Mechanism of the Oxidation of 3,5,3′,5′-Tetramethylbenzidine by Myeloperoxidase Determined by Transient- and Steady-State Kinetics†

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ABSTRACT: Earlier investigations of the oxidation of 3,5,3′,5′-tetramethylbenzidine (TMB) using horseradish peroxidase and prostaglandin H-synthase have shown the formation of a cation free radical of TMB in equilibrium with a charge-transfer complex, consistent with either a two- or a one-electron initial oxidation. In this work, we exploited the distinct spectroscopic properties of myeloperoxidase and its oxidized intermediates, compounds I and II, to establish two successive one-electron oxidations of TMB. By employing stopped-flow techniques under transient-state and steady-state conditions, we also determined the rate constants for the elementary steps of the myeloperoxidase-catalyzed oxidation of TMB at pH 5.4 and 20 °C. The second-order rate constant for compound I formation from the reaction of native enzyme with H_2O_2 is 2.6×10^7 M^{-1} s^{-1}. Compound I undergoes a one-electron reduction to compound II in the presence of TMB, and the rate constant for this reaction was determined to be $(3.6 \pm 0.1) \times 10^6$ M^{-1} s^{-1}. The spectral scans show that compound II accumulates in the steady state. The rate constant for compound II reduction to native enzyme by TMB obtained under steady-state conditions is $(9.4 \pm 0.6) \times 10^5$ M^{-1} s^{-1}. The results are applied to a new, more accurate assay for myeloperoxidase based upon the formation of the charge-transfer complex between TMB and its diimine final product.

Peroxidases catalyze the oxidation by peroxide of a wide variety of substrates, many of which have strong absorption bands or give colored oxidation products. Thus, assays for peroxidases frequently employ spectrophotometric measurements of the disappearance of chromophoric substrates or the appearance of colored products (Chance & Maehly, 1955). For example, the oxidation of benzidine to benzidine blue has been used to detect the presence of heme proteins like peroxidases (Maehly, 1954). The carcinogenic hazard posed by the use of benzidine has prompted its replacement with 3,5,3′,5′-tetramethylbenzidine (TMB)[1] (Clayson & Garner, 1975; Choudhury, 1996). Since it was shown that carcinogenicity was mainly caused by *o*-hydroxylation of aromatic amines, TMB proved to be a noncarcinogenic derivative because *o*-hydroxylation was prevented (Holland et al., 1974). The use of TMB in peroxidase activity assays not only has been proven safe but also has been shown to be of higher sensitivity compared to benzidine (Mesulam, 1978). Enzyme immunoassays (EIA) employing horseradish peroxidase and TMB as chromogen have been developed and widely used (Bos et al., 1981; Pujol et al., 1993; Illera et al., 1996). TMB oxidation has also been used to assay the mammalian enzymes eosinophil peroxidase and myeloperoxidase (Bozeman et al., 1990).

Myeloperoxidase (MPO; donor:H_2O_2 oxidoreductase, EC 1.11.1.7) in polymorphonuclear leukocytes and monocytes plays an important role in killing bacteria phagocytosed by these cells (Klebanoff & Clark, 1978). MPO is localized in azurophilic granules and is released into extracellular sites when the leukocytes are stimulated. Recently, TMB oxidation has been used to detect and quantitate MPO activity associated with the influx of neutrophils into sites of inflammation (Schneider & Issekutz, 1996) and MPO activity in renal tissue after ischemia/reperfusion (Laight et al., 1994).

Previous investigations on TMB oxidation using horseradish peroxidase, lactoperoxidase, and prostaglandin H synthase by Josephy et al. (1982a,b, 1983) utilized optical and electron spin resonance spectroscopies. The results showed that during the course of the reaction a cation free radical is in rapid equilibrium with a charge-transfer complex of the parent diamine and product diimine. The oxidation of *o*-dianisidine (3,3′-dimethoxybenzidine) was proposed to occur as a direct two-electron oxidation because a free radical intermediate was not detected using electron spin resonance spectroscopy (Claiborne & Fridovich, 1979). However, a free radical of *o*-dianisidine, less stable than that of TMB, was detected later (Josephy et al., 1982a), again in equilibrium with a charge-transfer complex. Either a two-electron or a one-electron initial oxidation is consistent with the above results (Yamazaki, 1977).

Myeloperoxidase generally follows the normal peroxidase cycle (Dunford, 1991; Poulos & Fenna, 1994):

$$\text{MPO} + \text{H}_2\text{O}_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{MPO-I} + \text{H}_2\text{O}$$

$$\text{MPO-I} + \text{AH}_2 \xrightarrow{k_2} \text{MPO-II} + \text{AH}^\bullet$$

$$\text{MPO-II} + \text{AH}_2 \xrightarrow{k_3} \text{MPO} + \text{AH}^\bullet + \text{H}_2\text{O}$$

where a single two-electron oxidation of native enzyme

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[1] Abbreviations: TMB, 3,5,3′,5′-tetramethylbenzidine; EIA, enzyme immunoassay; MPO, MPO-I, MPO-II, myeloperoxidase and the oxidized enzyme intermediate compounds I and II; RZ, reinheitzahl (purity number).

