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Peroxidase-Catalyzed Co-oxidation of 3,3′,5,5′-Tetramethylbenzidine with 2-Amino-4-nitrophenol, 4,4′-Dihydroxydiphenylsulfone, and Their Polydisulfides in Aqueous and Micellar Media

E. I. Karasyova[1], Yu. P. Losev[2], and D. I. Metelitza[1]*

[1]Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, ul. Kuprevicha 5/2, Minsk, 220141 Belarus; fax: (375)-(172) 63-7274; E-mail: enzyme@ns.iboch.ac.by
[2]School of Chemistry, Belorussian State University, Leningradskaya ul. 14, Minsk, 220050 Belarus; fax: (375)-(172) 26-5942; E-mail: organic@chem.bsu.unibel.by

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Abstract—The effects of different concentrations of 2-amino-4-nitrophenol (ANP) and of its polydisulfide (poly(ADSNP)) on peroxidase-catalyzed oxidation of 3,3′5,5′-tetramethylbenzidine (TMB) were studied at 20°C in reversed micelles of AOT (0.2 M) in heptane and in mixed reversed micelles of AOT (0.1 M)–Triton X-100 (0.1 M) in isooctane supplemented with 15% hexanol. The oxidation of TMB was activated nearly twofold in the presence of ANP and nearly fourfold in the presence of poly(ADSNP) in reversed micelles of AOT, whereas in the mixed micelles oxidation of the TMB–ANP pair was associated with inhibition of TMB conversion and poly(ADSNP) activated oxidation of TMB. The co-oxidation of TMB with 4,4′-dihydroxydiphenylsulfone (DDS) and with its polydisulfide (poly(DSDDS)) at different concentrations of phenol components was accompanied by activation of TMB conversion in 0.01 M phosphate buffer (pH 6.4) supplemented with 5% DMF and in reversed micelles of AOT in heptane. The effect of pH of the aqueous solution on the initial oxidation rate of the TMB–DDS and TMB–poly(DSDDS) pairs and also the effect of hydration degree of reversed micelles of AOT on conversion of the same pairs by peroxidase were studied. A scheme of peroxidase-dependent co-oxidation of "aromatic amine–phenol" pairs is proposed and discussed. A significant part of this scheme is a nonenzymatic exchange of phenoxyl radicals with amines and of aminyl radicals with phenols.

Key words: horseradish peroxidase, tetramethylbenzidine, 2-amino-4-nitrophenol, 4,4′-dihydroxydiphenylsulfone, phenol polydisulfides, co-oxidation, inhibition of peroxidase reactions

The co-oxidation of phenols with various amines is widely used for quantitative photometric determination of phenols and naphthols [1]. Especially interesting is the co-oxidation of "amine–phenol" pairs catalyzed by peroxidases [2-13].

The peroxidase-catalyzed oxidation of such pairs as 4-aminoantipyrine (AAP)–phenols [5-7] and luminol–phenols [8-13] is characterized by a highly increasing rate of amine oxidation, whereas the co-oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) with gallic acid [14], its polydisulfide (poly(DSG)) [15, 16], 2-amino-4-nitrophenol (ANP), and its polydisulfide [17] is characterized by a clearly pronounced inhibition of TMB oxidation. The degree of inhibition of the peroxidase-catalyzed oxidation of TMB depends on the phenol type and is much higher for polydisulfide inhibitors compared to analogs of their monomer units [14-17].

The peroxidase-catalyzed co-oxidation of aromatic amines and phenols is a complex of consecutive and parallel enzymatic and nonenzymatic reactions that we have considered and analyzed in previous works [5-7, 12-17]. We think that a particularly important role in the peroxidase-catalyzed co-oxidation belongs to the nonenzymatic exchange reaction discovered by Academician N. M.

Abbreviations: AOT) Aerosol OT, or sodium salt of di-(2-ethyl)hexyl ester of sulfosuccinic acid; ANP) 2-amino-4-nitrophenol; poly(ADSNP)) poly(2-aminodisulfide-4-nitrophenol); DDS) 4,4′-dihydroxydiphenylsulfone; poly(DSDDS)) polydisulfide of 4,4′-dihydroxydiphenylsulfone; TMB) 3,3′5,5′-tetramethylbenzidine; HP) horseradish peroxidase; $AmNH_2$) aromatic amine; *f*) stoichiometric inhibition coefficient; PhOH) phenols; W_o) hydration degree of reverse micelles of surfactants in organic solvents.

* To whom correspondence should be addressed.

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Emanuel and his coworkers [18, 19] in the liquid-phase oxidation of hydrocarbons inhibited by "amine–phenol" mixtures: $PhO^{\cdot} + AmNH_2 \rightleftarrows PhOH + AmNH^{\cdot}$.

The effect of phenol on the peroxidase-catalyzed oxidation of amine depends on the reaction direction: if the reaction goes from left to right, the amine oxidation is accelerated and the phenol is regenerated (first variant); if the reaction goes from right to left, the phenol oxidation is accelerated and the amine is regenerated (second variant).

Obviously, to predict the mutual effect of phenols and amines during their peroxidase-catalyzed co-oxidation, one should know rate constants of the exchange reaction in both directions for each of "amine–phenol" pairs. However, these rate constants are usually not determined. Therefore, "accelerators" of oxidation of aromatic amines on one hand and effective inhibitors of peroxidase-catalyzed conversion of amines on the other are still chosen empirically, especially because the general direction of co-oxidation in "amine–phenol" pairs depends not only on the nonenzymatic exchange reaction.

Thus, it was important to find such "amine–phenol" pairs for which changes in conditions during the peroxidase-catalyzed oxidation (the environment pH, organic co-solvent, micelles instead of buffer solution, etc.) would be associated with one of the components being either an accelerator or an inhibitor of the oxidation of the other component, i.e., it was necessary to study the co-oxidation of the same amine with different phenols under different conditions. For the amine we have chosen a widely used peroxidase substrate, TMB.

