted from the effects of electron-withdrawing (4-cyano-) and electron-donating (4-methyl-) substituents. When tested at identical concentrations of 5.0 mM, a series of three substituted pyridines behaved, in terms of their ability to boost HRP peroxidation of dianisidine, as would be predicted from their nucleophilic character. Neither 4-cyanopyridine (pK_a = 1.90) nor 3-bromopyridine (pK_a = 2.84) gave any appreciable stimulation when HRP was assayed at 3.4×10^{-5} M dianisidine and 1.5×10^{-4} M H_2O_2 in 10 mM potassium phosphate, pH 7.5. 4-Ethylpyridine, however, was nearly 30% more effective than pyridine (pK_a = 6.02, 5.17, respectively), which was itself an effective booster.

However, most of the experimental procedures employed to correlate catalysis with nucleophilicity do not make a clear distinction between nucleophilicity and basicity. Indeed, in most cases nucleophilicity may be largely dictated by pK_a (Bruice & Benkovic, 1966). At least three criteria have been developed to distinguish these two catalytic effects (Bruice & Benkovic, 1966) and were utilized here. First, general base catalysis is characterized by a significant deuterium solvent isotope effect, with values of k_H/k_D usually greater than 2.0. Second, if basicity is the only factor controlling the behavior of a known catalyst, then dibasic phosphate (HPO_4^{2-}) would be expected to perform more effectively than imidazole (pK_a = 7.2, 7.05, respectively). Third, general bases, since they only interact with protons, do not exhibit marked steric effects upon the substitution of bulky groups near the basic center. Nucleophiles, on the other hand, are sensitive to such effects, since they most often interact with positive centers on sp^2 hybridized carbon.

The application of these criteria to the stimulatory effects of nitrogenous compounds upon the peroxidation of dianisidine by HRP yielded the following results. First, as shown in Table I, the HRP-catalyzed reaction with dianisidine in the presence of four different nitrogenous compounds at pH(D) 7.5 showed

Table I: Kinetic Solvent Isotope Effects for the HRP-Catalyzed Peroxidation of Dianisidine in the Presence of Nitrogenous Activators[a]

activator	buffer	k_H/k_D
pyridine	10 mM phosphate	1.33
2,6-dimethylpyridine	10 mM phosphate	1.45
2-methylimidazole	20, 40 mM phosphate	1.33
imidazole	20 mM phosphate	1.48

[a] Assay conditions: 3.4×10^{-5} M dianisidine dihydrochloride, 1.5×10^{-4} M H_2O_2 in indicated buffers at pH(D) 7.5 with 0.02 nM HRP and nitrogenous compounds indicated at 5.0 mM (free base concentration). Assays were performed in triplicate over the first 20 s of reaction.

no solvent isotope effect (98–99% D_2O) greater than 1.48, considerably below the value of 2.0 set as a minimum for standard general base catalysis. The small effects observed could be explained by secondary isotope effects upon the enzyme. Second, raising the total phosphate concentration from 10 to 50 mM at pH 7.5 did not affect HRP activity with 1.35×10^{-4} M dianisidine and 8.68×10^{-4} M H_2O_2. Finally, when compared in 50% aqueous ethanol–50 mM potassium phosphate, pH 7.5, 2,6-diisopropylpyridine at 20 mM had no significant effect on the HRP–dianisidine reaction; whereas, pyridine at the same concentration boosted the reaction rate by 50–52%. This behavior held in spite of the fact that the former compound is a better base in 50% aqueous ethanol than is pyridine (pK_a = 5.34, 4.38, respectively; Brown & Kanner, 1953). We conclude that steric effects play a substantial role in the interaction of nitrogenous compounds with the HRP–dianisidine system. Furthermore, the lowering of pK_a for pyridine from 5.17 (aqueous) to 4.38 (50% ethanol) most probably explains the lower stimulatory effect observed with pyridine in 50% ethanol as compared with aqueous buffer.

All of the nitrogenous compounds reported by Fridovich (1963) to significantly boost activity could be classified as good nucleophiles; in addition, the observation that only the peroxidations of *o*-dianisidine and *p*-phenylenediamine by HRP were susceptible to such stimulation implicated a special class of HRP–substrate intermediates in this respect. A simple kinetic model for use in studies of this stimulation was developed in the original report (Fridovich, 1963), and presumes reversible formation of an enzyme–substrate–nitrogenous "ligand" complex under k_4-limited conditions; this model did account for the kinetic aspects of boosting by nitrogenous compounds.

