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Activation of Peroxidase-Catalyzed Oxidation of Aromatic Amines with 2-Aminothiazole and Melamine

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Abstract—The peroxidase-catalyzed oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB), *ortho*-phenylenediamine (PDA), and 5-aminosalicylic acid (5-ASA) is significantly accelerated in the presence of 2-aminothiazole (AT) and melamine (MA), and an increase in their concentrations is associated with a parallel increase in the k_{cat} and K_m values for TMB and PDA. The activation of the peroxidase-catalyzed oxidation of TMB and PDA is quantitatively characterized by a coefficient (degree) α (M^{-1}) which significantly depends on pH in the range 6.2-6.4, 6.4-7.4, and 6.0-7.4 for the TMB–AT, TMB–MA, and PDA–MA pairs, respectively. An increase in the coefficient α with increase in pH confirms nucleophilicity of activation of the peroxidase-catalyzed oxidation of the aromatic amines in the presence of AT and MA. Under optimal conditions the coefficients α for the TMB–AT, PDA–AT, TMB–MA, and PDA–MA pairs vary in the limits of $(1.90\text{-}3.53)\cdot10^3$ M^{-1}.

Key words: horseradish peroxidase, tetramethylbenzidine, *ortho*-phenylenediamine, 5-aminosalicylic acid, activation of oxidation, 2-aminothiazole, melamine, nucleophilic catalysis

The aromatic amines 3,3′,5,5′-tetramethylbenzidine (TMB), *ortho*-dianizidine (*o*-DA), *ortho*-phenylenediamine (PDA), and 5-aminosalicylic acid (5-ASA) are widely used as peroxidase (EC 1.11.1.7) substrates in current bioanalytical methods of high sensitivity: enzyme immunoassay (ELISA) [1], immunocytochemistry [2], chemiluminescent analysis and chemiluminescent ELISA [3], and in biosensor devices [4]. The sensitivity of these methods can be, in particular, increased by activation ("enhancement") of the peroxidase-catalyzed oxidation of the above-mentioned aromatic amines. Fundamentally different approaches for activation of the peroxidase-catalyzed oxidation of amines were analyzed in brief in our previous work [5]. First, the peroxidase-catalyzed oxidation of amines was accelerated by addition into the reaction medium of nitrogen-containing organic bases, such as ammonia, imidazole and its derivatives, and of pyridine which at pH ≥ 6.5 activated the enzyme as nucleophiles by changing p*K* of functional groups of the peroxidase active site and widening the pH optimum of their catalytic activity: the kinetics have been studied of the peroxidase-catalyzed oxidation of *o*-DA and of *para*-phenylenediamine activated with pyridine, imidazole [6, 7], and with various derivatives of the latter [8-10], and also with 1,2,4-triazole, 4-aminopyridine, and with substituted indoles [11].

We have recently shown a strong activating effect of poly(5-aminodisulfide of salicylic acid) on the peroxidase-catalyzed oxidation of TMB [5] and a similar effect of melamine (MA) on the oxidation of PDA [12]. This activating effect is quantitatively characterized by an activation coefficient (degree) α (M^{-1}) determined at various pH values which describes an increase in the kinetic parameters k_{cat} and K_m in the presence of 1 M activator in the reaction mixtures: under optimal conditions at pH 7.0 the coefficient α for the pair TMB–poly(5-aminodisulfide of salicylic acid) is $2.44\cdot10^5$ M^{-1} [5] and for the pair PDA–melamine at pH 7.4 it is $2.41\cdot10^3$ M^{-1} [12].

However, the activation mechanism of the peroxidase-catalyzed oxidation of aromatic amines is not completely clear [5]. Therefore, systematic studies on activation of the peroxidase-catalyzed oxidation of various amines with the same organic base, on one hand, and on activation of the oxidation of the same amine with various activators, on the other hand, are still important.

Abbreviations: A) activator of the peroxidase-catalyzed reaction; AT) 2-aminothiazole; 5-ASA) 5-aminosalicylic acid; MA) melamine (2,4,6-triamino-1,3,5-triazine); poly(ADST)) poly(2-aminodisulfidethiazole); HP) horseradish peroxidase; TMB) 3,3′,5,5′-tetramethylbenzidine; PDA) *ortho*-phenylenediamine; PCB) phosphate citrate buffer.

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The purpose of this work was to study the activation kinetics of the peroxidase-catalyzed oxidation of TMB, PDA, and 5-ASA with 2-aminothiazole (AT) and those of TMB and 5-ASA oxidation with melamine (MA) and to compare the resulting quantitative data. The peroxidase substrates (TMB, PDA, and 5-ASA) were chosen because of their importance in practice [1-4] and because of their structural differences. 2-Aminothiazole was chosen as an activator because of its aromatic amine features and clearly pronounced nucleophilicity at pH ≥ 6 and also because of its easy electrophilic replacement in position 5; melamine (2,4,6-triamino-1,3,5-triazine) was chosen because of a strong pH dependence of its nucleophilicity, of its molecular symmetry, and also because of its previously found strong activating effect on the peroxidase-catalyzed oxidation of PDA [12].

MATERIALS AND METHODS

Reagents. The acidic isoform of horseradish peroxidase (HP; EC 1.11.1.7), type A, with optical purity index *RZ* 2.25 or 2.40 was from Biolar (Olaine, Latvia). The enzyme concentration was determined spectrophotometrically using the molar absorption coefficient ε in the Soret band (403 nm) equal to 102,000 $M^{-1}\cdot cm^{-1}$ [13]. Diluted hydrogen peroxide solution was used as an oxidizer, the H_2O_2 concentration being determined spectrophotometrically using $\varepsilon_{230} = 72.1\ M^{-1}\cdot cm^{-1}$ [14]. TMB (Serva, Germany), PDA of chemical purity (Kharkov Chemico-Pharmaceutical plant, Ukraine) purified by sublimation in vacuum, and 5-ASA (Reakhim, Russia) with absorption maxima of the UV spectrum at 240 and 300 nm and the corresponding molar absorption coefficients ε equal to 3310 and 3470 $M^{-1}\cdot cm^{-1}$ were used as reducers of HP.