The present work was designed to study kinetics of the peroxidase-catalyzed co-oxidation of TMB with ANP and with its polydisulfide in reversed micelles of varied composition and also with 4,4′-dihydroxydiphenylsulfone (DDS) and with its polydisulfide (poly(DSDDS)) in aqueous medium and in reversed AOT micelles in heptane. These phenols and their polydisulfides were chosen because of their significant difference from the polyatomic phenols used by us earlier [14-16]. Changes in the structure of phenol components and in conditions of their co-oxidation with the same amine (TMB) should be promising with respect to new information upon the specific features of the peroxidase-catalyzed co-oxidation of "aromatic amine–substituted phenol" pairs.

MATERIALS AND METHODS

Reagents. The acid isoform of horseradish peroxidase (EC 1.11.1.7), type A, with optical purity index RZ 2.75 was from Biolar (Olaine, Latvia). The enzyme concentration was determined spectrophotometrically using the molar absorption coefficient in the Soret band maximum (403 nm) equal to 102,000 $M^{-1}\cdot cm^{-1}$ [20]. Diluted hydrogen peroxide solution was used as an oxidizer; the concentration of H_2O_2 was determined spectrophotometrically using ε (203 nm) = 72.1 $M^{-1}\cdot cm^{-1}$ [21]. TMB (Serva, Germany) was used for the reducing substrate of HP. Organic solvents (heptane, isooctane, dimethylformamide (DMF), and hexanol) were distilled before use. All salts and bases for preparation of buffer solutions were from Reakhim (Russia).

For preparation of reversed micelles in organic solvents, AOT (Serva) and Triton X-100 (Sigma, USA) were used.

Phenols and their polydisulfides. 2-Amino-4-nitrophenol (ANP) and 4,4′-dihydroxydiphenylsulfone (DDS) were from Reakhim. ANP polydisulfide (poly(ADSNP)) with average molecular weight ~1400 daltons (~7 monomer units) was prepared as described earlier [22]. DDS polydisulfide (poly(DSDDS)) with average molecular weight ~2350 daltons (7-8 monomer units) was synthesized as described earlier [23].

Absorption spectra of ANP, DDS, and of their disulfides were recorded in different media; the spectral characteristics of the substituted phenols are presented in Table 1. Based on comparison of molar absorption coefficients of ANP, DDS, and their polydisulfides, it was concluded that poly(ADSNP) and poly(DSDDS) contained 7.0-7.3 and ~7.8 monomer units, respectively; this was consistent with the element analysis of the polydisulfides.

Peroxidase-catalyzed oxidation of TMB in aqueous media without and with phenols. Stock solutions of 0.1 M phosphate buffer (pH 6.4), 0.2 M H_2O_2, and 0.2 μM HP were prepared in distilled water. Stock solutions of 0.2 M TMB, DDS, and their polydisulfides were prepared in distilled DMF.

TMB was oxidized without and with phenols at 20°C in 0.01 M phosphate buffer (pH 6.4) supplemented with 5% DMF (buffer A) and, as a rule, 1 nM HP, 1 mM H_2O_2, and 0.5 or 1.0 mM TMB. The total volume of the reaction mixture was 1 ml. The reaction was initiated by addition of H_2O_2 solution and was followed spectrophotometrically by recording the increase in optical density of the TMB oxidation product at the maximum of its absorption at 655 nm. It should be noted that the absorption bands of all four substituted phenols and of their conversion products (Table 1) did not overlap with the absorption band of the TMB oxidation product (655 nm), i.e., the determination of the TMB oxidation product was uncomplicated. The initial rate of TMB oxidation was calculated using the molar absorption coefficient of its product, 39,000 $M^{-1}\cdot cm^{-1}$ [20]. The reactions were carried out in a thermostatted cuvette of a Specol-211 device (Carl Zeiss, Germany).

Peroxidase-catalyzed oxidation of TMB in micellar media without phenols and in their presence. The initial solution of Triton X-100 was prepared using isooctane

Table 1. Spectral characteristics of 2-amino-4-nitrophenol (ANP), 4,4′-dihydroxydiphenylsulfone (DDS), and their polydisulfide derivatives (poly(ADSNP) and poly(DSDDS)) in different media

Medium	Phenols	Absorption maximums, nm			Molar absorption coefficients × 10^{-4}, $M^{-1}\cdot cm^{-1}$		
		λ_1	λ_2	λ_3	ε_1	ε_2	ε_3
0.01 M phosphate buffer (pH 6.4) supplemented with 10% DMF	ANP	257	314	433	0.83	0.45	0.54
	poly(ADSNP)	259	314	437	5.75	3.13	3.81
Reversed micelles of AOT (0.2 M) in heptane: 9% 0.001 M phosphate buffer (pH 6.4) supplemented with 1% DMF	ANP	261	310	383	0.98	0.49	0.44
	poly(ADSNP)	259	310	373	6.92	3.49	3.17
Mixed reversed micelles of AOT (0.1 M)–Triton X-100 (0.1 M) in isooctane supplemented with 15% hexanol: 9% 0.001 M phosphate buffer (pH 6.4) supplemented with 1% DMF	ANP	—	—	387	—	—	0.38
	poly(ADSNP)	—	—	388	—	—	2.77
0.01 M phosphate buffer (pH 6.4) supplemented with 10% DMF	DDS	262	—	—	1.96	—	—
	poly(DSDDS)	262	—	—	15.40	—	—

supplemented with 15% hexanol. The total volume of all micellar mixtures was 1.2 ml. The mixtures were prepared as follows: 0.012 ml of buffer A, ANP, DDS, or their polydisulfides were successively solubilized in 0.2 M AOT solution in heptane and H_2O was added depending on the preset value of the hydration degree W_o; the mixture was supplemented with 0.006 ml of TMB solution in DMF and maintained for 2 min at 20°C; then the mixture was supplemented with 0.006 ml of HP solution and intensively shaken for 15 sec; then 0.006 ml of H_2O_2 solution was added, and the mixture was shaken again for 15 sec, and this was believed to initiate the reaction.

