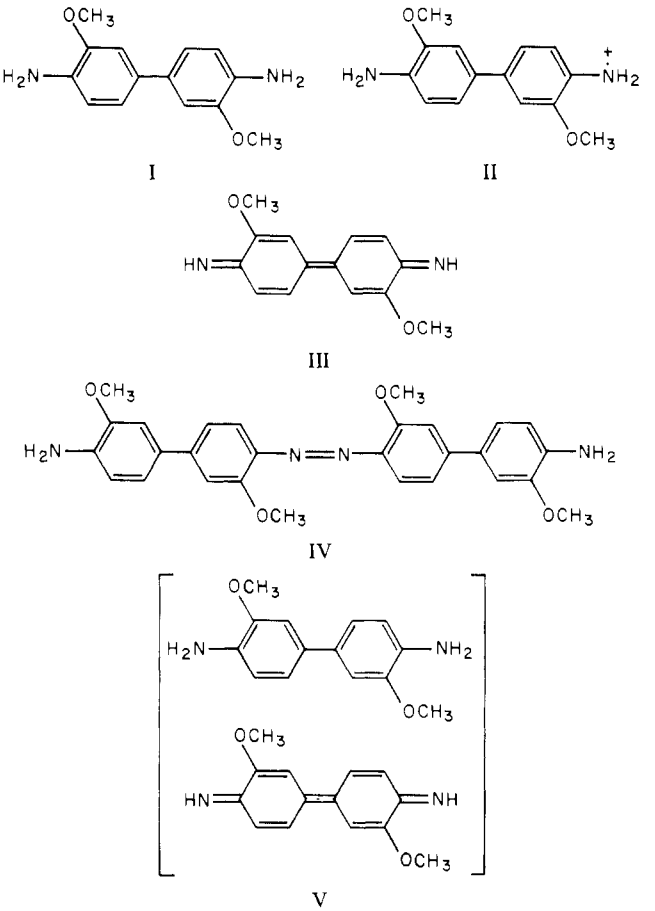
Chemical and Enzymatic Intermediates in the Peroxidation of *o*-Dianisidine by Horseradish Peroxidase. 1. Spectral Properties of the Products of Dianisidine Oxidation†

Al Claiborne and Irwin Fridovich*

ABSTRACT: Studies of the optical spectra of the products formed during peroxidation of *o*-dianisidine by horseradish peroxidase indicate at least three distinct species. At pH 3.7 and 4 °C, peroxidation of dianisidine at low concentrations yields the free dianisidine quinonediimine (the two-equivalent oxidized form) with λ_{max} 452 and 514 nm. At higher concentrations, the first detectable product is not the free quinonediimine but an intermolecular complex (meriquinone or charge-transfer complex) consisting of quinonediimine and parental diamine. This complex is freely reversible and is sensitive to simple dilution or acidification, either of which restores the spectrum of the free quinonediimine. Furthermore, at near-neutral pH, the quinonediimine appears to undergo irreversible self-coupling, yielding yet a different optical spectrum presumably characteristic of the bisazobiphenyl structure proposed by K. M. Møller & P. Ottolenghi [(1966) *C. R. Trav. Lab. Carlsberg 35*, 369–389]. Butylated hydroxyanisole was shown to react in the presence of peroxidase–H_2O_2 and dianisidine to yield a spectrum (λ_{max} 575 nm) nearly identical with that obtained when Gibbs' reagent (2,6-dichloroquinone 4-chloroimine) was incubated with butylated hydroxyanisole, thus suggesting that the free quinonediimine itself couples with the phenolic antioxidant. Finally, continuous-flow EPR studies of dianisidine oxidation both with HRP–H_2O_2 and with ceric sulfate were unable to detect any free dianisidine semiquinone radical in the steady state; we conclude that oxidation of dianisidine occurs in a rapid two-electron process in both the HRP–H_2O_2 and Ce(IV) systems.

Horseradish peroxidase is divalently oxidized to compound I by hydrogen peroxide. The reduction of compound I back to the initial state is generally believed to occur by two successive univalent interactions with reducing substrates. This mechanism requires the production both of free radicals of the reducing substrates and of a one-electron oxidized form of the enzyme, called compound II. Both optical and EPR[1] evidence have been adduced in support of this mechanism (George, 1952; Chance, 1952a,b; Roman & Dunford, 1973; Yamazaki et al., 1960; Piette et al., 1961; Yamazaki, 1971). There do, however, appear to be exceptions. The peroxidations of iodide and sulfite appear to proceed by the divalent reduction of compound I (Björkstén, 1970; Roman & Dunford, 1972, 1973; Araiso et al., 1976).