As effectors of the peroxidase-catalyzed oxidation of aromatic amines 2-aminothiazole (AT) and melamine (MA) (Reakhim) were used. MA of chemical purity had the absorption maxima at 212 nm in distilled water and at 223 nm in 0.015 M phosphate citrate buffer (PCB, pH 6.0). Poly(2-aminodisulfidethiazole) (poly(ADST)) with the average molecular weight of ~1850 daltons which was synthesized as described in [15] and kindly presented to us by Yu. P. Losev (School of Chemistry, Belorussian State University, Minsk) was also used. The absorption maximum of poly(ADST) in DMSO at 267 nm has extinction $\varepsilon = 57{,}400\ M^{-1}\cdot cm^{-1}$, and the comparison with the ε_{254} value (7000 $M^{-1}\cdot cm^{-1}$) for AT suggests that poly(ADST) contains about eight monomeric links.

Organic solvents dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were distilled before use. Initial solutions of phosphate and phosphate citrate buffers, H_2O_2, HP, and PDA were prepared with distilled water; solutions of TMB, 5-ASA, and 2-aminothiazole were prepared with DMF, and the solution of poly(ADST) was prepared with DMSO. The final concentrations of the reagents in different series of experiments are given in the legends to figures and tables and in the text.

The peroxidase-catalyzed oxidation of TMB, PDA, 5-ASA, and also of the pairs TMB–AT, TMB–MA, TMB–poly(ADST), PDA–AT, PDA–poly(ADST), 5-ASA–AT, and 5-ASA–MA was performed at 20°C using a Specol-211 spectrophotometer (Carl Zeiss, Germany) equipped with a thermostatted cuvette section.

Peroxidase-catalyzed oxidation of TMB and of the TMB–AT, TMB–poly(ADST), and TMB–MA pairs. To prepare the reaction mixture of 1 ml of the total volume, a tube was supplemented with 0.7 ml of distilled water, 0.1 ml of 0.1 M phosphate buffer, 0.05 ml of 0.02 M TMB solution in DMF, 0.05 ml of DMF in experiments with addition of AT or 0.05 ml of DMSO in experiments with addition of poly(ADST), 0.05 ml of 20 nM HP, and 0.05 ml of 0.02 M H_2O_2. Before addition of H_2O_2 the mixture was kept for 3 min at 20°C. In a typical experiment during the oxidation of TMB and of the pairs TMB–AT and TMB–poly(ADST) in 0.01 M phosphate buffer (pH prescribed) which contained 10% DMF or 5% DMF and 5% DMSO the final concentrations of the reagents were as follows: 1 nM HP, 1 mM TMB, 1 mM H_2O_2. The concentrations of effectors were varied.

The oxidation of the pair TMB–MA was performed in the reaction mixture of 1 ml in total which contained 0.015 M PCB (pH prescribed), 0.4 nM HP, 1 mM TMB, 1 mM H_2O_2, 5% DMF, and the melamine concentrations prescribed. In this case the reaction was initiated by addition of HP solution.

Reactions of the peroxidase-catalyzed oxidation of TMB in the presence and in the absence of activators were performed for 1-2 min, the absorption of the TMB oxidation product was recorded at its maximum of 655 nm (A_{655}), and the initial rate of the reaction was calculated using the molar absorption coefficient equal to 39,000 $M^{-1}\cdot cm^{-1}$ [13].

Peroxidase-catalyzed oxidation of PDA and of PDA–AT and PDA–poly(ADST) pairs. The reaction mixture with a total volume of 1 ml contained 0.7 ml H_2O, 0.1 ml 0.15 M PCB, 0.05 ml PDA (0.04 M), 0.05 ml HP (20 nM), 0.05 ml DMF in experiments with AT; and before the addition of 0.05 ml of 0.04 M H_2O_2 the mixture was kept for 3 min at 20°C. In a typical experiment the final concentrations of the reagents in 0.015 M PCB were as follows: 5% DMF, 1 nM HP, 2 mM PDA, 2 mM H_2O_2, the 2-aminothiazole concentrations were prescribed. The reaction was performed for 1-2 min and was recorded by an increase in the absorption band of the PDA oxidation product A_{455}; the initial rate of the reaction was calculated using the molar absorption coefficient of the product which at pH 6.4 was 16,400 $M^{-1}\cdot cm^{-1}$ [16].

Peroxidase-catalyzed oxidation of 5-ASA and of 5-ASA–AT and 5-ASA–MA pairs. The reaction mixture

with a total volume of 1 ml contained 0.7 ml H_2O, 0.1 ml 0.15 M PCB (pH 6.4), 0.05 ml 5-ASA (0.04 M) in DMF, 0.05 ml DMSO, and 0.05 ml HP (20 nM); and before the addition of 0.05 ml of 0.04 M H_2O_2 the mixtures were kept for 3 min at 20°C. This was considered to be the reaction start. The final concentrations of the reagents in 0.015 M PCB were as follows: 5% DMF, 5% DMSO, 1 nM HP, 1 mM H_2O_2, 2.5 mM 5-ASA. The concentration of AT was varied.