The final concentrations of the micellar system components calculated per their total volume were as follows: 0.001 M buffer, 1 mM TMB, 1 mM H_2O_2, 1 nM HP, and 1.5% DMF. In various experiments the preset concentrations of all four substituted phenols were varied, as it is mentioned in the figure captions and in the text.

Mixed micelles of AOT (0.1 M)–Triton X-100 (0.1 M) were similarly prepared in isooctane supplemented with 15% hexanol.

TMB was co-oxidized with phenols at 20°C in cuvettes of a KFK.3 photometer (Russia) equipped with a thermostatted cuvette section and with digital indication of optical density. The TMB oxidation was followed by the increase in the light absorption of the product at 674 nm. The initial rates of TMB oxidation were calculated similarly to calculations for aqueous media. Within 1-40 min, the absorption of the oxidation products failed to contribute to the absorption band of the TMB transformation product in reversed micelles of different composition, i.e., the determination of TMB oxidation rates in the micellar systems in all cases was uncomplicated.

RESULTS AND DISCUSSION

In our previous work [17], the peroxidase-catalyzed co-oxidation of TMP with ANP or with its polymer derivative poly(ADSNP) in 0.01 M phosphate buffer (pH 6.4) supplemented with 5% DMF (buffer A) was shown to markedly inhibit the amine conversion. To present and discuss the findings on the peroxidase-catalyzed co-oxidation of TMB with substituted phenols in reversed micelles of surfactants of varied composition, it seems necessary to consider certain specific features of the peroxidase-catalyzed oxidation of TMB itself in the micellar media chosen.

Peroxidase-catalyzed oxidation of TMB in reversed micelles of surfactants. We have shown earlier that the peroxidase-catalyzed oxidation of TMB in reversed micelles of AOT in heptane is described by the Michaelis–Menten equation over a wide range of the substrate concentration and strongly depends on the pH of the aqueous microphase of micelles [16]. In the present work the Michaelis–Menten dependences were obtained for the peroxidase-catalyzed oxidation of TMB in the mixed micelles of AOT (0.1 M)–Triton X-100 (0.1 M) in isooctane supplemented with 15% hexanol under the following conditions: 20°C, 0.001 M phosphate buffer (pH 6.4) as the aqueous phase, 1.5% DMF, 1 nM HP, 1 mM TMB, and 1 mM H_2O_2 (the concentrations are calculated for the total volume of the micellar system). The Michaelis–Menten equation was valid for different hydration degrees W_o (15.3-25.0) of the mixed micelles.

At pH values of the aqueous phase of micelles higher than 6.0, the rates of TMB oxidation decreased monotonically in the micelles of both types. The dependences of v_o on pH were similar in the micelles of both types at micelle

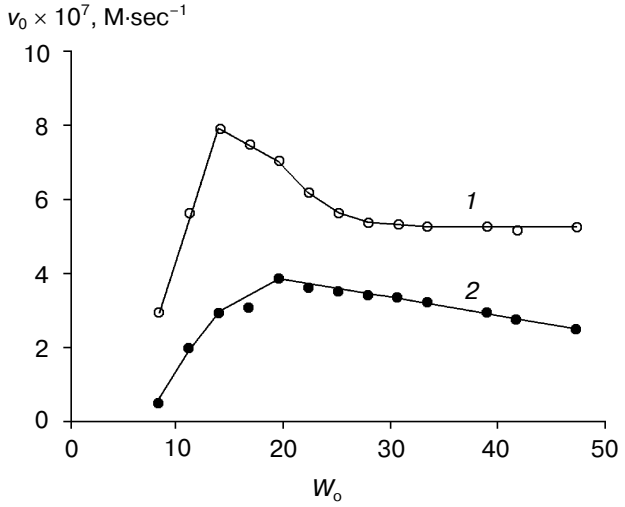

Fig. 1. Dependences of the initial rate of the peroxidase-catalyzed TMB oxidation in reversed micelles of AOT (0.2 M) in heptane (*1*) and in mixed micelles of AOT (0.1 M)–Triton X-100 (0.1 M) in isooctane supplemented with 15% hexanol (*2*) on the hydration degree of the micelles: HP (1 nM), TMB (1 mM), H_2O_2 (1 mM).

hydration degrees 15.3 and 25.0, but the oxidation rates in the mixed micelles were decreased nearly twofold.

It is known that the true pH values of the aqueous phase of micelles differ, as a rule, from the initial pH values of the solubilized solution [24, 25]. It was shown [25] that for reversed micelles of AOT, Eq. (1) gives the relation between pH_{mic} and the pH of the initial aqueous solution:

$$pH_{mic} = 0.66\ pH + 2.32. \quad (1)$$

It follows from the empirical equation (1) that pH in AOT micelles should increase at pH < 6.8 and decrease at pH > 6.8; at pH 6.8 of the aqueous solution, its value inside micelles is unchanged; thus, this pH value seems to be an inversion point. According to Eq. (1), in our case, if pH of the aqueous solution is 6.4, the pH inside the micelles is 6.5, and if the pH of the aqueous phase is 7.4, pH_{mic} is 7.2, i.e., changes in pH inside the micelles are relatively small.

The effect of hydration degree of reversed micelles on the initial rate of TMB oxidation is shown in Fig. 1. The activity maximum at $W_o = 14$ specific for micellar systems was found for AOT micelles in heptane (dependence *1*), and this is close to or in agreement with data for the solubilized peroxidase (for instance, see [26]). In the mixed micelles the activity maximum is shifted to $W_o = 19$ (dependence *2*).

Thus, the pH of the aqueous microphase of micelles and their hydration degree W_o are important factors that influence the peroxidase-catalyzed oxidation of an individual amine (TMB) in reversed micelles of AOT in heptane and in the mixed reversed micelles of AOT–Triton X-100 in isooctane supplemented with 15% hexanol. These factors should be taken in consideration for the peroxidase-catalyzed co-oxidation of TMB with substituted phenols in micellar systems.