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(MPO) to compound I (MPO-I) is followed by two successive one-electron reductions (by reducing substrate AH_2) to native enzyme via compound II (MPO-II). However, chloride reduces MPO-I directly to native enzyme by a two-electron process (Harrison & Schultz, 1976).

In this work, we have utilized stopped-flow techniques to obtain rapid-scan optical spectra, and transient- and steady-state kinetic data to show for the first time that TMB oxidation by peroxidase proceeds by two successive one-electron oxidations. We have measured the rate constants k_1, k_2, and k_3. We also present a more accurate MPO assay based upon TMB oxidation, and expressed enzyme activity in terms of the amount of H_2O_2 consumed. It is shown that TMB is more sensitive than guaiacol as an assay reagent (Chance & Maehly, 1955; Suzuki et al., 1983).

EXPERIMENTAL PROCEDURES

Materials. Bovine spleen myeloperoxidase was isolated and purified using a combination of modified procedures (Davis & Averill, 1981, 1984; Ikeda-Saito, 1985; Marquez et al., 1994). The enzyme preparation used in this study exhibited an RZ (A_{429}/A_{280}) of 0.84. The MPO concentration was determined spectrophotometrically using an extinction coefficient of 178 mM^{-1} cm^{-1} at 429 nm (Agner, 1958).

Hydrogen peroxide, obtained as 30% solution from BDH Chemicals, was diluted and the concentration determined by absorbance measurement at 240 nm where the extinction coefficient is 39.4 M^{-1} cm^{-1} (Nelson & Kiesow, 1972). The chemicals for the buffer solutions were used without further purification. Aqueous solutions were prepared using water purified through the Milli-Q system (Millipore Corp.). A stock solution of TMB (Sigma) was prepared in *N*,*N*′-dimethylformamide and kept in a dark bottle. The concentration of TMB was determined by measuring the absorbance at 285 nm where the extinction coefficient is 2.1×10^4 M^{-1} cm^{-1} (Josephy et al., 1982a). Further dilutions were made using Millipore water.

Methods. Routine absorbance measurements were made on a Beckman DU-650 spectrophotometer equipped with thermally jacketed 1-cm cuvette holders. Rapid spectral scans and kinetic measurements were performed using the SX.17 MV microvolume stopped-flow spectophotometer (Applied Photophysics). All experiments were carried out at 20.5 ± 0.5 °C and pH 5.4 (phosphate buffer) unless otherwise specified. All pH measurements were made using a Fisher Accumet Model 25 digital pH meter.

Transient State Experiments. Rapid spectral scans of MPO and its intermediates during the oxidation of TMB were conducted by taking time-dependent spectra from single-wavelength shots. One syringe contained 1 μM MPO and 50 μM TMB, for which no reaction was observed previously, while the other syringe contained 20 μM H_2O_2 in 0.2 M phosphate buffer at pH 5.4. The spectral region from 380 to 500 nm was scanned at 2 nm intervals for 200 ms. In another experiment, MPO-II, prepared by mixing 1 μM MPO with 50 μM H_2O_2, was placed in one syringe and 100 μM TMB in another. The region from 405 to 480 nm was scanned at 1 nm intervals. The spectra generated from the rapid scans were reconstructed using the GLint application software from Applied Photophysics.

Because of the inherent instability of MPO-I, the kinetics of the reaction of MPO-I with TMB were carried out using the sequential (multi-mixing) mode of the stopped-flow apparatus. One micromolar of myeloperoxidase was premixed with 20 μM H_2O_2 in 0.1 M buffer. After a delay time of 20 ms, the MPO-I formed was allowed to react with varying concentrations of TMB, the final concentrations of which were at least 20-fold in excess of the enzyme. The time course of the reaction was followed by monitoring the absorbance changes at 456 nm accompanying the formation of MPO-II. This wavelength is also the isosbestic point between native enzyme and MPO-I (Marquez et al., 1994). Pseudo-first-order rate constants were determined by using the single-exponential curve fit equation of the Applied Photophysics software on the the first phase of the time course. Six to eight determinations of rate constants were performed for each TMB concentration, and the mean values were plotted against the substrate concentration. The apparent second-order rate constant for the MPO-I reaction with TMB was calculated from the slope of the plot using linear least-squares regression analysis (Enzfitter, Elsevier-Biosoft).