Double-reciprocal plots of the increment $(v'-v)$ against the "ligand" concentration were shown to yield linear relationships with *y*-axis intercepts giving the reciprocal of a maximal increment extrapolated to infinite "ligand" concentration, and *x*-axis intercepts of $-(1/K_{N_i})$, with K_{N_i} representing the concentration of "ligand" required for half-maximal stimulation.[2] Figures 7A and 7B display the results of such plots obtained with imidazole and 2-ethylpyridine, respectively. The nonlinear deviations observed at high concentrations of both compounds appear to involve some inhibitory effect of these compounds; solubility limitations were easily dismissed as a possible cause of this behavior. Similar patterns were seen for most of the other compounds tested; Table II summarizes the K_{N_i} and $(v'-v)_{max}$ values so determined for several substituted pyridines and imidazoles.

[2] $(v'-v)$ represents the increment or increase in reaction velocity caused by the addition of a boosting compound. K_{N_i} represents the concentration of a given compound required for half-maximal boosting.

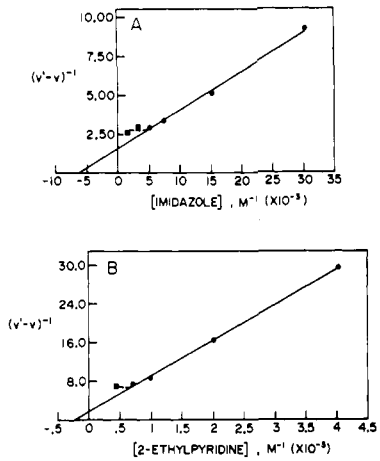

FIGURE 7: Double-reciprocal plots of $(v'-v)^{-1}$ vs $(N:)^{-1}$ for imidazole (A) and 2-ethylpyridine (B). Assays were performed in 50 mM potassium phosphate (pH 7.5) containing 1.35×10^{-4} M dianisidine dihydrochloride and 8.68×10^{-4} M H_2O_2 at 0.1 nM HRP and were recorded in triplicate during the first 10 s of reaction. 2-Ethylpyridine was added in small aliquots of 95% ethanol. Solid lines represent least-squares plots for points denoted (●).

Table II: $K_{N:}$ and $(v'-v)_{max}$ Parameters for Nitrogenous Activators with the HRP-Catalyzed Peroxidation of Dianisidine[a]

activator	pK_a	$K_{N:}$ (M)	$(v'-v)_{max}$ (min^{-1})
2-benzylpyridine	5.13	1.5×10^{-3}	0.260
pyridine	5.17	2.6×10^{-3}	0.317
4-phenylpyridine	5.55	6.2×10^{-4}	0.362
2-ethylpyridine	5.97	4.2×10^{-3}	0.579
4-*tert*-butylpyridine	5.99	4.1×10^{-4}	0.350
imidazole	7.05	1.7×10^{-4}	0.684
2-methylimidazole	7.85	5.4×10^{-5}	0.572

[a] Assay conditions: 1.35×10^{-4} M dianisidine dihydrochloride, 8.7×10^{-4} M H_2O_2 in 50 mM potassium phosphate (pH 7.5) with 0.1 nM HRP. Assays were performed in triplicate over the first 10 s of reaction; control rate was 0.095 min^{-1}.

An equation correlating rates of nucleophilic reactions with both nucleophilic strength and basicity was formulated by Edwards (1954)

$$\log \frac{K}{K_0} = \alpha E_n + \beta H \qquad (1)$$

where E_n is a nucleophilic constant based upon electron-donating ability, H is directly related to pK_a, and α and β represent sensitivity constants characteristic of the nucleophilic reaction center. Experiments limited to a single type of nucleophile should yield linear Edwards plots (Bruice & Benkovic, 1966) similar to the Brönsted plots, relating nucleophilicity and pK_a, used by Jencks & Carriuolo (1960). Figure 8 demonstrates the result of an Edwards plot for several of the pyridine compounds listed in Table II. In this case, the value of K/K_0, which represents the ratio of reaction rates with the nucleophile and water, respectively, is substituted for by the value of $(v'-v)_{max}/v$. For at least four of these compounds, a satisfactory linear correlation is observed. The sole exception of 4-*tert*-butylpyridine is not understood, although this compound did not exhibit the apparent inhibitory behavior seen at higher concentrations of 2-ethylpyridine or imidazole (Figure 7). We tentatively conclude that the presence of a bulky substituent in the 4-position somehow precludes proper orientation of this compound in the catalytic complex, and thus renders it less effective as a nucleophile toward the HRP–dianisidine complex.