The peroxidase-catalyzed co-oxidation of TMB with ANP and with its polymer derivative in reversed micelles of surfactants. The effect of increasing concentrations of ANP on the initial rate of the peroxidase-catalyzed TMB oxidation in reversed micelles of AOT in heptane is shown in Fig. 2a: the oxidation of TMB is activated within the whole range of ANP concentrations, and thus the initial rate of this process increases more than twofold at $[ANP]_o = 0.1$ mM and at TMB concentration 10 times higher than the concentration of phenol added. Figure 2b presents the

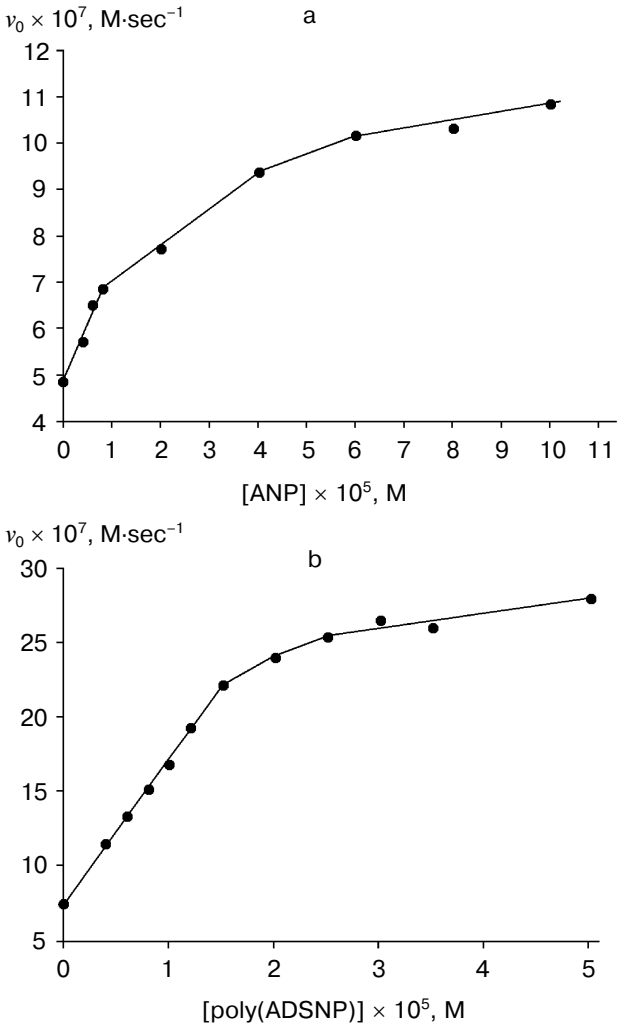

Fig. 2. Dependences of the initial rate of the peroxidase-catalyzed TMB oxidation in reversed micelles of AOT (0.2 M) in heptane on concentrations of ANP (a) and poly(ADSNP) (b): $W_o = 20.85$, HP (1 nM), TMB (1 mM), H_2O_2 (1 mM); 0.001 M phosphate buffer (pH 6.4) supplemented with 1.5% DMF as the aqueous phase.

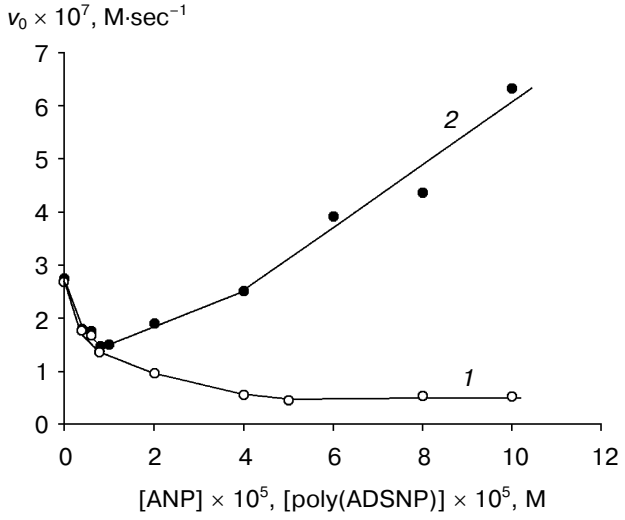

Fig. 3. Dependences of the initial rate of the peroxidase-catalyzed TMB oxidation in mixed reversed micelles of AOT (0.1 M)–Triton X-100 (0.1 M) in isooctane supplemented with 15% hexanol (W_o = 20.85) on concentrations of ANP (*1*) and of poly(ADSNP) (*2*); 0.001 M phosphate buffer (pH 6.4) with 1.5% DMF as the aqueous phase supplemented with HP (1 nM), TMB (1 mM), and H_2O_2 (1 mM).

effect of poly(ADSNP) on the TMB oxidation in AOT micelles in heptane: in this case the process is activated approximately fourfold at the polyphenol concentration of $5.0 \cdot 10^{-5}$ M that is 20 times lower than the substrate concentration ($[TMB]_o$ = 1.0 mM).

Thus, the features of TMB co-oxidation with both phenols in reversed micelles of AOT in heptane are opposite to the features of its co-oxidation in an aqueous phase, where ANP and poly(ADSNP) significantly inhibit the oxidation of TMB [17].

For the next step, it was reasonable to study the co-oxidation of TMB–ANP and TMB–poly(ADSNP) pairs in reversed micelles of varied composition. Figure 3 presents the effects of ANP and its polymer derivative concentrations on the peroxidase-catalyzed TMB oxidation in the mixed micelles of AOT–Triton X-100 (dependences *1* and *2*, respectively). ANP over the whole range of its concentrations inhibited the TMB oxidation, whereas poly(ADSNP) at concentrations above 10^{-5} M activated the TMB oxidation (about twofold at the concentration of 0.1 mM, tenfold lower than the concentration of TMB). Thus, changes in the composition of micelles have a large effect on the peroxidase-catalyzed co-oxidation of both pairs, TMB–ANP and TMB–poly(ADSNP) (compare to Fig. 2, a and b).