During the peroxidase-catalyzed oxidation of the 5-ASA–MA pair 1 ml of the reaction mixture contained in 0.015 M PCB: 1 nM HP, 2 mM 5-ASA, 2 mM H_2O_2, 0.6 mM MA, and 5% DMF. In this case the reaction was initiated by addition of HP solution. An increase in the absorption of the reaction product A_{455} was recorded spectrophotometrically 1-2 min later, and the initial rates of the oxidation were calculated using the molar absorption coefficient $\varepsilon_{455} = 15{,}000\ M^{-1}\cdot cm^{-1}$ [13]. The reaction mixture temperature was 20°C.

Note that the absorption bands of the oxidation products of 2-aminothiazole and of melamine were not overlapping with the absorption bands of the oxidation products of TMB (655 nm), PDA (455 nm), and 5-ASA (455 nm), i.e., the monitoring of the peroxidase-catalyzed oxidation of the substrates was sufficient for a correct determination of the reaction rates in all cases. The initial rates v_0 of the peroxidase-catalyzed oxidation of TMB, PDA, and 5-ASA were determined only by the strictly linear initial regions of the kinetic dependences of the A_{655} and A_{455} increase.

RESULTS AND DISCUSSION

Activating effect of 2-aminothiazole on peroxidase-catalyzed oxidation of TMB, PDA, and 5-ASA. Figure 1 presents dependences of the reaction rates expressed in relative units on the increasing concentration of AT during the oxidation of TMB (*1*), PDA (*2*), and 5-ASA (*3*): all dependences have a saturating type, the oxidation of TMB, PDA, and 5-ASA is activated maximally fourfold, more than twofold, and 1.4-fold, respectively. The kinetic features were studied in detail during the peroxidase-catalyzed oxidation of the TMB–AT pair at different concentrations of the substrates (TMB and H_2O_2) and at pH varied from 6.2 to 7.4. Figure 2a shows in double reciprocal coordinates dependences of the initial rate of the TMB oxidation on its concentration in the absence of AT (*1*) and in its presence (*2-6*) at concentrations of $(0.7\text{-}5.0)\cdot10^{-4}$ M. From the dependences shown in Fig. 2a the kinetic parameters k_{cat} and K_m were calculated in the absence of AT (*1*) and the values of k^a_{cat} and K^a_m were calculated for data taken in its presence (*2-6*). The values of both kinetic parameters changed strictly in parallel (Fig. 2b) and were directly proportional to the AT concentration that suggested a noncompetitive type of the 2-aminothiazole-induced activation of the peroxidase-catalyzed oxidation of TMB. Based on the data presented in Fig. 2b and in Table 1 the kinetic parameters k^a_{cat} and K^a_m of the activated oxidation are expressed by empirical equations (1) and (2):

$$k^a_{cat} = k_{cat}\,(1 + \alpha[A]_0)\ sec^{-1}, \quad (1)$$

$$K^a_m = K_m\,(1 + \alpha[A]_0)\ M, \quad (2)$$

where $[A]_0$ is the initial concentration of the activator AT, and α is the activation coefficient equal to $1.83\cdot10^3\ M^{-1}$ at pH 6.4. The efficiency of HP expressed as k_{cat}/K_m does not change in the presence of activator and is $5.37\cdot10^6\ M^{-1}\cdot sec^{-1}$ (Table 1).

Dependences of the initial rate of the peroxidase-catalyzed oxidation of 1 mM TMB on the initial concentration of H_2O_2 in the range of 0.01-0.60 mM were studied in the absence and in the presence of AT at increasing concentrations (up to 0.4 mM). These dependences were transformed in Lineweaver–Burk coordinates and kinetic parameters of the process were calculated (Table 2). In this case the k_{cat} and K_m values also increased in parallel with the increase in the AT concentration. Table 2 presents the coefficients α which are equal to $6.07\cdot10^3\ M^{-1}$ and $7.25\cdot10^3\ M^{-1}$ calculated from the k_{cat} dependence on $[AT]_0$ and from that of K_m on $[AT]_0$, respectively. The

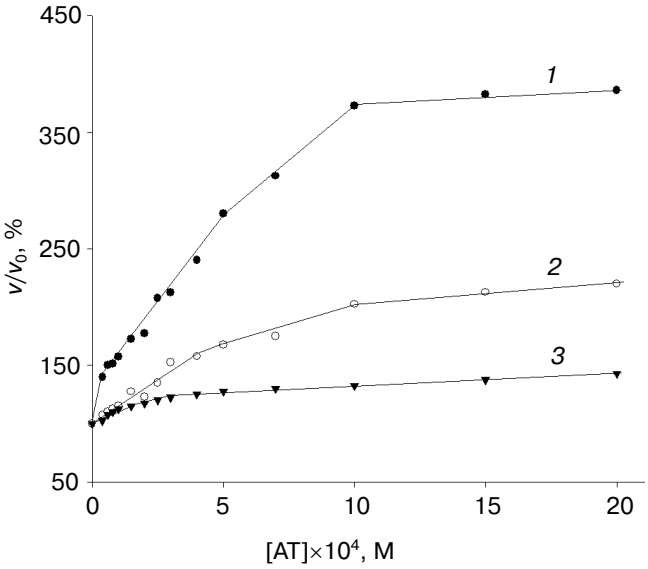

Fig. 1. Dependences of the oxidation rates of 1 mM TMB (*1*), 2 mM PDA (*2*), and 2.5 mM 5-ASA (*3*) at 20°C in the presence of 1 nM peroxidase on the 2-aminothiazole (AT) concentration: *1*) 0.01 M phosphate buffer (pH 6.4) containing 10% DMF and 1 mM H_2O_2; *2*) 0.015 M PCB (pH 6.0) containing 5% DMF and 2 mM H_2O_2; *3*) 0.01 M phosphate buffer (pH 6.4) containing 10% DMF and 2 mM H_2O_2.