Steady-State Experiments. The oxidation of TMB by the MPO/H_2O_2 system was also investigated by following the initial rates of product formation under steady-state conditions. Preliminary wavelength scans using the Beckman spectrophotometer showed that when 0.25 nM MPO is added to a solution containing 0.15 mM TMB and 0.3 mM H_2O_2, an absorbance peak at 652 nm steadily increases with time. Thus, the rate of oxidation of TMB can be determined by monitoring the absorbance at 652 nm as a function of time. Steady-state experiments were performed using the single-mixing mode of the stopped-flow apparatus. One syringe contained 10−250 μM H_2O_2 in 0.2 M phosphate buffer, pH 5.4. The other syringe contained a solution of 20 nM MPO and 20−200 μM TMB in water. The initial rate of the reaction was determined from the slope of the first linear portion of the kinetic trace obtained after mixing all the components within about 1.5 ms, the dead time of the instrument. Usually six or more kinetic traces were recorded, and the mean values of the the initial rates were calculated. Mean initial rates were plotted against H_2O_2 concentration for the various concentrations of TMB used. A nonlinear regression data analysis program (Enzfitter) was used for the curve fittings and calculations.

Enzyme Assays. We modified the published assay for myeloperoxidase using TMB (Andrews & Krinsky, 1986; Suzuki et al., 1983) by excluding phosphate-buffered saline solution from the reaction mixture and by measuring the initial rate of product formation at 652 nm and 20 °C instead of the final absorbance at 655 nm after 3 min of incubation at 37 °C. The 1-mL assay solution contained 80 mM phosphate, pH 5.4, 0.3 mM H_2O_2, and 1.6 mM TMB. A blank reading was taken at 652 nm using a 1-cm cuvette, and then 10 μL of the enzyme solution to be assayed was added. The initial increase in absorbance at 652 nm was used to calculate the amount of TMB oxidation product by using an extinction coefficient of 3.9×10^4 M^{-1} cm^{-1} (Josephy et al., 1982a).

The applicability of TMB oxidation as a quantitative assay for myeloperoxidase was also compared with the guaiacol assay (Chance & Maehly, 1955) which we have routinely used in our lab to determine myeloperoxidase activity. In the guaiacol assay, the assay solution was prepared by mixing 26.9 mL of distilled water, 3.0 mL of 0.1 M phosphate buffer at pH 7.0, 0.10 mL of 0.1 M H_2O_2, and 0.048 mL of guaiacol

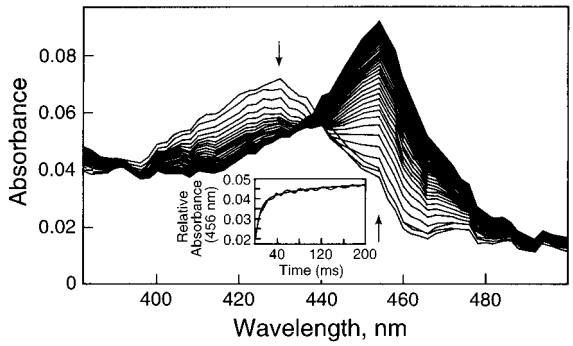

FIGURE 1: Rapid spectral scan of the oxidation of TMB by the MPO/H_2O_2 system. One syringe contained 1.0 μM MPO and 50 μM TMB while the other had 20 μM H_2O_2 in 0.2 M phosphate buffer at pH 5.4. The arrows show the direction of absorbance changes with time. The first, second, and last scans were taken 1.6, 2.6, and 100 ms after mixing. The inset shows a typical kinetic trace and curve fit (solid line) of MPO-I reduction by TMB followed at 456 nm using the sequential mixing mode. Final concentrations were 0.25 μM MPO, 5 μM H_2O_2 in 0.1 M phosphate buffer at pH 5.4.

(Sigma). Three milliliters of the assay solution was placed in a 1-cm cuvette, and a blank reading at 470 nm was taken. Ten microliters of the enzyme solution to be assayed was added and the initial change in absorbance at 470 nm measured. Calculations of the enzyme activity are based on the assumption that the only product formed during the oxidation reaction is tetraguaiacol whose extinction coefficient is 26.6 mM^{-1} cm^{-1} at 470 nm (George, 1953).

The concentrations of protein in milligram per milliliter of the enzyme samples were determined using the Bio-Rad Coomassie brilliant blue G-250 dye-protein assay with bovine serum albumin as standard.

RESULTS

Rapid spectral scans of the reaction between native MPO and H_2O_2 in the presence of TMB are shown in Figure 1. The absence of a single isosbestic point indicates that there exists an admixture of native enzyme and the oxidized intermediates MPO-I and MPO-II. In the presence of a 20-fold excess of H_2O_2, native MPO is rapidly converted to MPO-I (Marquez et al., 1994). The scans in Figure 1 demonstrate the fast reduction of MPO-I by TMB to MPO-II, characterized by the steadily increasing peak at 456 nm.