The peroxidase-catalyzed co-oxidation of TMB with DDS and with its polymer derivative in aqueous solution and in reversed micelles of AOT in heptane. In 0.01 M phosphate buffer (pH 6.4) supplemented with 5% DMF the initial rate of the peroxidase-catalyzed oxidation of TMB (1 mM) by hydrogen peroxide (1 mM) is directly proportional to HP concentration within the range of 0.1-1.5 nM. Under these conditions, DDS at the concentrations of 0.05 and 0.1 mM increases the rate of TMB oxidation ~2- and 3.2-fold, respectively, i.e., this substituted phenol activates the peroxidase-catalyzed TMB oxidation in aqueous medium. Figure 4a presents the dependences of the initial rate of TMB oxidation on the increasing concentration of DDS in 0.01 M phosphate buffer supplemented with 5% DMF at different pH values within the range of 5.9-7.2. In all cases, the initial rate of TMB oxidation increased 8-9 times, up to its limiting value at DDS concentration about 0.1 mM. During the co-oxidation of the TMB–DDS pair the initial rate of TMB conversion significantly decreased with increasing pH from 5.9 to 7.2 at different concentrations of the phe-

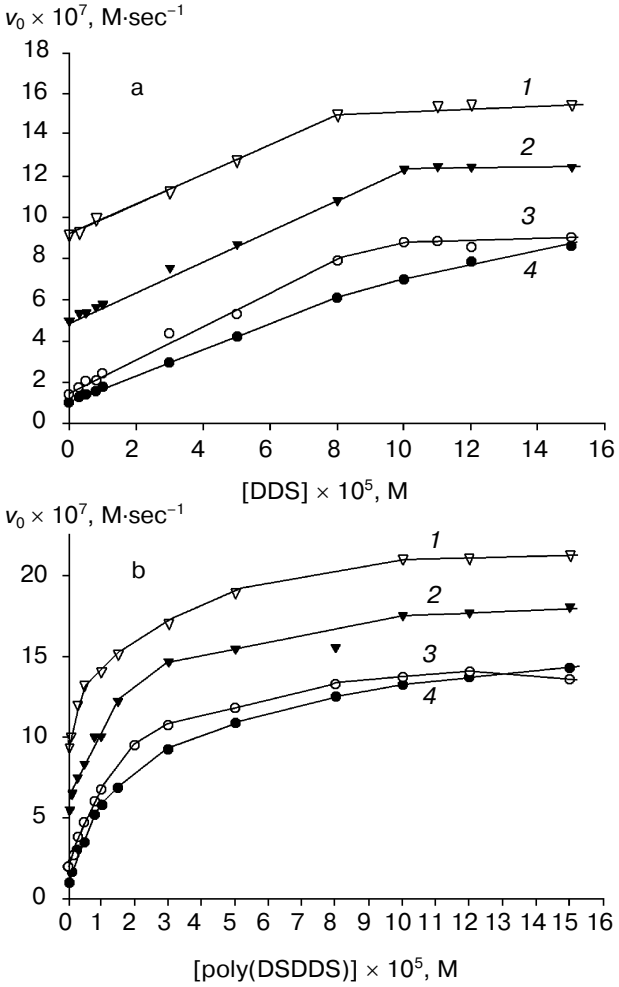

Fig. 4. Dependences of the initial rate of the peroxidase-catalyzed TMB oxidation in 0.1 M phosphate buffer with 5% DMF on concentrations of DDS (a) and of poly(DSDDS) (b) at different pH values: 5.9 (*1*), 6.4 (*2*), 7.0 (*3*), and 7.2 (*4*); HP (1 nM), TMB (1 mM), H_2O_2 (1 mM).

nol component. Recall that the peroxidase-catalyzed oxidation of the TMB alone similarly depends on pH.

The dependences of the initial rate of TMB oxidation on increasing concentration of poly(DSDDS) in 0.01 M phosphate buffer supplemented with 5% DMF at various pH values within the range of 5.9-7.2 are presented in Fig. 4b. In all cases, the polymer phenol activates the oxidation of TMB up to limiting values at nearly 0.1 mM poly(DSDDS). Values of v_o decrease with increasing pH at different concentrations of poly(DSDDS). Thus, in buffer solution at pH values within the range of 5.9-7.2 the peroxidase-catalyzed co-oxidation of TMB–DDS and TMB–poly(DSDDS) pairs is characterized by a significantly activated TMB conversion: it was increased 8-9-fold for DDS and ~12-fold for poly(DSDDS).

Figure 5 presents the dependences of the initial rate of the peroxidase-catalyzed TMB oxidation on the increasing concentrations of DDS and of poly(DSDDS) in reversed micelles of AOT in heptane at their hydration degree $W_o = 25$ (*1*, *2*) and 19.4 (*3*, *4*). Both DDS (dependences *1*, *3*) and its polymer derivative (dependences *2*, *4*) activated the oxidation of TMB in reversed AOT micelles, but this activation was significantly lower than in aqueous solution during the co-oxidation of the same pairs (Fig. 4, a and b). The hydration degree W_o of reversed micelles is a very important characteristic that determines the micelle size, the activity and stability of enzymes solubilized by them, and other features of micellar systems [24-27]. Therefore, the effect of hydration degree of reversed micelles of AOT in heptane on the co-oxidation parameters of TMB–DDS and TMB–poly(DSDDS) pairs was studied in detail. The dependences of the initial rate of TMB oxidation on the hydration degree of AOT micelles are shown in Fig. 6 for the pair of TMB–DDS (a) and for the pair of TMB–poly(DSDDS) (b).