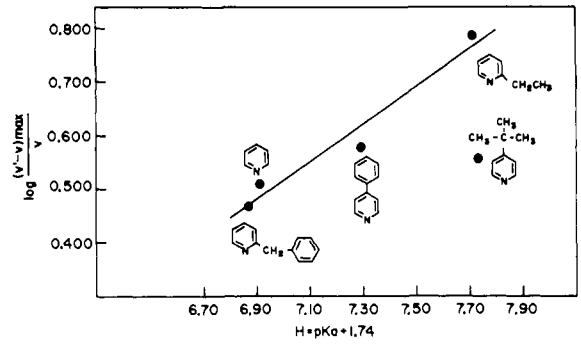

FIGURE 8: Edwards plot for nucleophilic catalysis by a series of pyridine compounds. $(v'-v)_{max}$ values for these compounds were taken from Table II and plotted as log $[(v'-v)_{max}/v]$ vs. $(pK_a + 1.74)$, as explained by Edwards (1954).

A final criterion for nucleophilic catalysis not mentioned previously involves detection and isolation of a corresponding covalent intermediate of nucleophile and substrate, as has been demonstrated with the detection of *N*-acetylimidazole during nucleophilic catalysis of acetate ester hydrolyses (Bender & Turnquest, 1957). Similar studies were attempted in the nucleophilic catalysis of HRP-catalyzed dianisidine peroxidation. However, neither imidazole nor 2,6-dimethylpyridine showed any spectral changes at micromolar concentrations during oxidation of dianisidine with high levels of HRP at pH 7.5. It has been shown by Staab (1957) that *N*-acetylbenzimidazole exhibits a half-life in neutral aqueous solution of 21 h, compared with a half-life of 40 min for *N*-acetylimidazole under identical conditons. We therefore hoped that the visible spectrum of the dianisidine product formed at pH 7.5 in the presence of HRP–H_2O_2 plus 0.50 mM benzimidazole might reflect such an intermediate; however, this proved not to be the case. We conclude that any covalent adduct of nucleophile and dianisidine product must be labile in aqueous solution.

Role of Active-Site Heme. In the original report, Fridovich (1963) suggested that the nitrogenous "ligands" stimulated dianisidine peroxidation by HRP via ligation to active site heme. The observation that $K_{N:}$ for ammonia decreased in 50% aqueous ethanol suggested that hydroxide ion competed against this stimulatory ligation. In addition, the dual effects of cyanide and hydrazine as both activators and potent inhibitors were interpreted in terms of two different binding sites, possibly the two sides of the heme plane. In their study of coordination of imidazoles with ferrimesoporphyrin, Cowgill & Clark (1952) demonstrated that steric hindrance played a substantial role in determining the ability of a compound to ligate to heme. 2-Methylimidazole was among a number of apparently sterically hindered imidazoles which were shown by spectroscopic methods not to bind ferrimesoporphyrin. However, as shown in Table II, 2-methylimidazole is a very effective agent in the stimulation of HRP activity with dianisidine, although perhaps slightly less effective than imidazole (pK_a = 7.85, 7.05, respectively). Similarly benzimidazole (pK_a = 5.4) stimulated dianisidine peroxidation 3.2-fold at only 0.50 mM and yet, was shown by Cowgill & Clark not to bind ferrimesoporphyrin. It would appear that an alternative nucleophilic site in the enzyme–substrate complex exists, apart from the heme itself; we conclude that this second site is the dianisidine radical moiety of the rate-limiting ES complex.

Discussion

Previous investigations have led to two mechanisms of peroxidase action: (1) the compulsory univalent mechanism

Scheme I[3]

$$H_2\ddot{N}Ar\ddot{N}H_2 + [Fe(IV)\ddot{\pi}^+] \rightleftharpoons (H_2\ddot{N}Ar\ddot{N}H_2^+, [Fe(IV)\ddot{\pi}]) \quad (a)$$

$$(H_2\ddot{N}Ar\ddot{N}H_2^+, [Fe(IV)\ddot{\pi}]) \rightarrow (H_2\ddot{N}Ar\ddot{N}H_2^+, [Fe(IV)\ddot{\pi}]) \quad (b)$$

$$(H_2\ddot{N}Ar\ddot{N}H_2^+, [Fe(IV)\ddot{\pi}]) \xrightarrow{\text{slow}} (HN{=}Ar{=}NH, [Fe(III)\ddot{\pi}H_2^{2+}]) \quad (c)$$

$$(HN{=}Ar{=}NH, [Fe(III)\ddot{\pi}H_2^{2+}]) \rightarrow HN{=}Ar{=}NH + [Fe(III)\ddot{\pi}H_2^{2+}] \quad (d)$$

(George, 1952; Chance, 1952a,b) in which the rate-determining process involves a reaction of HRP–compound II with a second mole of reducing substrate (for example, ascorbate) generating free HRP and substrate-free radical; (2) the simple divalent mechanism (Roman & Dunford, 1972, 1973; Araiso et al., 1976) in which HRP–compound I and reducing substrate (for example, sulfite) react to form oxidized substrate and free HRP without detectable intermediates.