In order to determine the rate of reduction of MPO-I by TMB, it was necessary to employ sequential mixing stopped-flow techniques. MPO and H_2O_2 are premixed, and the MPO-I formed is allowed to react with TMB within 20 ms before MPO-I spontaneously decays to MPO-II. The time course for the reaction was followed at 456 nm, the isosbestic point between native MPO and MPO-I. Pseudo-first-order rate constants (k_{obs}) were obtained by fitting the kinetic traces to a single-exponential equation as shown in the inset to Figure 1. When the reaction was monitored at longer times, it was observed that the fast exponential rise in absorbance was followed by a slower linear increase. A spectral scan of the reaction system revealed a strongly absorbing species at 453 nm which coincided with the formation of MPO-II. Thus, only the initial increase in absorbance was fit to a single-exponential equation to determine k_{obs} values. The apparent second-order rate constant obtained from the slope of the plot of k_{obs} against TMB concentration was (3.6 $\pm$ 0.1) $\times$ 10^6 M^{-1} s^{-1}; the intercept of the plot was 11.4 $\pm$ 4.5 s^{-1}.

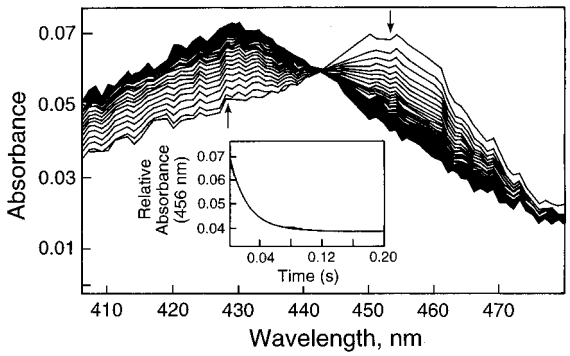

FIGURE 2: Rapid spectral scans of MPO-II reduction to native enzyme by TMB. One syringe contained MPO-II, prepared by adding 50 μM H_2O_2 to 1 μM MPO in 0.2 M phosphate, pH 5.4, while the other syringe contained 100 μM TMB. The arrows show directions of absorbance changes with time. The first, second, and last scans were taken 3, 8, and 500 ms after mixing. The inset is a typical trace of the absorbance change at 456 nm fit to a single exponential equation (solid line).

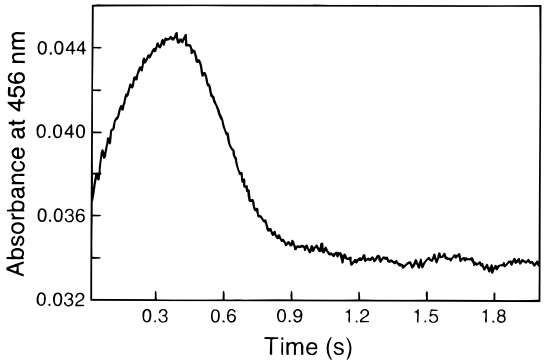

FIGURE 3: Time course of the two-step conversion of MPO-I to native enzyme. The kinetic trace was monitored at 456 nm using the sequential mixing mode of the stopped-flow apparatus. One micromolar of MPO was premixed with 20 μM H_2O_2, and after a delay time of 20 ms, the MPO-I formed is allowed to react with 50 μM TMB. The initial exponential rise is due to the one-electron reduction of MPO-I to MPO-II and the later exponential decline to the one-electron reduction of MPO-II to native enzyme.

A stable preparation of MPO-II is obtained by adding a 50-fold excess of H_2O_2 to pure native MPO. Upon addition of TMB, MPO is rapidly reduced to native enzyme (Figure 2 and inset) with an isosbestic point at 442 nm. In the presence of excess H_2O_2, the native enzyme formed can be rapidly converted to MPO-II until a steady-state concentration is attained. Thus, it is not possible to accurately determine the rate of the single-step conversion of MPO-II to MPO under transient-state conditions.

A kinetic trace of the two successive one-electron reductions of MPO-I to MPO-II followed by MPO-II to native enzyme is shown in Figure 3. The time course was monitored at 456 nm, the Soret peak of MPO-II. The fast exponential increase corresponds to the conversion of MPO-I to MPO-II while the slower exponential decrease shows the return of MPO-II to native enzyme.