The oxidation of *o*-dianisidine (3,3′-dimethoxybenzidine, I) by HRP was originally studied as the basis of a convenient and specific assay for glucose. Thus, glucose oxidase was used to oxidize glucose to gluconolactone plus H_2O_2, while HRP utilized this H_2O_2 in the oxidation of dianisidine to a colored product (Keston, 1956). The stable product of the peroxidation of dianisidine was shown to be a bisazobiphenyl (IV) which was considered to be produced by condensation of two molecules of the dianisidine quinonediimine (III) (Møller & Ottolenghi, 1966). The existence of a semiquinone intermediate (II) preceding the quinonediimine has been debated. Oldfield & Bockris (1951), in a study of the redox properties of dianisidine in acidic media, concluded on the basis of potentiometric titration data with aqueous bromine that oxidation did proceed via a semiquinone intermediate; their observation of a transient green color in partially oxidized solutions was used to confirm the presence of a semiquinone radical. A green intermediate was also seen during the HRP-catalyzed peroxidation of *N,N,N′,N′*-tetramethylbenzidine by Naylor & Saunders (1950) and was similarly interpreted as a semiquinone radical. Similar intermediates were encountered during the oxidation of benzidine and *o*-tolidine, but it was suggested that these were charge-transfer



† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received January 8, 1979.* This work was supported and made possible by research grants from the National Institutes of Health, Bethesda, MD (GM-10287), from the United States Army Research Office, Research Triangle Park, NC (DRXRO-PR-P-15319-L), and from Merck Sharp & Dohme, Rahway, NJ.

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

0006-2960/79/0418-2324$01.00/0 © 1979 American Chemical Society

complexes or meriquinones (V) formed by association of quinonediimine with the parental diamine (Clark et al., 1926; Clark, 1972; Hughes & Hush, 1947). The meriquinone structure was proposed much earlier by Willstätter & Piccard (1908) to account for the properties of Wurster's salts and by Schlenk & Knorr (1908) to explain the redox properties and concentration-dependent characteristics of the color of oxidized benzidines. Semiquinone intermediates in the oxidation of benzidines were sought, but not found, by Michaelis & Hill (1933) and by Michaelis (1935). Piette et al. (1962) were similarly unable to obtain EPR evidence for the formation of a semiquinone during the electrolytic oxidation of *o*-dianisidine, under a variety of conditions.

Our own studies with the *o*-dianisidine peroxidase of *Escherichia coli* B have shown it to possess substantial catalytic activity; in fact, it appears that its peroxidatic activity is of little physiological significance (Claiborne, 1978; Claiborne & Fridovich, 1979a). Our interest in the dual hydroperoxidatic activities of this enzyme led to the question of whether both catalytic and peroxidatic reactions proceeded via divalent processes (Chance & Fergusson, 1954), or whether the peroxidation of dianisidine indeed followed the compulsory univalent mechanism suggested by George (1952) and Chance (1952a,b) for HRP. As a model for the peroxidatic activity of *E. coli* hydroperoxidase I, this study focuses upon the mechanism of *o*-dianisidine peroxidation by horseradish peroxidase. The results, which form the basis of this report, support the occurrence of an intermediate meriquinone, but not of a semiquinone, during the HRP-catalyzed peroxidation of *o*-dianisidine.

Materials and Methods

All optical spectra were recorded with an Aminco DW-2 UV–visible spectrophotometer at 4 °C, unless otherwise specified. Samples (3.0 mL) in quartz cuvettes were chilled for 5 min at 0 to −5 °C in an ice–salt bath prior to transfer to the thermostated cell compartment. When Yankeelov cuvettes were used, samples of 1.0 mL were placed into each of the cuvette compartments. Samples were positioned against the face of the phototube to minimize the light-scattering effects of moisture condensing onto the optical faces of the cuvettes. pH measurements were made at 23 °C and were not corrected for the effects of chilling. EPR spectra were recorded with a Varian E-9 spectrometer at 100 kHz field modulation, which was coupled with the two-stream continuous flow system of Smith et al. (1975, 1977), employing a mix-and-flow cell of the type described by Borg (1964).

o-Dianisidine was purchased from Sigma Chemical Co. and from Eastman Organic Chemicals and was recrystallized as the dihydrochloride according to Talbot et al. (1940). H_2O_2 (30%, AR grade) was purchased from Mallinckrodt, Inc.; its concentration was determined at 240 nm by using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} (Hildebrandt & Roots, 1975). Sodium L-ascorbate, butylated hydroxyanisole, and butylated hydroxytoluene were purchased from Sigma. $Ce(SO_4)_2 \cdot 4H_2O$ was from Fisher Scientific Co., and 2,6-dichloroquinone 4-chloroimine (Gibbs' reagent) was purchased from Aldrich Chemical Co. Horseradish peroxidase (HPOD grade, RZ = 1.0) was a product of Worthington Biochemical Corp. BHA, BHT, and 2,6-DCQCI were added to buffered reaction mixtures in small volumes of 95% ethanol.

Results

Intermediates in the Peroxidation of o-Dianisidine. Figure 1 compares the visible spectra of the dianisidine oxidation products formed in the presence of HRP and H_2O_2 at pH 6.0 (Figure 1A) and at pH 3.7 (Figure 1B). Absorbance maxima at 386 and 704 nm became progressively less evident at pH 6.0 as a stable product absorbing at 476 nm accumulated; at pH 3.7, the level of the primary intermediate continued to increase without subsequent development of the 476-nm species. The deep green color of the primary species formed at pH 3.7 was reminiscent of the intermediate observed by Oldfield & Bockris (1951); however, EPR studies at the temperature of liquid nitrogen detected no organic radical signal.