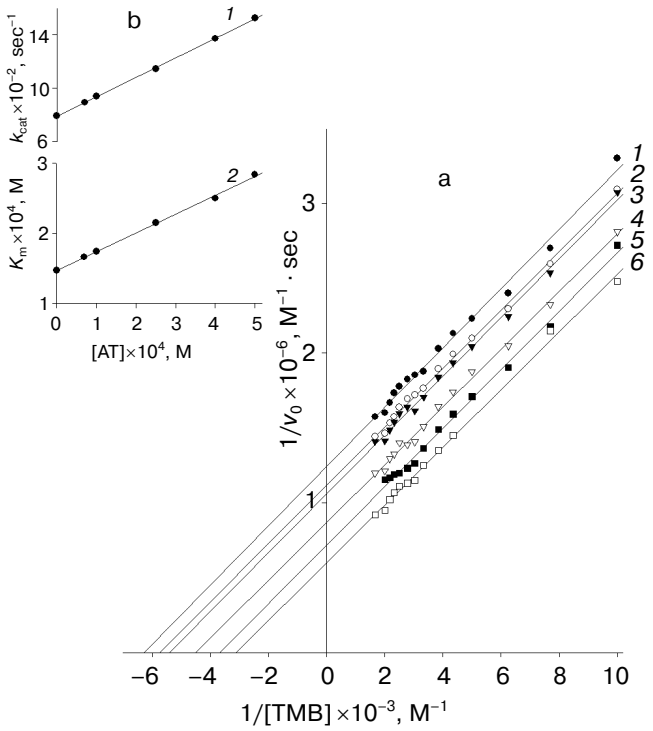

Fig. 2. a) Dependences in double reciprocal coordinates of the initial rate (v_0) of the peroxidase-catalyzed oxidation of TMB on its concentration in the presence of increasing concentrations of AT: 0.07 (*2*), 0.1 (*3*), 0.25 (*4*), 0.4 (*5*), and 0.5 mM (*6*), and in the absence of AT (*1*); 0.01 M phosphate buffer (pH 6.4), 10% DMF, 1 nM HP, 1 mM H_2O_2. b) Dependences of k_{cat} (*1*) and of K_m (*2*) during the peroxidase-catalyzed oxidation of TMB on the AT concentration (under the same conditions).

resulting values are higher than those obtained in the experiments with the TMB concentration varied at constant [H_2O_2] (Table 1), but this is not unexpected because catalytic constants of the peroxidase-catalyzed oxidation of many substrates determined on varying concentrations of substrates and reducers and of the H_2O_2 concentration are, as a rule, different due to an increase in the nonproductive expense of H_2O_2 for enzyme inactivation along with the increase in oxidizer concentration [17].

The nucleophilic properties of 2-aminothiazole are strongly dependent on pH of the medium. Therefore, the kinetics of the activated peroxidase-catalyzed oxidation of TMB was studied depending on the substrate concentration in 0.01 M phosphate buffer at pH varied from 6.2 to 7.4. The AT concentration in all cases was 1.3 mM. From the k_{cat} and K_m dependences on $[AT]_0$ for media with different pH values the coefficients α were calculated, and they are presented in Table 3 along with other kinetic parameters. The pH dependence of α during the activated peroxidase-catalyzed oxidation of TMP is presented in Fig. 3, and it shows that at pH varied from 6.2 to 6.8 α changed insignificantly and is ~$1.87\cdot10^3$ M^{-1}; however, at pH > 6.8 this coefficient sharply decreases that we think to be associated with a dramatic fall of the catalytic activity of HP on changing the pH from 6.2 (k_{cat} = 957 sec^{-1}) to 7.4 (k_{cat} = 172 sec^{-1}) (Table 3).

Figure 4a shows dependences of the initial rate of the peroxidase-catalyzed oxidation of PDA in double reciprocal coordinates in the absence of AT (*1*) and in its presence (0.1-0.5 mM) (*2-4*). Figure 4b shows dependences

Table 1. Kinetic characteristics of the peroxidase-catalyzed oxidation of TMB (0.01-0.60 mM) in the presence of 2-aminothiazole (AT) and in its absence at 20°C in 0.01 M phosphate buffer (pH 6.4) containing 10% DMF, 1 nM HP, and 1 mM H_2O_2

[AT] × 10^4, M	k_{cat}, sec^{-1}	α × 10^{-3}, M^{-1} (by k_{cat})	K_m × 10^4, M	α × 10^{-3}, M^{-1} (by K_m)	(k_{cat} / K_m) × 10^{-6}, $M^{-1}\cdot sec^{-1}$
0	790	—	1.47	—	5.37
0.7	891	1.83	1.66	1.85	5.37
1.0	937	1.86	1.74	1.84	5.39
2.5	1143	1.79	2.15	1.85	5.32
4.0	1371	1.84	2.50	1.75	5.48
5.0	1525	1.86	2.84	1.86	5.37

Table 2. Kinetic characteristics of the peroxidase-catalyzed oxidation of TMB (1 mM) in the presence of 2-aminothiazole (AT) and in its absence at 20°C in 0.01 M phosphate buffer (pH 6.4) containing 10% DMF, 1 nM HP, and H_2O_2 (0.01-0.60 mM)

[AT] × 10^4, M	k_{cat}, sec^{-1}	α × 10^{-3}, M^{-1} (by k_{cat})	K_m × 10^4, M	α × 10^{-3}, M^{-1} (by K_m)	(k_{cat} / K_m) × 10^{-6}, $M^{-1}\cdot sec^{-1}$
0	782	—	1.46	—	5.36
0.7	1095	5.72	2.16	6.85	5.07
1.0	1272	6.27	2.50	7.12	5.09
2.5	1909	5.76	4.00	6.96	4.77
4.0	2688	6.09	5.70	7.26	4.71