While the rapid spectral scans under transient-state conditions allowed us to see the interconversion between MPO and its oxidized intermediates, we were also interested in the spectral changes accompanying the oxidation of the substrate TMB. Steady-state conditions were achieved by adding 0.25 nM MPO to a solution of 0.15 mM TMB and 0.3 mM H_2O_2. Figure 4 shows that upon addition of the enzyme, new peaks appeared at 370 and 652 nm, the

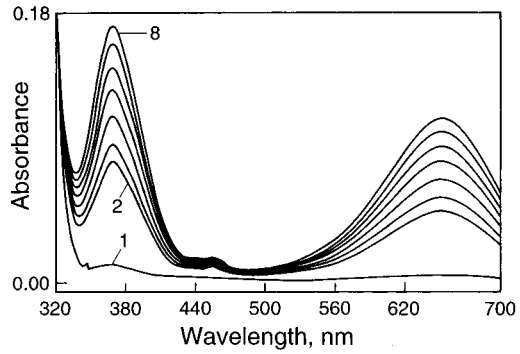

FIGURE 4: Spectral scans of the oxidation of TMB by MPO/H_2O_2 under steady-state conditions. The initial scan (1) was taken from a solution of 0.15 mM TMB and 0.3 mM H_2O_2 in 0.1 M phosphate buffer at pH 5.4. The next scan (2) was taken immediately after 0.25 nM was added to the reaction mixture; the succeeding scans were taken at 3 min intervals at a scan rate of 20 nm/s.

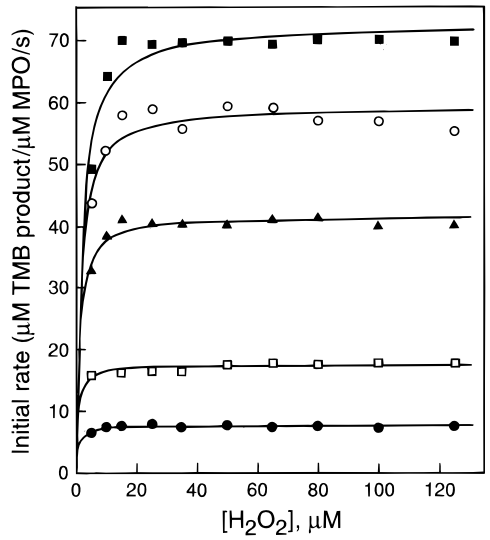

FIGURE 5: Steady-state kinetics of TMB oxidation: dependence of the initial rate of TMB oxidation on H_2O_2 and TMB concentrations. The reaction mixture contained 10 nM MPO, and the final concentrations of TMB were 10 μM (●), 20 μM (□), 40 μM (▲), 60 μM (○), and 80 μM (■).

absorbance of which increased steadily with time. A shoulder at 450 nm is also apparent but does not contribute significantly to the optical spectra of the oxidation product of TMB.

Initial rates of TMB oxidation were measured by following the rate of increase of absorbance at 652 nm where the molar extinction coefficient of the TMB charge-transfer complex formed between TMB and its diimine oxidation product was determined to be 3.9×10^4 M^{-1} cm^{-1} (Josephy et al., 1982a). The initial rates were determined as a function of [H_2O_2] at different TMB concentrations, yielding a family of hyperbolic curves (Figure 5). The initial rate and substrate concentrations were fitted to the hyperbolic equation:

$$\frac{\text{d[TMB oxidation product]/d}t}{[\text{E}]_0} = \frac{A[\text{H}_2\text{O}_2]}{B + [\text{H}_2\text{O}_2]}$$

where $[E]_0$ is the total MPO concentration and A and B are steady-state parameters.

The initial rate equation derived from the classical peroxidase mechanism and the putative disproportionation of the radical cation $TMB^{\bullet+}$ to form the charge-transfer complex

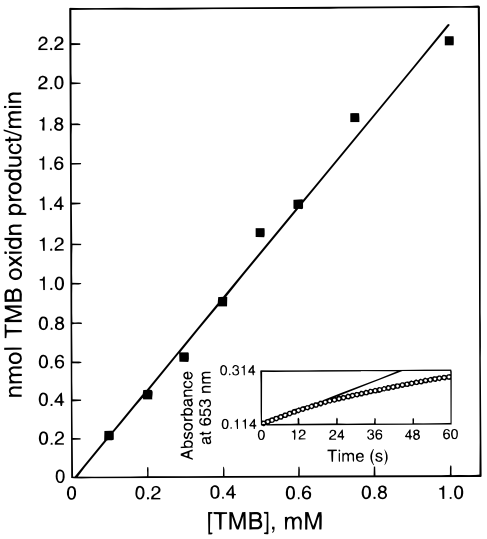

FIGURE 6: Dependence of the rate of TMB oxidation on TMB concentration. The 1-mL assay mixture contained 80 mM phosphate buffer, pH 5.4, 0.3 mM H_2O_2, 0.74 nM MPO, and TMB concentrations ranging from 0.1 to 1.0 mM. The initial rate was calculated from the slope of the early phase of the time course (inset) monitored at 652 nm.