Chemical Trapping with BHA. Butylated hydroxyanisole has been shown to react with an intermediate of dianisidine peroxidation in the presence of H_2O_2 and *E. coli* hydroperoxidase I (Claiborne & Fridovich, 1979a) and works equally well in the HRP/dianisidine system. Figure 2 demonstrates the spectrum (λ_{max} 575 nm) of the product formed when HRP and H_2O_2 are incubated in the presence of dianisidine and BHA at pH 6.5. The ultraviolet spectrum (λ_{max} 287 nm) of BHA undergoes pronounced change only when dianisidine is added to solutions already containing HRP and H_2O_2, thus indicating that BHA is not itself peroxidized by HRP. BHA and dianisidine do not react in the absence of HRP, nor will BHA react with the stable product of dianisidine oxidation at pH 6.5. A spectral species nearly identical with that shown in Figure 2 is generated at pH 3.7 when BHA is added to a solution of the enzymatically generated primary intermediate.

Nature of the Primary Intermediate. Oldfield & Bockris (1951) showed that the dianisidine quinonediimine could be produced in a stable form by oxidation of dianisidine with ceric sulfate in sulfuric acid; this intermediate was characterized by its cherry red color. Møller & Ottolenghi (1966) later showed that the quinonediimine rapidly dimerized at near-neutral pH to yield the covalent bisazo compound identical with the product of HRP-catalyzed dianisidine peroxidation at neutral pH. Dianisidine meriquinone, generated by the action of HRP at pH 3.7, as described in Figure 1B, gave a spectrum, upon treatment with H_2SO_4 (Figure 3), which was identical with that obtained by oxidation of dianisidine in 1.5 M H_2SO_4 with Ce(IV). The first detectable intermediate of dianisidine oxidation by HRP is thus the quinonediimine.

In their original study of the meriquinones formed upon partial oxidation of *o*-tolidine and *o,o'*-dichloro-*o*-tolidine, Schlenk & Knorr (1908) noted sharp color changes upon simple dilution of samples; these observations led to the suggestion of reversible intermolecular associations between the quinonediimines and their parental diamines. Figure 4 demonstrates the effect of twofold dilution upon the spectrum of the primary intermediate of dianisidine peroxidation at pH 3.7. The new spectrum (λ_{max} 452, 514 nm) closely resembles that produced by ceric sulfate oxidation of dianisidine and the spectrum produced by acidification in Figure 3. The near identity of these quinonediimine spectra obtained by both dilution and acidification suggests that pH changes may only play an indirect role in inducing spectral transitions between primary intermediate and quinonediimine; the concentration-dependence demonstrated in Figure 4 indicates that intermolecular association may be the factor directly responsible for these spectral changes.

Addition of BHA to the dilute quinonediimine, produced by allowing HRP to act on 0.04 mM dianisidine plus 0.86 mM H_2O_2 at pH 3.7, resulted in a rapid reaction yielding a spectrum (λ_{max} 533 nm) similar to that in Figure 2. This observation would suggest that the free dianisidine quinonediimine reacts directly with BHA; this possibility was further explored with a model system by using 2,6-dichloroquinone

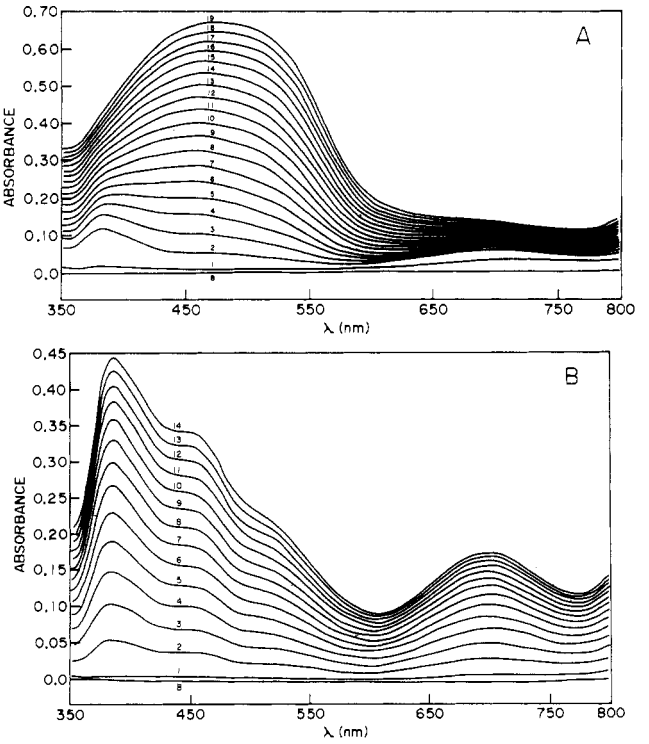


FIGURE 1: The peroxidation of *o*-dianisidine by HRP at pH 6.0 and 3.7. (A) Reaction mixtures contained 0.135 mM dianisidine, 0.29 mM H_2O_2, and 33 ng/mL HRP in 10 mM potassium phosphate at pH 6.0. (B) Reaction mixtures contained 0.135 mM *o*-dianisidine, 0.29 mM H_2O_2, and 13 ng/mL HRP in 14 mM acetic acid, 10 mM potassium phosphate at pH 3.7. Reactions at 4 °C were initiated by addition of HRP, and spectra were recorded at a scan rate of 20 nm/s. A complete spectrum was thus recorded every 46 s. Spectra designated B represent base lines in these and all other figures.