Table 3. Kinetic parameters of the peroxidase-catalyzed oxidation of TMB (0.01-0.60 mM) in the presence of 1.3 mM 2-aminothiazole (AT) (*2*) and in its absence (*1*) at 20°C in 0.01 M phosphate buffer (pH varied) containing 10% DMF, 1 nM HP, and 1 mM H_2O_2

pH	k_{cat}, sec^{-1}		$\alpha \times 10^{-3}$, M^{-1} (by k_{cat})	$K_m \times 10^4$, M		$\alpha \times 10^{-3}$, M^{-1} (by K_m)	$(k_{cat} / K_m) \times 10^{-6}$, $M^{-1} \cdot sec^{-1}$	
	1	2		1	2		1	2
6.2	957	3301	1.88	1.30	4.54	1.92	7.36	7.27
6.4	751	2571	1.86	1.21	4.14	1.86	6.21	6.21
6.6	651	2222	1.86	1.11	3.85	1.90	5.86	5.77
6.8	435	1504	1.89	0.93	3.25	1.92	4.68	4.63
7.0	321	1053	1.75	0.95	3.13	1.76	3.37	3.36
7.4	172	476	1.39	0.89	2.50	1.39	1.94	1.90

Table 4. Kinetic parameters of the peroxidase-catalyzed oxidation of PDA (0.15-1.0 mM) in the presence of 2-aminothiazole (AT) at 20°C in 0.015 M PCB, pH 6.2, containing 5% DMF, 1 nM HP, and 2 mM H_2O_2

[AT] $\times 10^4$, M	k_{cat}, sec^{-1}	$\alpha \times 10^{-3}$, M^{-1} (by k_{cat})	$K_m \times 10^4$, M	$\alpha \times 10^{-3}$, M^{-1} (by K_m)	$(k_{cat} / K_m) \times 10^{-6}$, $M^{-1} \cdot sec^{-1}$
0	528		3.82		1.38
1.0	826	—	5.96	—	1.39
3.0	1056	3.33	7.75	3.43	1.36
5.0	1462	3.54	10.74	3.62	1.36

of k_{cat} and of K_m on the increasing concentration of AT: these dependences are linear and confirm the parallel growth of k_{cat} and K_m with an increase in $[AT]_0$ and allow us to calculate the coefficient α which at pH 6.4 is $3.53 \cdot 10^3$ M^{-1} and $3.62 \cdot 10^3$ M^{-1} (from the dependences of k_{cat} and K_m, respectively). Kinetic parameters of the peroxidase-catalyzed oxidation of the PDA–AT pair at 20°C in 0.015 M PCB (pH 6.4) containing 5% DMF are presented in Table 4.

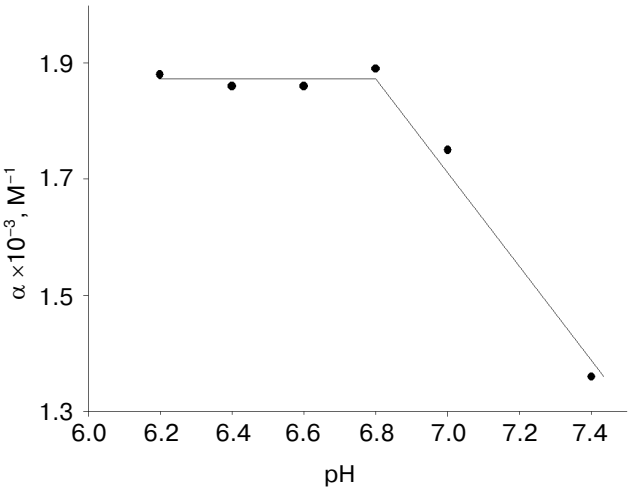

Fig. 3. Dependence of the coefficient α during the peroxidase-catalyzed oxidation of TMB in the presence of AT on pH value of 0.01 M phosphate buffer containing 10% DMF (under the same conditions as in Fig. 2a).

The effect of poly(2-aminodisulfidethiazole) (poly(ADST)) on the peroxidase-catalyzed oxidation of TMB and PDA was studied under conditions described in the legend to Fig. 5. Polydisulfide is shown to inhibit the peroxidase-catalyzed oxidation of both substrates, i.e., 2-aminothiazole in the polymer completely loses its activating properties that seems to be due to two causes: first, the nucleophilicity of AT is determined by its amino group and is lost during the polycondensation because of its modification to –NH–S–S–; second, the dimensions of poly(ADST) promote the competition of the polymer with the TMB and PDA substrates for the binding to HP in the hydrophobic channel of the enzyme that partially inhibits the oxidation of both substrates. Note that the peroxidase-catalyzed oxidation of TMB is inhibited by the competitive mechanism with polydisulfides of gallic acid [18], 2-amino-4-nitrophenol [19], resorcinol, and 2,4-dinitrosoresorcinol [20], i.e., poly(ADST) is one of the polydisulfide inhibitors, but it has a lower antiradical activity because of the absence in its molecule of amino and HO-groups responsible for antiradical properties.