(Josephy et al., 1982a) is given by

$$\frac{\text{d[TMB oxidation product]/d}t}{[\text{E}]_0} = \frac{k_3[\text{TMB}][\text{H}_2\text{O}_2]}{(k_3/k_1)[\text{TMB}] + [\text{H}_2\text{O}_2]}$$

Therefore, $A = k_3$[TMB] and $B = (k_3/k_1)$[TMB].

The secondary plot of parameter A against TMB concentration was linear from which k_3 was obtained: $(9.4 \pm 0.6) \times 10^5$ M^{-1} s^{-1}. The plot of parameter B against TMB concentration was also linear and yielded a k_1 value of 2.6×10^7 M^{-1} s^{-1}. The value of k_{-1}, 58 ± 4 s^{-1}, has been measured previously and does not affect the determination of k_1 (Marquez et al., 1994).

The TMB assay for myeloperoxidase was performed under previously optimized conditions of 1.6 mM TMB, 0.3 mM H_2O_2, pH 5.4, and incubation time of 3 min at 37 °C (Suzuki et al., 1983). Single absorbance measurements at 655 nm were taken after stopping the reaction with cold acetate buffer at pH 3.0. When we employed these conditions to measure the activity of our purified myeloperoxidase preparations, we found that the rate of TMB oxidation was linear from 0.25 to 1.5 nM enzyme but began to level off at higher enzyme concentrations. We also noted that the oxidation reaction continued to proceed even after addition of cold acid. Moreover, the absorbance at 655 nm did not increase linearly during the 3-min incubation period. While the published TMB assay procedure proved to be very sensitive in detecting myeloperoxidase activity particularly in polymorphonuclear leukocyte supernatants (Andrews & Krinsky, 1986), we modified the procedure for the purpose of quantifying the activity of highly purified enzyme preparations (RZ $\geq$ 0.80). In order to slow down the reaction, we performed the assay at 20.5 ± 0.5 °C. To obtain a more accurate rate of product formation, we monitored the absorbance change at 652 nm within 1 min after initiating the reaction and determined the slope of the initial linear phase of the time course (Figure 6, inset). The enzyme activity in terms of nanomoles of TMB oxidation product (extinction coefficient of 3.9×10^4 M^{-1}

Table 1: Enzyme Activities of Three MPO Preparations As Assayed Using TMB (pH 5.4) and Guaiacol (pH 7) at 20 °C[a]

RZ of MPO sample	TMB assay		guaiacol assay	
	ΔA_{652}/min	units/mL	ΔA_{470}/min	units/mL
0.72	0.6152	243	0.0694	241
0.82	0.8920	369	0.0848	309
0.84	1.0165	458	0.0954	377

[a] Enzyme activities are defined both in terms of changes in absorbance as a function of time and in terms of the amount of enzyme which consumes 1 μmol of H_2O_2 per minute. The protein concentration in each sample is 1 mg/mL.

cm^{-1}) formed per minute was linearly dependent on the concentrations of TMB (Figure 6) and MPO (data not shown).

Our lab has routinely used the guaiacol method (Maehly, 1954) to assay MPO activity. On oxidation with peroxidase and H_2O_2, guaiacol forms the amber-colored product tetraguaiacol whose extinction coefficient is 26.6 mM^{-1} cm^{-1} at 470 nm (George, 1953). We assayed three different preparations of bovine spleen MPO using the TMB and guaiacol assays. Both these assays for MPO should reflect the amount of H_2O_2 reacted. Thus, instead of defining the activity of the enzyme in terms of the magnitude of the absorbance change at a specified wavelength per unit time, we defined 1 unit (U) of enzyme activity as the amount of enzyme that utilizes 1 μmol of H_2O_2 per minute at 20 °C. In the case of the TMB assay, since 1 mol of H_2O_2 produces 0.5 mol of the charge-transfer complex that absorbs at 652 nm (Josephy et al., 1982a), and 10 μL of enzyme sample is added to 1 mL of assay solution in a 1-cm cuvette, then

$$\frac{\text{U}}{\text{mL}} = \left(\frac{2 \times \Delta A_{652\text{ nm}}/\text{min}}{3.9 \times 10^4\ \text{M}^{-1}}\right)\left(\frac{10^3\ \mu\text{mol}}{\text{mmol}} \times 1\ \text{mL} \times \frac{1}{10\ \mu\text{L}} \times \frac{1000\ \mu\text{L}}{\text{mL}}\right)$$

$$= \Delta A_{652\text{ nm}}/\text{min} \times 5.13$$

On the other hand, in the case of the guaiacol assay since 4 mol of H_2O_2 is used to produce 1 mol of the tetraguaiacol product (Maehly, 1954), and 10 μL of enzyme sample is added to 3 mL of assay solution in a 1-cm cuvette, then the enzyme activity is calculated as

$$\frac{\text{U}}{\text{mL}} = \left(\frac{4 \times \Delta A_{470\text{ nm}}/\text{min}}{26.6\ \text{mM}^{-1}}\right)\left(3\ \text{mL} \times \frac{1}{10\ \mu\text{L}} \times \frac{1000\ \mu\text{L}}{\text{mL}}\right)$$

$$= \Delta A_{470\text{ nm}}/\text{min} \times 45.1$$

Table 1 shows the results of the enzyme activity calculations for three MPO samples of different purity (RZ) using both the TMB and guaiacol assays.