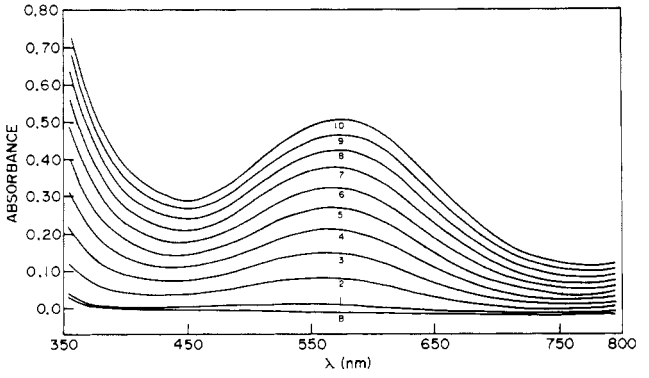
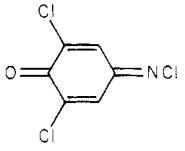


FIGURE 2: Peroxidation of *o*-dianisidine in the presence of BHA. Reaction mixtures contained 0.33 mM dianisidine, 0.855 mM H_2O_2, 0.33 mM BHA, and 33 ng/mL HRP in 10 mM potassium phosphate at pH 6.5 and 4 °C. The reaction was initiated and the spectra were recorded as in Figure 1.

4-chloroimine (Gibbs' reagent VI). This compound is a known coupler of phenols (Gibbs et al., 1925), including BHA, with which it develops a blue–violet color similar to that of the product shown in Figure 2 (van der Heide, 1966). Its structural similarity with the dianisidine quinonediimine can be shown by comparing structures III and VI. Figure 5 shows



VI

the spectrum (λ_{max} 582 nm) of the product formed when BHA

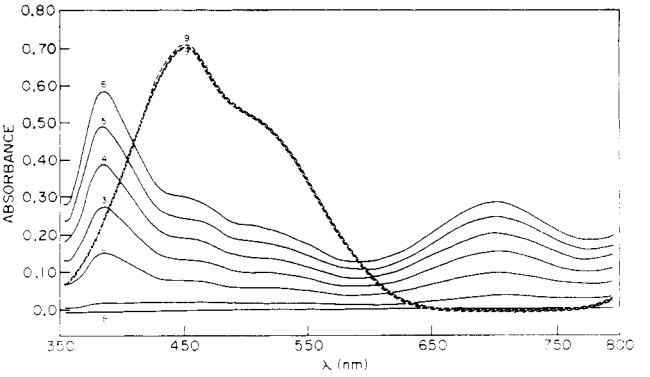


FIGURE 3: Effect of acidification of the meriquinone intermediate. The primary intermediate of dianisidine oxidation was generated and spectra were recorded as described in Figure 1B, but with 33 ng/mL HRP (solid lines no. 1–6). H_2SO_4 was then added to 50 mM, and spectral scanning was continued. Dashed lines no. 7–9 represent three spectra recorded at 46-s intervals after acidification. The net dilution occasioned by addition of H_2SO_4 was less than 4% and so may be ignored.

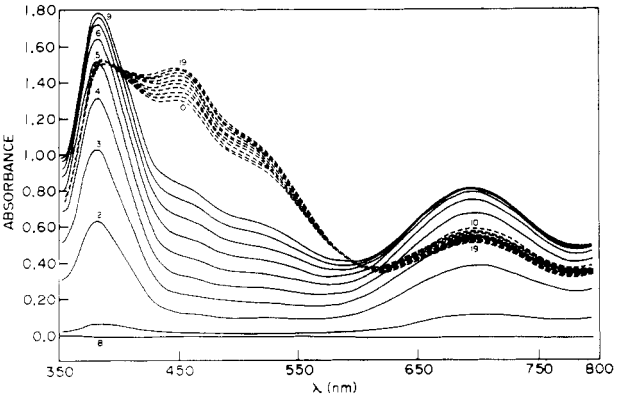


FIGURE 4: Effect of dilution upon the spectrum of the meriquinone intermediate. The reaction mixture contained 0.32 mM dianisidine, 0.86 mM H_2O_2, and 97 ng/mL HRP and 14 mM acetic acid, 10 mM potassium phosphate, pH 3.7, in a volume of 1.03 mL and was placed in one chamber of a Yankeelov cuvette. The second compartment contained 1.0 mL of the buffer mixture. The reaction was followed by recording spectra at 46-s intervals (solid lines no. 1–9). The cuvette was then inverted and mixed before spectra 10–19 (dashed lines) were recorded. Because of the tandem geometry of the Yankeelov cuvette, the twofold dilution was compensated by a twofold increase in path length and the changes shown relate entirely to dilution-induced chemical changes in the reaction mixture.