The activating effect of melamine (MA) on the peroxidase-catalyzed oxidation of TMB and 5-ASA. Figure 6a presents the dependence of HP activity expressed in con-

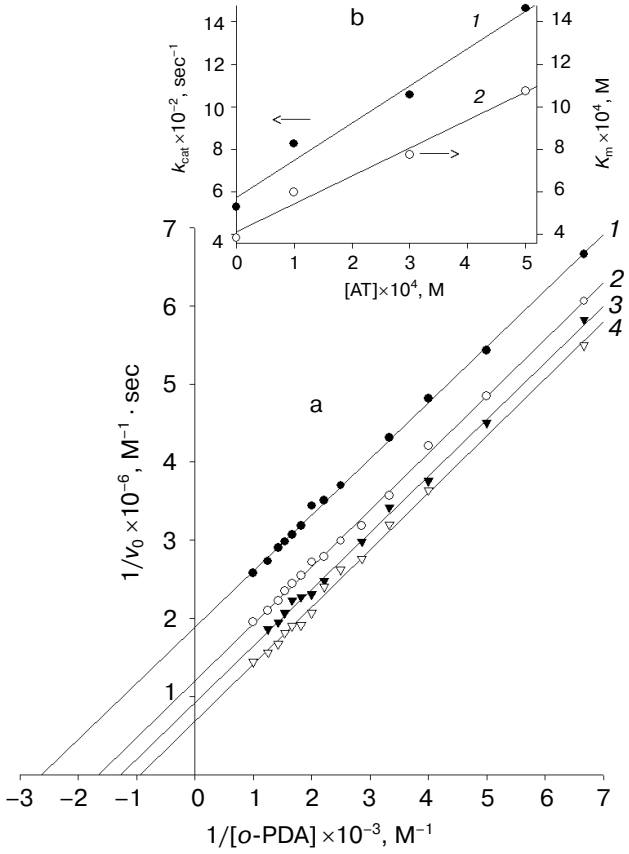

Fig. 4. a) Dependences of the initial rate of the peroxidase-catalyzed oxidation of PDA on its concentration in double reciprocal coordinates in the absence of AT (*1*) and with its increasing concentrations: 0.1 (*2*), 0.3 (*3*), 0.5 mM (*4*); 0.015 M PCB (pH 6.4) contained 5% DMF, 1 nM HP, and 2 mM H_2O_2. b) Dependences of k_{cat} (*1*) and of K_m (*2*) during the peroxidase-catalyzed oxidation of PDA on the AT concentration (the same conditions).

ventional units on the increasing concentration of MA during the oxidation of TMB: the oxidation of this substrate (1 mM) is activated about twofold at equimolar concentration of MA (20°C, 0.015 M PCB (pH 6.4) containing 5% DMF). Profiles of dependences of the initial rate of the peroxidase-catalyzed oxidation of TMB (*1*) and of the TMB–MA pair (*2*) on the pH value of PCB in the range of 2.6-7.7 are shown in Fig. 6b. In both cases the maximal values of v_0 are in the pH range of 3.5-4.5 that corresponds to p*I* of the acidic isoform of HP which is ~5 [21]. The oxidation of 1 mM TMB in the presence of 0.6 mM MA is activated only at pH > 5.2, i.e., only with the nucleophilicity of melamine.

Figure 7a presents in double reciprocal coordinates dependences of the initial rate of TMB oxidation on its concentration in the absence of MA (*1*) and in its presence at concentrations from 0.3 to 1.0 mM (*2-4*). These dependences suggest a noncompetitive activation of the peroxidase-catalyzed oxidation of TMB with melamine in 0.015 M PCB (pH 6.4) containing 5% DMF. Figure 7b shows linear dependences of k_{cat} and K_m on the increasing concentration of MA; they are strictly parallel and allow us to determine the coefficient α at pH 6.4, and the coefficient value is $0.86\cdot10^3$ M^{-1} in both cases. Dependences

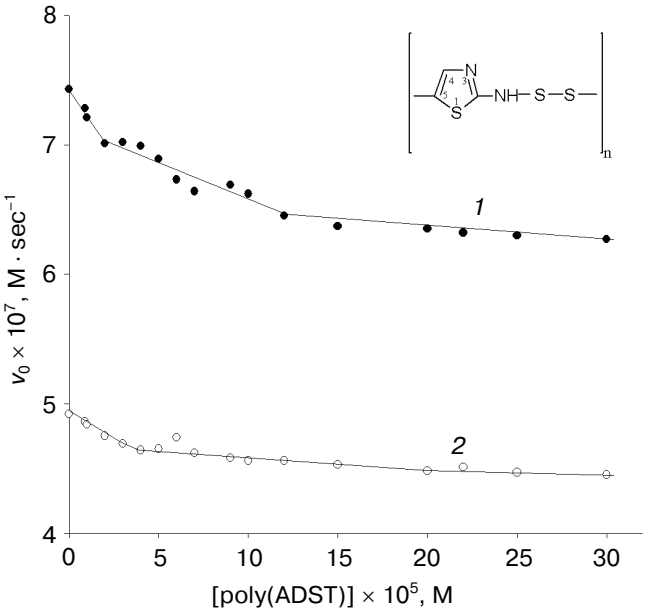

Fig. 5. Dependences of the initial rate of the peroxidase-catalyzed oxidation of 1 mM TMB (*1*) and of 2 mM PDA (*2*) on the initial concentration of poly(ADST): *1*) 0.01 M phosphate buffer (pH 6.4), 5% DMF, 5% DMSO, 1 nM HP, 1 mM H_2O_2; *2*) 0.015 M PCB (pH 6.2), 5% DMSO, 1 nM HP, 2 mM H_2O_2.