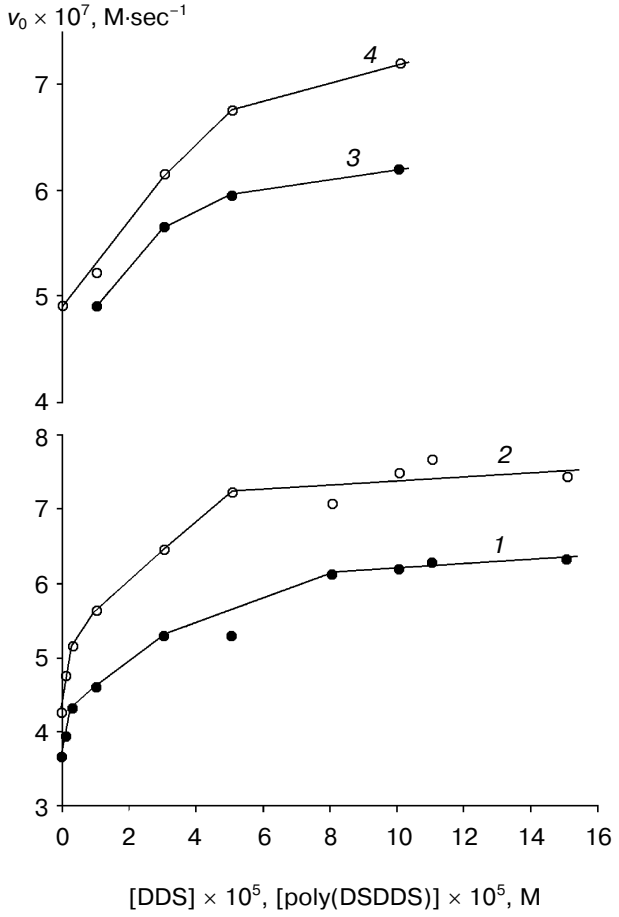

Fig. 5. Dependences of the initial rate of the peroxidase-catalyzed TMB oxidation in reversed micelles of AOT (0.2 M) in heptane on the initial concentrations of DDS (*1*, *3*) and of poly(DSDDS) (*2*, *4*): $W_o = 25$ (*1*, *2*) and 19.4 (*3*, *4*); 0.001 M phosphate buffer (pH 6.4) as the aqueous phase supplemented with HP (1 nM), TMB (1 mM), H_2O_2 (1 mM), and 1.5% DMF.

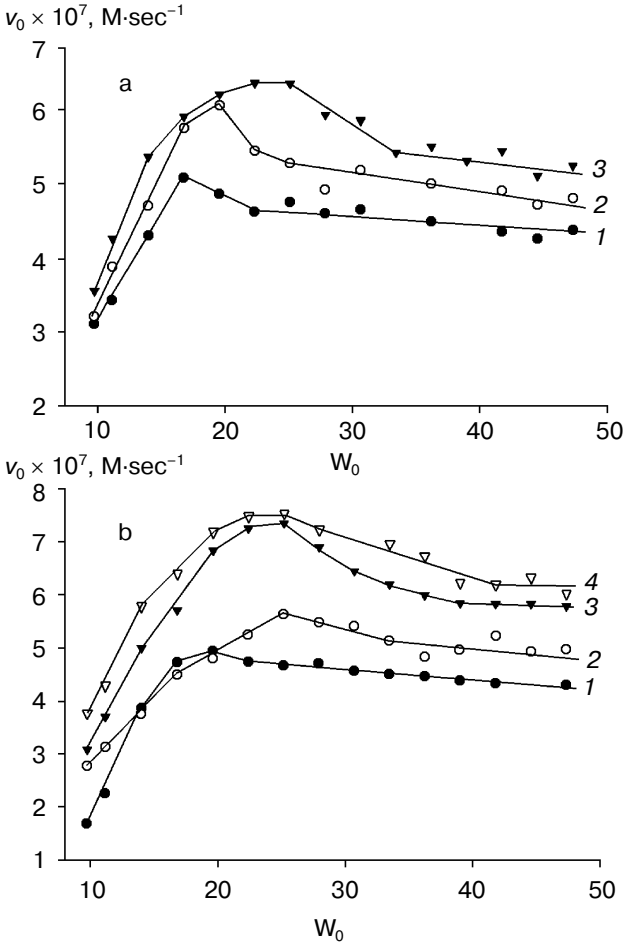

Fig. 6. Dependences of the initial rate of the peroxidase-catalyzed TMB oxidation in reversed micelles of AOT (0.2 M) in heptane on the micelle hydration degree W_o in the presence of DDS (a) and of poly(DSDDS) (b): a) 5 μM (*1*), 50 μM (*2*), and 100 μM (*3*) DDS; b) without phenols (*1*), 5 μM (*2*), 50 μM (*3*), and 100 μM (*4*) poly(DSDDS); 0.001 M phosphate buffer (pH 6.4) as the aqueous phase supplemented with HP (1 nM), TMB (1 mM), H_2O_2 (1 mM), and 1.5% DMF.

Figure 6a shows that during the co-oxidation of the TMB–DDS pairs the profile of dependences v_o–W_o is specific for peroxidase-catalyzed processes in AOT micelles [16, 26, 27]: the oxidation rate of TMB is maximal at certain hydration degrees of the micelles. It is interesting that this maximum position is determined by the DDS concentration in the micellar system: with an increase in the DDS concentration this maximum is shifted from 17 at 5 μM to 22.5 at 0.1 mM. As expected, increasing concentrations of DDS provide an increase in v_o at all hydration degrees of reversed AOT micelles in heptane. Figure 6b presents the dependences of the initial rate of TMB oxidation on the micelle hydration degree for the individual amine (*1*) and for the TMB–poly(DSDDS) pair with increasing concentrations of the phenol component (*2-4*). At different degrees of micelle hydration poly(DSDDS) clearly activated the TMB oxidation. The profiles of v_o–W_o dependences are specific for micellar peroxidase-catalyzed reactions, but the maximum position depends on the presence of the polymer phenol in the system: from 19.5 this maximum is shifted to 25 at poly(DSDDS) concentrations from 5 μM to 0.1 mM.

Thus, the general character of the dependences v_o–W_o is not changed during the TMB oxidation for the TMB–DDS and TMB–poly(DSDDS) pairs compared to oxidation of the individual amine in reversed micelles of AOT in heptane; however, the maximum position on the dependences v_o–W_o depends on the phenol concentration. The activating effects of DDS and of its polymer derivative occur at all hydration degrees of reversed micelles of AOT in heptane.