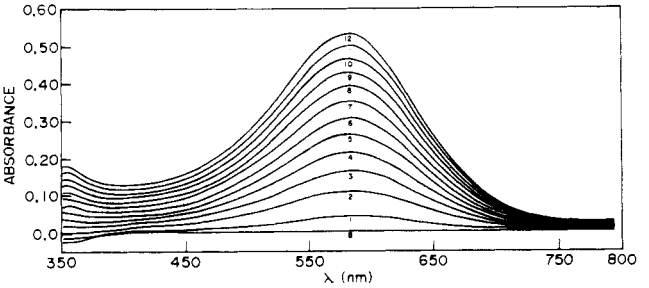


FIGURE 5: Reaction of Gibbs' reagent with BHA. 2,6-DCQCI (0.68 mM) was mixed with BHA (0.33 mM), in 10 mM potassium phosphate at pH 6.5, and spectra were recorded at 46-s intervals.

and 2,6-DCQCI are allowed to react at pH 6.5, which is very similar to that shown in Figure 2 during BHA reaction in the HRP–dianisidine system at the same pH. We conclude that BHA indeed reacts with the free quinonediimine of dianisidine.

Further Reaction of the Quinonediimine at Neutral pH. Figure 6 demonstrates that the quinonediimine of dianisidine formed enzymatically at pH 3.7 is induced to further reaction

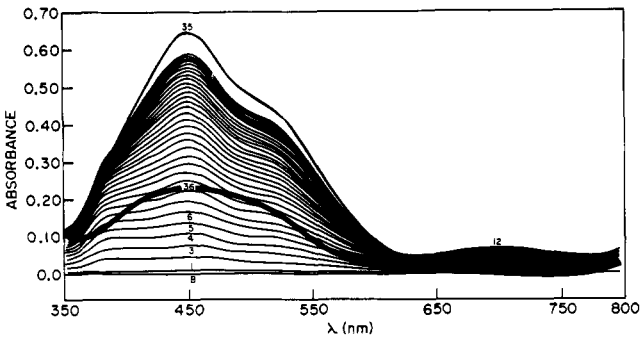


FIGURE 6: Effect of neutralizing the quinonediimine intermediate. A reaction mixture containing 0.041 mM dianisidine, 0.29 mM H_2O_2, and 33 ng/mL HRP in 14 mM acetate–10 mM phosphate buffer at pH 3.7 was followed by repetitive spectral scans at 46-s intervals (solid lines no. 1–35). The pH was rapidly raised to 6.8 by addition of NaOH and the spectrum again recorded (solid line no. 36). The net dilution caused by addition of NaOH was less than 4%.

upon neutralization of the solution. The spectrum (λ_{max} 453 nm) so generated is identical with that seen at pH 6.0 when reaction is initiated with 4.6×10^{-5} M dianisidine and HRP; the insensitivity of this spectrum to acidification back to pH 3.7 and to reaction with BHA is an indication of the irreversibility of the reaction and the stability of the product.

Continuous-Flow EPR Studies. The dianisidine semiquinone intermediate suggested by Oldfield & Bockris would be predicted to show considerable stability (Piette et al., 1962; Adams, 1969); however, Piette et al. (1962) were unable to detect any EPR signal during electrolytic oxidation of dianisidine under a wide range of conditions. Our own studies have shown that the green intermediate of the dianisidine redox system is nonparamagnetic at the temperature of liquid nitrogen; however, we were still hesitant to dismiss the possibility of transient semiquinone radical formation by HRP and H_2O_2 without further experimental testing.

Yamazaki et al. (1960) were able to unequivocally demonstrate the production of free radicals of ascorbate and of hydroquinone by peroxidase–H_2O_2 by using a continuous-flow EPR system in which mixing of substrate and enzyme occurred within the spectrometer cavity at ambient temperature. With the generous cooperation of Dr. Peter Smith of the Duke University Department of Chemistry, a similar system was used to investigate the oxidation of dianisidine. These results are shown in Figure 7. Line 1 represents a scan taken during continuous mixing of dianisidine and $Ce(SO_4)_2$ in 0.75 M H_2SO_4 and is virtually superimposable upon the spectrum of a buffer blank. Ce(IV) is presumed to represent a one-equivalent oxidant (Higginson and Marshall, 1957); we would certainly have anticipated detection of dianisidine radical. However, scans at a variety of flow rates and scanning ranges did not reveal any signal. Line 2 represents a scan during which ascorbate–H_2O_2 and HRP were mixed continuously at pH 3.9. This spectrum is identical with that reported by Borg (1964) for ascorbate radical; its intensity was substantially lowered in the absence of HRP. Line 3 represents a scan during continuous mixing of dianisidine–H_2O_2 and HRP at pH 2.8 and was duplicated when the pH was raised to 3.7 by using one-tenth the amount of dianisidine dihydrochloride. Again, the absence of dianisidine radical signal is in agreement with the findings of Piette et al. (1962).