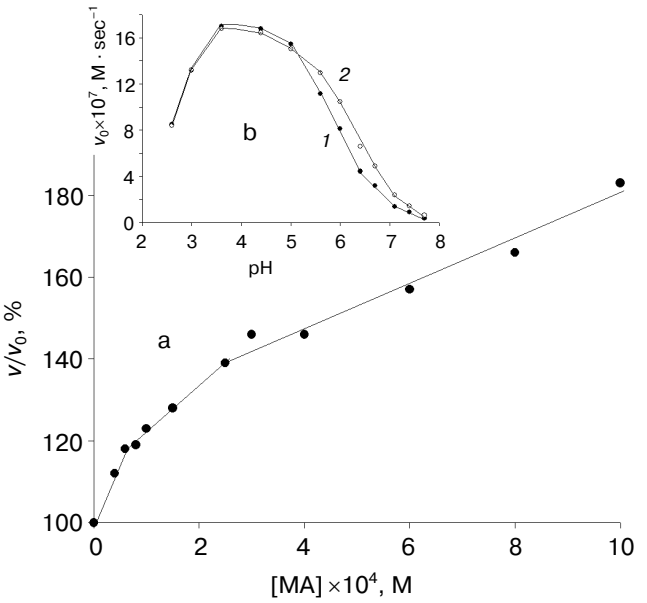

Fig. 6. Dependences of the rate of the peroxidase-catalyzed oxidation of 1 mM TMB on the MA concentration (a) and on the pH value of PCB containing 5% DMF (b). a) PCB (pH 6.4) with 5% DMF, 0.4 nM HP, 1 mM H_2O_2; b) in the absence (*1*) and in the presence of 0.6 mM MA (*2*).

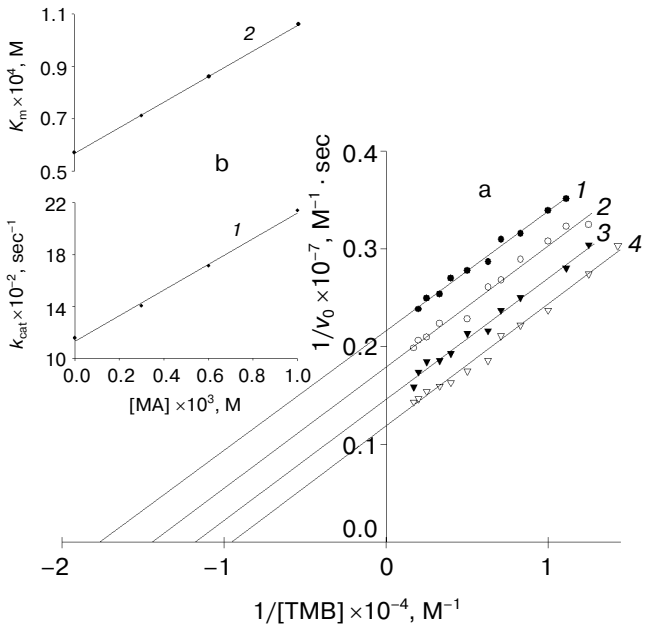

Fig. 7. a) Dependences in double reciprocal coordinates of the initial rates of the peroxidase-catalyzed oxidation of TMB on its concentration in the absence of MA (*1*) and in the presence of 0.3 (*2*), 0.6 (*3*), and 1.0 mM MA (*4*); 0.015 M PCB (pH 6.4), 5% DMF, 0.4 nM HP, and 1 mM H_2O_2. b) Dependences of k_{cat} (*1*) and of K_m (*2*) during the peroxidase-catalyzed oxidation of TMB on the concentration of MA (under the same conditions).

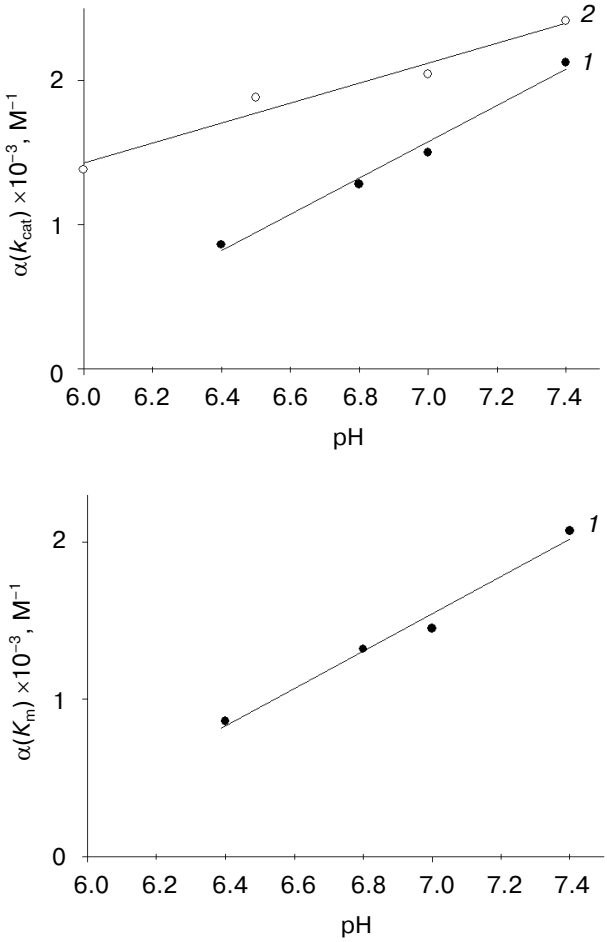

Fig. 8. Dependences of the coefficients α during the peroxidase-catalyzed oxidation of TMB (*1*) and of PDA (*2*) on the pH value of 0.015 M PCB at 20°C: *1*) 5% DMF, 1 mM H_2O_2, 0.07-0.6 mM TMB; 0.4 nM HP at pH values 6.4 and 6.8, and 0.8 nM HP at pH 7.0 and 7.4; *2*) 1 mM H_2O_2, 0.06-1.0 mM PDA; 1 nM HP at pH values 6.0 and 6.5, and 3 nM HP at pH 7.0 and 7.4.

similar to those presented in Fig. 7 (a and b) were also obtained in PCB with pH 6.8, 7.0, and 7.4. Conditions of the peroxidase-catalyzed oxidation of TMB and of the TMB–MA pair and the resulting kinetic parameters are presented in Table 5.