Table 2 compares the effects of monophenol (ANP), diphenol (DDS), triatomic phenol (gallic acid (GA)), and their polydisulfide derivatives on the peroxidase-catalyzed oxidation of the same amine (TMB) in buffer solutions and in reversed micelles of surfactants in organic solvents. Gallic acid and its polydisulfide (poly(DSG)) effectively inhibited the peroxidase-catalyzed TMB oxidation in both aqueous medium and reversed AOT micelles in heptane [14-16]. Diphenol (DDS) and its polydisulfide effectively activated the oxidation of the same amine in both aqueous medium and reversed AOT micelles. The monophenol (ANP) and its polydisulfide are especially interesting because they inhibited the TMB oxidation in aqueous solution but significantly activated the conversion of this amine in reversed micelles of AOT in heptane and at certain concentrations they also inhibited the TMB oxidation in the mixed reversed micelles of AOT–Triton X-100, i.e., their effect on the peroxidase-catalyzed transformation of TMB changed depending on the medium from strong inhibition of the reaction to its significant activation.

Table 2. Effects of substituted phenols and of their polydisulfides on the peroxidase-catalyzed oxidation of TMB in aqueous and micellar media

Medium	Phenols	Effect	References
0.01 M phosphate buffer (pH 6.4) supplemented with 5% DMF	ANP poly(ADSNP)	activation*, inhibition inhibition	[17] [17]
Reversed micelles of AOT (0.2 M) in heptane: 0.001 M phosphate buffer (pH 6.4) supplemented with 1.5% DMF	ANP poly(ADSNP)	activation ~2-fold activation ~4-fold	present work
Mixed reversed micelles of AOT (0.1 M)–Triton X-100 (0.1 M) in isooctane supplemented with 15% hexanol: 0.001 M phosphate buffer (pH 6.4) supplemented with 1.5% DMF	ANP poly(ADSNP)	inhibition 5-6-fold inhibition**, activation	present work
0.01 M phosphate buffer (pH 6.4) supplemented with 5% DMF	DDS poly(DSDDS)	activation up to 8-9-fold activation up to 12-fold	present work
Reversed micelles of AOT (0.2 M) in heptane: 0.001 M phosphate buffer (pH 6.4) supplemented with 1.5% DMF at different hydration degree	DDS poly(DSDDS)	activation activation	present work
0.01 M phosphate buffer (pH 6.4) supplemented with 10% DMF	GA poly(DSG)	inhibition inhibition	[14] [15]
Reversed micelles of AOT (0.2 M) in heptane: 0.001 M phosphate buffer (pH 6.4) supplemented with 1% DMF	GA poly(DSG)	inhibition inhibition	[14] [16]

Note: GA) gallic acid; poly(DSG)) polydisulfide of gallic acid.
* Activation only at low concentrations of ANP.
** Inhibition only at low concentrations of poly(ADSNP).

Based on the findings presented here and on results of our previous works [14-17], we unambiguously conclude that the peroxidase-catalyzed TMB oxidation is both inhibited and activated at much lower concentrations of the substituted phenols than of the amine concentration, i.e., this is a cyclic, or more strictly, chain process; therefore, it cannot be explained without taking into account the exchange reaction of phenoxyl radicals with amine that was mentioned in the introduction to this article. To explain the data presented in Table 2, it is necessary to consider a simple scheme of the peroxidase-catalyzed co-oxidation of "amine–phenol" pairs which is based on many of our works [5-7, 12-17] and on data of other authors [8-11]. In the scheme the following abbreviations are used: E) peroxidase; E_1 and E_2) active peroxidase forms; I and II) the corresponding complexes; P_1 and P_2) oxidation products of phenols and amines, respectively; P_3) product of phenol and amine co-oxidation. The scheme includes enzymatic and nonenzymatic stages.

Enzymatic stages

$$E + H_2O_2 \rightleftarrows E_1 + H_2O\ ; \quad (1)$$

$$PhOH + E_1 \rightarrow E_2 + PhO^{\bullet}\ ; \quad (2)$$

$$AmNH_2 + E_1 \rightarrow E_2 + AmNH^{\bullet}\ ; \quad (2^*)$$

$$PhOH + E_2 \rightarrow E + PhO^{\bullet}\ ; \quad (3)$$

$$AmNH_2 + E_2 \rightarrow E + AmNH^{\bullet}\ . \quad (3^*)$$

Nonenzymatic stages

$$2\ PhO^{\bullet} \rightarrow P_1\ ; \quad (4)$$

$$2\ AmNH^{\bullet} \rightarrow P_2\ ; \quad (4^*)$$

$$PhO^{\bullet} + AmNH^{\bullet} \rightarrow P_3\,(PhO{-}HNAm)\ ; \quad (4^{**})$$

$$PhO^{\bullet} + AmNH_2 \rightleftarrows PhOH + AmNH^{\bullet}\ . \quad (5)$$

Progress in protein engineering and the determination of the tertiary structures of peroxidases during recent years [28-31] has resulted in a detailed elucidation of the molecular mechanism of the peroxidase-catalyzed process. By methods of protein engineering, HP was shown to be directly involved in the binding and heterolysis by H_2O_2 of residues of the distal histidine [32] and of the distal arginine [33]. The phenols and their polydisulfides used by us (Table 2) could not affect the first reaction of the peroxidase-catalyzed process that goes irreversibly and with high rate constant k_1 (~10^7 $M^{-1}\cdot cm^{-1}$) [10, 34].

Phenols are likely to compete with TMB for the binding to PMB in the distal region that determines the competitive inhibition during the oxidation of TMB, which we showed for GA acid and for poly(DSG) [14-16] and also for ANP and poly(ADSNP) in both aqueous solutions [17] and mixed reversed micelles of AOT–Triton X-100 in isooctane (Table 2). Polymer phenols are significantly more active inhibitors than their monomer analogs, i.e., an increase in the size of polymer phenols does not prevent their penetration into the distal region of peroxidase through a hydrophobic channel.