Discussion

These results suggest a direct two-electron oxidation mechanism for horseradish peroxidase acting on *o*-dianisidine, which yields the dianisidine quinonediimine as the first free

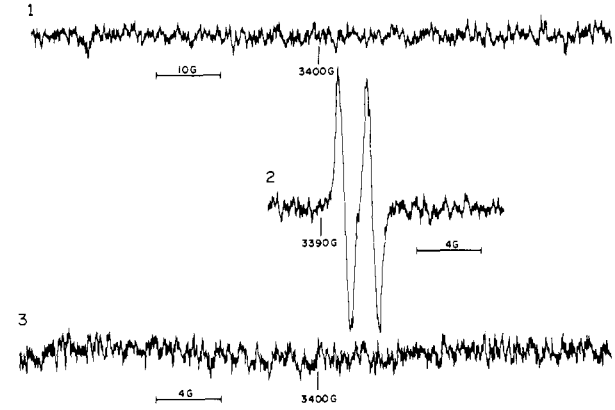


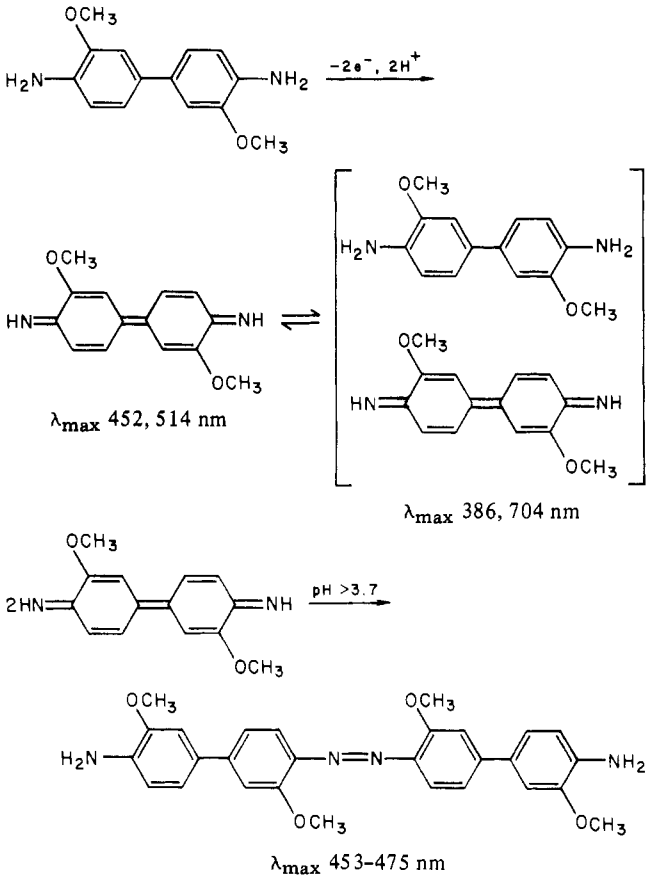
FIGURE 7: Continuous-flow EPR spectra. (Line 1) *o*-Dianisidine dihydrochloride (39 mM in water) and $Ce(SO_4)_2 \cdot 4H_2O$ (5 mM in 1.5 M H_2SO_4) each at a flow rate of 75 mL/min were mixed in the EPR cavity. Microwave power = 10 mW, modulation amplitude = 5G, and receiver gain = 2.5×10^4. (Line 2) Sodium ascorbate (40 mM), H_2O_2 (40 mM), and HRP (0.0125 g/L); each solution buffered at pH 3.9 with 10 mM potassium acetate–acetic acid and each at a flow rate of 75 mL/min were mixed in the EPR cavity. Microwave power = 20 mW, modulation amplitude = 1.0 G, and receiver gain = 2.5×10^4. (Line 3) *o*-Dianisidine dihydrochloride (39 mM), H_2O_2 (40 mM), HRP (0.0125 g/L), and potassium phosphate (10 mM), each at a flow rate of 75 mL/min, were mixed in the EPR cavity. The final pH was 2.8. Microwave power = 30 mW, modulation amplitude = 1.0 G, and receiver gain = 3.2×10^4. This experiment was repeated with the concentration of *o*-dianisidine dihydrochloride decreased by a factor of 10 and the final pH raised to 3.7 without any effect on the results. In all cases, the time constant was 1.0 s and the scan time was 8 min, while the temperature was 22–24 °C.

product. The quinonediimine, identified here by its spectral and chemical properties, appears to complex reversibly with dianisidine to yield the corresponding meriquinone; this equilibrium is quite sensitive to changes in concentration and pH. The long-wavelength (704 nm) absorbance band characteristic of the meriquinoid species is indicative of possible charge-transfer interaction in the complex (Foster, 1969). In the highly acidic medium afforded by 1.5 M H_2SO_4, protonation of amino and imino groups causes dissociation of the meriquinoid complex, presumably due to electrostatic repulsion.[2] At pH 3.7, the extent of protonation is considerably less, thus allowing complex formation, but still sufficient to prevent the irreversible reaction of quinonediimine to yield the final bisazobiphenyl product. Neutralization of solutions containing either free quinonediimine or high concentrations of the meriquinone leads to rapid irreversible reaction; the product thus formed appears identical with that formed from HRP/H_2O_2 with dianisidine at near-neutral pH. These results are summarized in Scheme I.