The former mechanism appears to be specific for peroxidase, while the latter closely resembles the mechanism for coupled oxidation of ethanol by liver catalase (Chance & Fergusson, 1954; Brill, 1966). Our studies of the peroxidation of *o*-dianisidine by horseradish peroxidase suggest a mechanism (summarized in Scheme I) intermediate to those of 1 and 2 above, in which dianisidine radical is not readily released from the enzyme, but rather serves as electron donor to yield, in a distinct second step, free HRP and dianisidine quinonediimine.

Initial indications that the classical univalent mechanism was not involved in the peroxidation of dianisidine were given in the preceding paper (Claiborne & Fridovich, 1979). Thus, optical and EPR studies showed that no free dianisidine semiquinone accumulated even as a transient oxidation product in reaction mixtures containing dianisidine, HRP, and H_2O_2. However, there remained the possibility of a divalent interaction within this enzyme–substrate pair. Roman & Dunford (1972) and Araiso et al. (1976) were able to demonstrate the absence of HRP–compound II in steady-state reaction mixtures of HRP, H_2O_2, and sulfite at mildly acidic pH, and Roman & Dunford (1973) were able to demonstrate similar behavior in a kinetic study of iodide peroxidation by HRP. These authors concluded that this reaction proceeded largely via simple divalent reduction of the oxidized enzyme. The spectrum of the reaction mixture obtained in the steady state was indeed shown to be that of HRP–compound I. However, our own studies with dianisidine peroxidation clearly demonstrate a different enzyme intermediate, not identifiable with either compound I or compound II. We conclude that this difference is attributable to some contribution from a partially oxidized substrate molecule still bound at the active site since neither the quinonediimine nor dianisidine induce these spectral changes with HRP.

Our suggestion that the dianisidine radical contributes substantially to the structure of the HRP—dianisidine intermediate is supported by the following.

First, it provides a chemical basis for understanding the nature and specificity of the stimulation of horseradish peroxidase activity by nitrogenous compounds. Manning et al. (1969) demonstrated that nucleophiles such as pyridine assisted electron-transfer oxidation of the 9,10-diphenylanthracene cation radical as measured with the rotated disc electrode in anhydrous solvents; the sequence proposed to account for this nucleophilic effect was

$$DPA \rightleftharpoons DPA^{+\cdot} + e^- \quad (2)$$

$$DPA^{+\cdot} + N: \rightarrow DPA{:}N^{+\cdot} \quad (3)$$

$$DPA{:}N^{+\cdot} \rightleftharpoons DPA{-}N^{2+} + e^- \quad (4)$$

and resulted in covalent adduct formation between N: (nucleophile) and DPA (9,10-diphenylanthracene). The boost in cation radical oxidation was explained through incorporation of the electron-rich nucleophile. The authors concluded that the hypothesis (Lund, 1957) favoring transfer of the DPA electron pair during oxidation was untenable. Our characterization of the boosting of HRP-catalyzed dianisidine peroxidation by nitrogenous compounds has demonstrated the following:

(a) Substitution of the pyridine ring with strongly electron-withdrawing substituents such as 4-cyano- and 3-bromo- completely deactivates pyridine as a booster, while substitution with electron-donating groups such as 4-ethyl- increases the boosting effect of pyridine.

(b) Comparison of the maximal increments for a series of pyridine compounds obtained by graphical extrapolation to infinite nucleophile concentration gives a strong correlation with the nucleophilicity equation of Edwards (1954).

(c) The possibility that the nitrogenous compounds were acting as general base catalysts in our aqueous system was excluded, since deuterium solvent isotope effects were smaller than 1.50, dibasic phosphate did not boost the HRP—dianisidine reaction (despite its higher pK_a than imidazole), and since steric hindrance around the basic nitrogen in 2,6-diisopropylpyridine rendered it ineffective despite the higher pK_a of this 2,6-disubstituted pyridine.

(d) The effectiveness of pyridine as a booster was substantially diminished in 50% aqueous ethanol, in keeping with the influence of pK_a in determining nucleophilicity.