DISCUSSION

The oxidation of amines such as benzidine and its derivatives by inorganic catalysts as well as enzymatic systems has been the subject of numerous investigations [reviewed by Saunders (1973)]. The elucidation of the oxidation mechanism is important in predicting the toxicological effects of xenobiotics which are substrates of peroxidases (Yamazaki, 1977; Mason, 1979).

The optical spectra of peroxidases and their oxidized intermediates make them amenable for mechanistic studies using absorption spectroscopy. By employing rapid-scan stopped-flow techniques, it is possible to probe the mechanism of the reaction by following the interconversion between native enzyme and its oxidized intermediates.

Previous investigations on the oxidation of TMB were performed using horseradish peroxidase (Josephy et al., 1982a) and prostaglandin H synthase (Josephy et al., 1982b). In both cases, a cation free radical was identified using electron spin resonance spectroscopy while a charge-transfer complex was observed using optical spectroscopy. The charge-transfer complex is formed when the two-electron oxidation product, the diimine, interacts with the original diamine substrate (Lakshmi et al., 1994). Since the free radical is in rapid equilibrium with the charge-transfer complex, the detection of free radicals does not establish whether the initial enzymatic oxidation proceeds via a one-electron or a two-electron mechanism. An alternative possibility suggested by Wise et al. (1984) for the benzidine reaction is that peroxidases may oxidize the diamine directly by two electrons to the diimine and then the diimine can undergo a one-electron reduction to the cation free radical. In addition, some reducing substrates are capable of donating two electrons to compound I and one electron to compound II (Dunford, 1995). By monitoring the formation and disappearance of enzyme intermediates during the oxidation reaction, it is possible to rule out one mechanism over another.

Our experiments were performed at pH 5.4 which was found to be the optimal pH for measuring enzyme activity using the TMB assay (Suzuki et al., 1983). We also found that the TMB oxidation products are more stable at acid pH. Moreover, while myeloperoxidase is stable from pH 2.5 to 12.8 (Maehly, 1955), the pH inside a phagocyte has been reported to be within pH 4−6 (Bainton, 1973); thus, our experiments were carried out under optimal and physiological pH conditions.

The spectral scans and kinetic trace at 456 nm (Figures 1 and 3) reveal the rapid reduction of MPO-I to MPO-II in the presence of TMB. This is indicative of a one-electron reaction. We were able to dismiss the possibility that the species at 456 nm is the two-electron oxidation product diimine based on the extinction coefficient (Josephy et al., 1982a) as well as the absence of native enzyme which should be formed accompanying a two-electron process from MPO-I. The kinetic trace at 429 nm, the isosbestic point between MPO-I and MPO-II, does not show any change in absorbance with time. Since 429 nm is the Soret peak of the native enzyme, the result indicates that MPO-I was not directly reduced to the native enzyme via a two-electron step. Instead, TMB undergoes a one-electron oxidation, and concurrently MPO-I is reduced to MPO-II. Our results thus support the formation of a cation free radical formed as a result of the one-electron oxidation of TMB. MPO-II consequently goes back to native enzyme via a second one-electron oxidation of TMB (Figure 3). The single isosbestic point at 442 nm suggests that MPO-II does not pass through any other intermediate when it goes back to native enzyme.

To the best of our knowledge, there has not been any published data on the kinetics of the peroxidase-catalyzed oxidation of TMB despite the widespread use of this substrate as chromogen in EIA (Bos et al., 1981; Pujol et al., 1993;