Butylated hydroxyanisole appears to react specifically with the quinonediimine; this can be demonstrated either in solutions of nearly homogeneous quinonediimine or in solutions of the dianisidine meriquinone complex. In the latter case, dissociation of the complex as monitored at 386 and 704 nm can be easily explained on the basis of the expected decrease in free quinonediimine concentration resulting from reaction with BHA. The specificity of the BHA–quinonediimine reaction is also supported by model studies with 2,6-chloroquinone 4-chloroimine. This compound, which is a well-recognized coupler of phenols, and which is structurally similar to the dianisidine quinonediimine, reacts with BHA to yield a visible spectrum nearly identical with that resulting from the reaction of BHA with the dianisidine quinonediimine (λ_{max} 582 nm with

[2] Oldfield & Bockris (1951) report a pK_{a2} for dianisidine of 3.24.

Scheme I



2,6-DCQCI; 575 nm with HRP/dianisidine). Controls showed that BHA does not react with dianisidine alone; nor is it directly peroxidizable by HRP. It would be expected that the imino nitrogen of the dianisidine quinonediimine should be a good electrophile, as suggested by Oldfield & Bockris (1951), as is the imino nitrogen of 2,6-DCQCI. The reaction would probably lead to aromatic substitution upon the side of the aromatic ring away from the *tert*-butyl substitution, since the presence of a second *tert*-butyl group in BHT (2,6-di-*tert*-butyl substitution) prevents reaction with the dianisidine quinonediimine by a presumed steric effect.

Finally, we conclude, in agreement with Piette et al. (1962), that oxidation of dianisidine does not proceed via any detectable semiquinone radical intermediate. The suggestion of such a free radical intermediate as advanced by Oldfield & Bockris (1951) was based on two criteria: (1) a two-stage color change during oxidation of dianisidine; and (2) potentiometric titration data for the reversible dianisidine/quinonediimine system at pH 1.9–4.0. As stated before, it seems quite probable that the meriquinoid complex of dianisidine, which is a characteristic deep green in color, was responsible for the color change observed by Oldfield & Bockris. Our own EPR studies, both under static conditions at liquid nitrogen temperature and by using continuous-flow at ambient temperature, failed to detect paramagnetic species even when the green intermediate had accumulated to high concentrations. We conclude that the green intermediate is the meriquinoid complex. With respect to the potentiometric titrations of dianisidine by Oldfield & Bockris, it should be recalled that Michaelis himself cautioned against direct interpretation of such data in the absence of corroborating evidence, when distinguishing oxidative mechanisms (Michaelis, 1935). The standard of the two-stage color change, as employed by Oldfield & Bockris, can indeed be misleading as corroborating evidence. Also, although Oldfield & Bockris stated that dye concentration has no effect upon midpoint potential as it should in a meriquinoid system, they do not specify the pH for this determination and only show data for the benzidine oxidation system.

It is surprising that no EPR spectrum is obtained when dianisidine is oxidized with Ce(IV) in H_2SO_4 since the latter is considered to be a one-equivalent oxidizing agent. We would have anticipated that dianisidine radical would have been formed, albeit transiently, in this system and would accumulate to detectable steady-state levels. Though Michaelis showed that semiquinone radicals of phenanthrene-3-sulfonate can reversibly dimerize to give a valence-saturated compound, he also concluded that ionization of the radical would substantially diminish its tendency to associate because of electrostatic repulsion (Michaelis & Fetcher, 1937); the presence of 0.75 M H_2SO_4 in our continuous-flow experiments with Ce(IV) would maintain any oxidized dianisidine species in their fully charged forms, thus retarding any such dimerization or other self-association. In addition, Oldfield & Bockris had shown that oxidation by $Ce(SO_4)_2$ of dianisidine in H_2SO_4 solutions greater than 3 M did proceed by a simple divalent mechanism. Our data appear to support this conclusion, although we cannot rule out the possibility of a univalent reaction of Ce(IV) with a transient dianisidine radical in a rapid second step. Swain & Hedberg (1950) suggested that Ce(IV) oxidiation of leucomalachite green proceeded via a complex of two ceric ions and one molecule of dye. Our data could be accommodated by such a mechanism.

These studies of the intermediates involved during the oxidation of dianisidine also raise fundamental questions concerning the mechanism of dianisidine peroxidation catalyzed by horseradish peroxidase, which will be considered in detail in the following paper (Claiborne & Fridovich, 1979b).

Acknowledgments

We wish to thank Dr. Peter Smith of the Duke University Department of Chemistry for his generous cooperation with the continuous-flow EPR experiments and Carolyn Weathers for her skillful operation of the system during these studies. We also wish to acknowledge Dr. K. V. Rajagopalan for his assistance during preliminary EPR studies.