Phenols and TMB are oxidized by Complexes I and II by reactions (2), (3) and (2*), (3*) with production of phenoxyl and aminyl radicals, respectively. The most reactive component is oxidized faster: in the case of phenol, $k_2 >> k_{2^*}$ and $k_3 >> k_{3^*}$, and phenol will be mainly spent initially. The phenoxyl radicals generated recombine to the product P_1, and in the presence of amine (TMB) can interact with it by the exchange reaction (5). If the activity of phenoxyls is sufficiently high, the reaction (5) goes from left to the right and additionally accelerates the expenditure of the amine associated with regeneration of the phenol component, which is again oxidized by active HP forms E_1 and E_2. This variant of the co-oxidation is a repeated activation of the amine (TMB) oxidation by the phenol oxidized. Such an activation occurs during the TMB co-oxidation with DDS and poly(DSDDS) in aqueous medium and in reversed micelles of AOT in heptane, i.e., phenoxyl radicals $DDS^{\bullet}$ and $poly(DSDDS)^{\bullet}$ are sufficiently active to oxidize TMB to the aminyl radical $(TMB)^{\bullet}$, and the recombination of the latter results in the product P_2 (reaction (4*)).

If amine is oxidized faster, i.e., if $k_{2^*} >> k_2$ and $k_{3^*} >> k_3$, the aminyl radicals generated not only recombine to the product P_2 but at sufficient activity can attack the phenol by reaction (5) with production of phenoxyls and regeneration of the initial amine (TMB). In this case, the amine oxidation is inhibited until the complete expenditure of the phenol component. This second variant of the TMB co-oxidation occurs in the presence of GA and poly(DSG) in aqueous medium and in reversed micelles of AOT in heptane [14-17] and also in the presence of ANP and of poly(ADSNP) in the mixed reversed micelles of AOT–Triton X-100.

Note that the effect of substituted phenols and of their polydisulfides on the peroxidase-catalyzed oxidation also depends on the concentration of the phenol component or on the ratio of concentrations [PhOH]/[TMB]. At low concentrations of ANP in aqueous medium, the peroxidase-catalyzed oxidation of TMB is slightly activated, but with an increase in [ANP] inhibition occurs [17]. In the present work low concentrations of poly(ADSNP) inhibited the TMB oxidation in the mixed reversed micelles, whereas high concentrations activated it (Fig. 3).

Based on our previous data [5-7, 12-17], the findings of the present work, and on the scheme of co-oxidation of "aromatic amine–phenol" pairs, we conclude that the general features of the peroxidase-catalyzed oxidation of such pairs depends on many factors: first, on the reactivities of amines and phenols relative to peroxidase Complexes I and II (the rate constants of reactions (2) and (2*), (3) and (3*)); second, on the initial concentrations of PhOH and $AmNH_2$ or on their ratio; third, on the reactivities of phenoxyl ($PhO^{\bullet}$) and aminyl ($AmNH^{\bullet}$) radicals in the exchange reaction (5), i.e., on values of the rate constants k_5 (the reaction from left to right) and k_{-5} (the reaction from right to left); fourth, on the steady state concentrations of Complexes I and II in a particular peroxidase-catalyzed co-oxidation of "amine–phenol" pairs; and, fifth, on the medium of the peroxidase-catalyzed co-oxidation of amines and phenols, as we have shown in the present work for the pairs of "TMB–ANP" and "TMB–poly(ADSNP)" (Table 2).

The goal of many researchers to predict the character of the peroxidase-catalyzed co-oxidation of "amine–phenol" pairs is quite reasonable because such processes are very important for analysis and immune diagnosis. However, even with a knowledge of the rate constants of the unit reactions (1), (2) and (2*), (3) and (3*), it is difficult to choose an "amine–phenol" pair that would deliberately have one of the components either as a stimulator or an inhibitor of the oxidation of another component because the role of the nonenzymatic reaction (5) is extremely high; however, as a rule, there are no data on its rate constants in both directions. Moreover, it is shown in the present work that the very direction of the exchange reaction (5) can be changed by changing one medium for another, as occurred during the oxidation of TMB–ANP and TMB–poly(ADSNP) pairs (Table 2).

The effect of micellar medium on peroxidase-catalyzed oxidation of TMB–phenol pairs is especially interesting because it is promising for new information about radical processes in the micelle environment. Based on the most general concepts, the exchange reaction (5) should be decelerated with a decrease in pH of the aqueous solution or of the aqueous microphase of reversed micelles because protonated phenols and amines are more slowly oxidized by radicals than their nonprotonated forms. This means that the effect of pH on oxidation of TMB separately or inside the pair with DDS and poly(DSDDS) (Figs. 4 and 5) is not associated with the effect of hydrogen ions on the exchange reaction (5) but is most likely explained by a decrease in the catalytic activity of HP with an increase in pH, because we used the acidic form of peroxidase with pI = 5.0 with maximum catalytic activity in AOT micelles at about pH 6 [16].

The displacement of the catalytic activity maximum of HP in reversed micelles of AOT in heptane in the dependences v_o–W_o at varied concentrations of phenol components (Fig. 6) is suggested to be associated with a possible involvement of high concentrations of DDS and especially of poly(DSDDS) in the production of reversed micelles and in changing their properties.

Thus, it is shown that the peroxidase-catalyzed co-oxidation of TMB with various phenols and their polydisulfides is a summation of successive and parallel enzymatic and nonenzymatic reactions. A special role is played by the nonenzymatic exchange reaction of phenoxyl radicals with amines or of aminyl radicals with phenols, and the direction of this reaction significantly effects the character of the process: it can accelerate (activate) the oxidation of one of the components or decelerate (inhibit) the conversion of one of the components. The exchange reaction can change direction with changes in the microenvironment of the peroxidase, as shown for oxidation of TMB–ANP and TMB–poly(ADSNP) pairs in aqueous solution and in reversed micelles of varied composition. And when the phenol component inhibited the peroxidase-catalyzed oxidation of the amine (TMB), this inhibition was increased in the micellar environment compared to the aqueous medium [16]. In all cases, the effects of phenol polydisulfides on the peroxidase-catalyzed oxidation of TMB were stronger than the effects of monomer analogs of their units.

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