Illera et al., 1996) and in measuring MPO activity (Bozeman et al., 1990; Suzuki et al., 1983; Andrews & Krinsky, 1986). The second-order rate constant k_2 [$(3.6 \pm 0.1) \times 10^6$ M^{-1} s^{-1}] for the MPO-I reaction with TMB is comparable to the rate constant of MPO-I reaction with chloride [$(4.7 \pm 0.1) \times 10^6$ M^{-1} s^{-1}] (Marquez & Dunford, 1995), the physiological MPO substrate. While extreme care was taken to ensure that strictly MPO-I reaction with TMB was being measured, i.e., by using sequential mixing stopped-flow and by operating at the isosbestic point of MPO and MPO-I (456 nm), the appearance of an absorbing species at 453 nm complicates data analysis of the kinetic traces and therefore gives at best an estimate of the k_2 value. The intercept of the plot of k_{obs} against the concentration of TMB in its reaction with MPO-I (11.4 ± 4.5 s^{-1}) corresponds to the rate of spontaneous reduction of MPO-I to MPO-II in the absence of extraneous reducing substrates. In an earlier work using tyrosine as reducing substrate (Marquez & Dunford, 1995), we measured this rate to be 2.2 ± 1.2 s^{-1}. The value we report in the present work may be slightly overestimated because of the interfering absorbance at 453 nm, but we minimized this effect by measuring the rates at the very early stages of the reaction.

The second-order rate constant for the reaction of MPO-II with TMB, k_3, was determined under steady-state conditions [$(9.4 \pm 0.6) \times 10^5$ M^{-1} s^{-1}]. The steady-state experiments also yielded a k_1 value of 2.6×10^7 M^{-1} s^{-1} (at pH 5.4) which is within the range of previously reported rate constants: 1.8×10^7 M^{-1} s^{-1} (at pH 6.5) (Marquez et al., 1994) and 3.3×10^7 M^{-1} s^{-1} (at pH 4.7) (Marquez & Dunford, 1994), and reflects the effect of pH on MPO-I formation (Marquez et al., 1994).

The spectral scan of the TMB oxidation products (Figure 4) for the MPO-catalyzed reaction is similar to that of the prostaglandin H synthase-catalyzed reaction (Josephy et al., 1982b), with peaks at 370 and 652 nm, characteristic of the charge-transfer complex (Josephy et al., 1982a). The optical spectra of the horseradish peroxidase-catalyzed reaction reveal a peak at 450 nm due to the diimine. The difference is not due to a change in mechanism but can be accounted for by the different concentrations of TMB in the two systems. An equilibrium analysis of the concentrations of various species in the reaction system (Josephy et al., 1982a) predicts that at low TMB concentration, as in the case of the HRP-catalyzed reaction, the diimine is the predominant species.

The modified assay procedure we have presented in this work is suggested as a substitute to previously published procedures (Andrews & Krinsky, 1986; Suzuki et al., 1983). The rationale behind our modifications is as follows: we omitted the phosphate-buffered saline in the assay mixture because it contains 0.12 M chloride which is also a MPO substrate, but which reacts by a direct two-electron mechanism (Harrison & Schultz, 1976; Marquez & Dunford, 1995). We lowered the assay temperature from 37 °C to 20 °C because we found the reaction to be too fast, especially for very pure MPO preparations. While a sufficiently diluted enzyme preparation could also slow down the rate, excessive dilution of the enzyme increases the chance of denaturation. Instead of measuring a single absorbance reading at 655 nm after a 3 min incubation, we continually monitored the absorbance change at 652 nm for the first minute of reaction. This is because we noted that the absorbance change is not linear for 3 min but starts to level off (Figure 6, inset). The 652 nm absorbance is caused exclusively by the charge-transfer complex; it has an extinction coefficient of 3.9×10^4 M^{-1} cm^{-1} (Josephy et al., 1982a).

Compared to the guaiacol assay, the TMB assay gives a bigger absorbance change due to the higher extinction coefficient of the TMB oxidation product. TMB is also more sensitive to the degree of purity of the enzyme preparation (Table 1). Moreover, there is still some contention as to the nature of the absorbing species in the guaiacol assay. It has been assumed that the species which absorbs at 470 nm is tetraguaiacol (Maehly, 1954). However, Booth and Saunders (1956) argue that tetraguaiacol could not account for the amber-colored product and proposed that the species absorbing at 470 nm is more likely 3,3′-dimethoxybiphenoquinone. Another disadvantage of the use of the guaiacol assay is the unexpected side reaction that occurs when catechol is present even as a minute impurity (Taurog et al., 1992). For these reasons, we prefer to use the TMB assay for the quantitative determination of MPO activity. We have expressed the units of enzyme activity using both TMB and guaiacol in terms of the amount of H_2O_2 consumed per unit time. This is a better definition of enzyme activity because it allows the comparison of enzyme activity units no matter which chromophoric substrate or product is being measured in any given assay.

In conclusion, transient-state spectral and kinetic measurements allowed us to establish the two successive one-electron oxidations of TMB by the MPO/H_2O_2 system. The rate constants determined for the elementary steps suggest that MPO-II is the enzyme intermediate that accumulates in the steady state. The cation free radical formed during the peroxidatic oxidation of TMB is derived from a one-electron oxidation process. A modified TMB assay for MPO offers distinct advantages over the guaiacol assay.

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