References

Adams, R. N. (1969) *Electrochemistry at Solid Electrodes*, p 362, Marcel Dekker, New York.

Araiso, T., Miyoshi, K., & Yamazaki, I. (1976) *Biochemistry 15*, 3059–3063.

Björkstên, F. (1970) *Biochim. Biophys. Acta 212*, 396–406.

Borg, D. C. (1964) *Nature (London) 201*, 1087–1090.

Chance, B. (1952a) *Arch. Biochem. Biophys. 41*, 404–415.

Chance, B. (1952b) *Arch. Biochem. Biophys. 41*, 416–424.

Chance, B., & Fergusson, R. R. (1954) in *The Mechanism of Enzyme Action* (McElroy, W. D., & Glass, B., Eds.) pp 389–398, Johns Hopkins Press, Baltimore, MD.

Claiborne, A. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1513.

Claiborne, A., & Fridovich, I. (1979a) *J. Biol. Chem.* (in press).

Claiborne, A., & Fridovich, I. (1979b) *Biochemistry 18* (following paper in this issue).

Clark, W. M. (1972) *Oxidation-Reduction Potentials of Organic Systems*, pp 396–402, Krieger Publishing Co., New York.

Clark, W. M., Cohen, B., & Gibbs, H. D. (1926) *Public Health Rep., Suppl. No. 54*, 1–61.

Foster, R. (1969) *Organic Charge-Transfer Complexes*, pp 33–36, Academic Press, New York.
George, P. (1952) *Nature (London) 169*, 612–613.
Gibbs, H. D., Cohen, B., & Cannan, R. K. (1925) *Public Health Rep. 40*, 649–663.
Higginson, W. C. E., & Marshall, J. W. (1957) *J. Chem. Soc.*, 447–458.
Hildebrandt, A. G., & Roots, I. (1975) *Arch. Biochem. Biophys. 171*, 385–397.
Hughes, G. K., & Hush, N. S. (1947) *J. Proc. R. Soc. N.S.W. 81*, 48–59.
Keston, A. S. (1956) *Abstracts*, 129th National Meeting of the American Chemical Society, Dallas, TX, April 1956, 31–32c.
Michaelis, L. (1935) *Chem. Rev. 16*, 243–286.
Michaelis, L., & Hill, E. S. (1933) *J. Am. Chem. Soc. 55*, 1481–1494.
Michaelis, L., & Fetcher, E. S. (1937) *J. Am. Chem. Soc. 59*, 2460–2467.
Møller, K. M., & Ottolenghi, P. (1966) *C. R. Trav. Lab. Carlsberg 35*, 369–389.
Naylor, F. T., & Saunders, B. C. (1950) *J. Chem. Soc.*, 3519–3523.
Oldfield, L. F., & Bockris, J. O'M. (1951) *J. Phys. Colloid Chem. 55*, 1255–1274.
Piette, L. H., Yamazaki, I., & Mason, H. S. (1961) in *Free Radicals in Biological Systems* (Blois, M. S., Brown, H. W., Lemmon, R. M., Lindblom, R. O., & Weissbluth, M., Eds.) pp 195–208, Academic Press, New York.
Piette, L. H., Ludwig, P., & Adams, R. N. (1962) *Anal. Chem. 34*, 916–921.
Roman, R., & Dunford, H. B. (1972) *Biochemistry 11*, 2076–2082.
Roman, R., & Dunford, H. B. (1973) *Can. J. Chem. 51*, 588–596.
Schlenk, W., & Knorr, A. (1908) *Justus Liebigs Ann. Chem. 363*, 313–339.
Smith, P., Kaba, R. A., Smith, T. C., Pearson, J. T., & Wood, P. B. (1975) *J. Magn. Reson. 18*, 254–264.
Smith, P., Kaba, R. A., Dominguez, L. M., & Denning, S. M. (1977) *J. Phys. Chem. 81*, 162–167.
Swain, C. G., & Hedberg, K. (1950) *J. Am. Chem. Soc. 72*, 3373–3375.
Talbot, N. B., Wolfe, J. K., MacLachlan, E. A., Karush, F., & Butler, A. M. (1940) *J. Biol. Chem. 134*, 319–330.
van der Heide, R. F. (1966) *J. Chromatogr. 24*, 239–243.
Willstätter, R., & Piccard, J. (1908) *Ber. 41*, 1458–1475.
Yamazaki, I. (1971) *Adv. Biophys. 2*, 33–76.
Yamazaki, I., Mason, H. S., & Piette, L. (1960) *J. Biol. Chem. 235*, 2444–2449.

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

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received January 8, 1979.* This work was supported and made possible by research grants from the National Institutes of Health, Bethesda, MD (GM-10287), from the United States Army Research Office, Research Triangle Park, NC (DRXRO-PR-P-15319-L), and from Merck Sharp & Dohme, Rahway, NJ.

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

0006-2960/79/0418-2329$01.00/0 © 1979 American Chemical Society