Figure 8 presents dependences of the coefficients α on pH at the melamine-activated peroxidase-catalyzed oxidation of TMB (*1*) and PDA (*2*) [12]. In both cases the dependences are linear and describe an increase in α with increase in pH from 6.0 to 7.4, i.e., the activating effect of MA on the peroxidase-catalyzed oxidation of two aromatic amines being directly associated with changes in the nucleophilicity of melamine with an increase in the medium pH. Figure 8 shows that at all pH values the coefficient α for PDA is higher than for TMB, i.e., that the activation efficiency of the peroxidase-catalyzed oxidation with the same nucleophile MA depends on the aromatic amine.

The profiles of pH dependences of the initial rates of the peroxidase-catalyzed oxidation of 5-ASA (*1*) and of the 5-ASA–MA pair (*2*) are compared in Fig. 9. The rate of the 5-ASA oxidation is maximal at pH 6.0 and it is not shifted in the presence of 0.6 mM MA at the substrate concentration of 2.0 mM. The oxidation of 5-ASA is activated with melamine at pH > 6. Obviously, an increase in

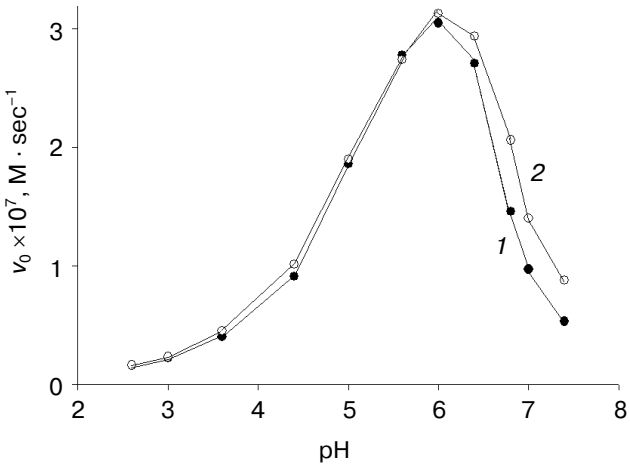

Fig. 9. The pH dependences of the initial rate of the peroxidase-catalyzed oxidation of 5-ASA in 0.015 M PCB containing 5% DMF at 20°C in the absence of MA (*1*) and in the presence of 0.6 mM MA (*2*) (1 nM HP, 2 mM H_2O_2).

Table 5. Kinetic characteristics of the peroxidase-catalyzed oxidation of TMB at 20°C in the presence of melamine (MA) at different pH values of 0.015 M phosphate citrate buffer containing 5% DMF and 1 mM H_2O_2

Conditions	pH	[MA] × 10^3, M	k_{cat}, sec^{-1}	α (k_{cat}) × 10^{-3}, M^{-1}	K_m × 10^4, M	α (K_m) × 10^{-3}, M^{-1}
[HP], 0.4 nM		0	1158		0.57	
[TMB], 0.07-0.6 mM	6.4	0.3	1405	0.86	0.71	0.86
		0.6	1713		0.86	
		1.0	2138		1.06	
[HP], 0.4 nM		0	588		0.33	
[TMB], 0.07-0.4 mM	6.8	0.3	776	1.28	0.44	1.32
		0.6	1130		0.64	
		1.0	1320		0.75	
[HP], 0.8 nM		0	536		0.42	
[TMB], 0.07-0.4 mM	7.0	0.6	1008	1.5	0.77	1.45
		1.0	1374		1.05	
[HP], 0.8 nM		0	162		0.45	
[TMB], 0.06-0.3 mM	7.4	0.6	345	2.12	0.94	2.07
		1.0	543		1.5	

Table 6. Activation of the peroxidase-catalyzed oxidation of aromatic amines at 20°C

Activator	Substrate	pH (max)	α, M^{-1}	References
Poly(ADSSA)*	TMB	7.0	$2.44 \cdot 10^5$	[5]
2-Aminothiazole	TMB	6.2-6.8	$1.90 \cdot 10^3$	present study
2-Aminothiazole	PDA	6.4	$3.53 \cdot 10^3$	present study
Melamine	PDA	7.4	$2.12 \cdot 10^3$	present study
Melamine	TMB	7.4	$2.41 \cdot 10^3$	[12]

* Poly(ADSSA) is poly(5-aminodisulfide of salicylic acid).

the MA concentration will be associated with an increase in the activation of the 5-ASA oxidation.

The totality of our findings confirms a nucleophilicity of the 2-aminothiazole- and melamine-induced activation of the peroxidase-catalyzed oxidation of TMB, PDA, and 5-ASA: parallel changes in the kinetic parameters k_{cat} and K_m in all cases are directly proportional to concentrations of the activators (Figs. 2b, 4b, and 7b); the coefficient α which quantitatively characterizes the activation of the peroxidase-catalyzed oxidation is strongly dependent on pH (Figs. 3 and 8), this suggesting changes in the activator nucleophilicity with an increase in pH. Table 6 compares the coefficient α values during the activated peroxidase-catalyzed oxidation of TMB and PDA: these values are little changed for 2-aminothiazole and melamine and are of the same order: $(1.90\text{-}3.53)\cdot10^3$ M^{-1}. Thus, at the pH values used AT and MA affect peroxidase as nucleophiles with similar strength. Notwithstanding numerous studies on the nucleophilic activation of peroxidase-catalyzed reactions [6-11] its mechanism is still unclear in detail. Many nitrogen-containing bases are well known to interact with HP not by the heme [7, 22, 23]: this is confirmed by the absence of spectral changes in HP and in its acidic isoform during the interaction with imidazole and pyridine [7]; studies by NMR also show the impossibility of a direct contact of these compounds with the heme [22], i.e., the polypeptide chain of HP has a channel providing the electron transfer from the substrate onto hemin and on the iron atom in the heme. Most likely, nucleophilic activators interact with this channel and this favors the catalysis.

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