We conclude that the nitrogenous compounds act as nucleophilic catalysts interacting with the dianisidine radical moiety of the rate-limiting HRP–dianisidine complex and thereby facilitating electron-transfer oxidation of this species by the enzyme. The specificity of the nitrogenous compound effect of the HRP-catalyzed peroxidations of *o*-dianisidine and *p*-phenylenediamine can be explained in terms of their electrochemical similarities; both participate in reversible two-electron oxidations (Piette et al., 1962). Enzymatically, it would appear that both substrates are oxidized via enzyme-bound radical intermediates at mildly alkaline pH, since both peroxidations with HRP are sensitive to nitrogenous boosting under these conditions (Fridovich, 1963).

Second, a reevaluation of the proposal by Fridovich (1963) that the nitrogenous boosters acted as heme ligands suggests that the active site heme of HRP is not the nucleophilic site suggested in our studies. 2-Methylimidazole and benzimidazole, therefore, are capable of powerfully stimulating dianisidine peroxidation by HRP at pH 7.5; yet, both compounds were shown not to ligate ferrimesoporphyrin by Cowgill & Clark (1952) due to steric hindrances from their respective substituents. In addition, it would appear that the proposed competition against nitrogenous compound "ligation" due to OH^-, which was also interpreted in terms of a heme–ligand interaction, can be explained by the effect of 50% ethanol upon the pK_a of the nucleophile and the corresponding decrease in nucleophilicity. Thus $K_{NH_3+NH_4^+}$ appeared higher when measured in an aqueous buffer than in the presence of 50% ethanol (Fridovich, 1963). We therefore conclude that the nitrogenous compounds do not interact by direct ligation with the active site heme of HRP and that the "second heme site" suggested by Fridovich is actually the dianisidine radical moiety in the HRP–dianisidine complex at mildly alkaline pH. This interpretation, which also explains the dual actions of cyanide and hydrazine as inhibitors (via heme ligation) and

[3] The electronic structure of compound I is taken to be the Fe(IV) π-cation radical suggested by Dolphin et al. (1971).

activators (as nucleophiles) of the HRP-catalyzed peroxidation of dianisidine, offers a second line of support for the existence of a dianisidine radical moiety in the HRP–dianisidine complex.

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Mechanism of *Lactobacillus leichmannii* Ribonucleotide Reductase Studied with *Coα*-[*α*-(Aden-9-yl)]-*Coβ*-adenosylcobamide (Pseudocoenzyme B_{12}) as Coenzyme†

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ABSTRACT: *Coα*-[*α*-(Aden-9-yl)]-*Coβ*-adenosylcobamide (pseudocoenzyme B_{12}) purified from *Clostridium tetanomorphum* has been reacted with ribonucleotide reductase purified from *Lactobacillus leichmannii* under various conditions, and the properties of the products obtained have been compared by electron paramagnetic resonance (EPR) with those previously reported for products formed from the normal coenzyme (adenosylcobalamin). The rapidly formed intermediate and the slowly formed "doublet" species from the pseudocoenzyme have EPR spectra identical with those formed from the normal coenzyme. This and other considerations make it less likely that the unusual magnetic properties of the rapidly formed intermediate are due to strongly distorted octahedral symmetry about Co(II) as previously postulated. Instead it is probable that the EPR spectrum is due to interaction of the radical pair by both exchange coupling and magnetic dipole–dipole coupling. Although *Coα*-[*α*-(aden-9-yl)]cob(II)amide in solution does not show superhyperfine splitting in the EPR spectrum because of its base-off configuration, the cob(II)amide formed by degradation of the pseudocoenzyme within the catalytic site of the enzyme did show triplets due to a nitrogen axially coordinated to cobalt. This suggests that binding of the cob(II)amide to the reductase catalytic site causes a shift to the base-on form.

The ribonucleoside-triphosphate reductase of *Lactobacillus leichmannii* (EC 1.17.4.2) differs from the reductase in *Escherichia coli* and in cells of vertebrate tissues in several important respects (Blakley, 1978; Hogenkamp & Sando, 1974; Follmann, 1974). Inter alia the *Lactobacillus* enzyme requires adenosylcobalamin as coenzyme and has no metal component or other requirement, whereas the *E. coli* and vertebrate enzymes contain nonheme iron and have no B_{12} requirement. Nevertheless, evidence is accumulating that these two classes of ribonucleotide reductase share some important features in common since both appear to catalyze reactions involving a radical mechanism.

In the presence of dGTP or other allosteric activators (which

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