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Activation of Peroxidase-Catalyzed Oxidation of 3,3′,5,5′-Tetramethylbenzidine with Poly(salicylic acid 5-aminodisulfide)

D. I. Metelitza* and E. I. Karasyova

Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, ul. Akademika Kuprevicha 5/2, Minsk, 220141 Belarus; fax: (375)-(172) 63-7274; E-mail: metelitza@iboch.bas-net.by

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Abstract—5-Aminosalicylic acid (5-ASA) inhibited by a mixed mechanism the peroxidase catalyzed oxidation of tetramethylbenzidine (TMB) in 0.015 M phosphate-citrate buffer (pH 6.4) supplemented with 5% DMSO and 5% DMF. Poly(salicylic acid 5-aminodisulfide) (poly(SAADS)) in 0.01 M phosphate buffer (pH 6.2-7.4) supplemented with 5% DMSO and 5% DMF effectively activated the peroxidase-catalyzed oxidation of TMB. The activation was quantitatively characterized by coefficients α (M^{-1}) determined at different pH values: α increased linearly with increase in pH up to the maximal value of $2.44 \cdot 10^5$ M^{-1} at pH 7.0. The activating effect of poly(SAADS) on the peroxidase-catalyzed oxidation of TMB is explained by the activator properties of polyelectrolyte, with its anionic form interacting with peroxidase sites responsible for the acid-base catalysis.

Key words: horseradish peroxidase, tetramethylbenzidine, 5-aminosalicylic acid, poly(salicylic acid 5-aminodisulfide), activation, co-oxidation, inhibition of peroxidase-dependent reactions

Soluble aromatic amines such as 3,3′,5,5′-tetramethylbenzidine (TMB), *o*-dianisidine (*o*-DA), *o*-phenylenediamine (*o*-PDA), 5-aminosalicylic acid (5-ASA) are widely used as peroxidase (EC 1.11.1.7) substrates in modern highly sensitive analytical methods (enzyme immunoassay, immunocytochemistry [1, 2]), whereas luminol, isoluminol, and their derivatives are used for chemiluminescent analysis, chemiluminescent enzyme immunoassay, and biosensors [3, 4]. Activation ("enhancement") of peroxidase-dependent oxidation of the above-mentioned amines is promising for increasing the sensitivity of these methods.

At present, three fundamentally different approaches for activation of peroxidase-dependent oxidation of amines are known. The first is realized on addition to the reaction mixture of nitrogen-containing organic bases (ammonia, imidazole and its derivatives, pyridine) at pH > 6.5 and is provided by the influence on the enzyme of these activators which as nucleophiles can change the p*K* values of functional groups of the active site of horseradish peroxidase (HP) and broaden the pH optimum of the peroxidase catalytic activity: the kinetics have been studied of the peroxidase-catalyzed oxidation of *o*-DA, *p*-phenylenediamine (*p*-PDA) activated with pyridine, imidazole [5, 6], and with many derivatives of the latter [7-9], and also with 1,2,4-triazole, 1,2,3-benzotriazole, 4-aminopyridine, and substituted indoles [10].

The second type of activation of peroxidase-dependent oxidation of aromatic amines occurs during their coupled conversion with substituted phenols and polyphenols. Oxidation of such pairs as 4-aminoantipyrine (AAP)—phenols [11-13] and luminol—phenols [3, 4, 14-16] is characterized by a great increase, sometimes 100-200-fold and more, in the oxidation rate of amines. However, the coupled oxidation of amine—phenol pairs with involvement of peroxidase is not always accompanied by an increase in the rate of amine conversion: thus, on the co-oxidation of TMB with gallic acid [17] and its polydisulfide (poly(GADS)) [18, 19], with 2-amino-4-nitrophenol (ANP) and its polydisulfide (poly(ANPDS)) [20, 21], and also with 2,4-dinitrosoresorcinol (DNR) and its polydisulfide (poly(DNRDS)), and with resorci-

Abbreviations: A) activator; 5-ASA) 5-aminosalicylic acid; poly(SAADS)) poly(salicylic acid 5-aminodisulfide); HP) horseradish peroxidase; TMB) 3,3′,5,5′-tetramethylbenzidine; $AmNH_2$) aromatic amines; PhOH) phenols; DMSO) dimethylsulfoxide; DMF) dimethylformamide; AAP) 4-aminoantipyrine.

* To whom correspondence should be addressed.

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nol polydisulfide (poly(RDS)) [22] the TMB conversion was inhibited, and this inhibition, as a rule, was competitive and especially effective in the case of poly(GADS) [18, 19] and of poly(RDS) [22]. However, the peroxidase-catalyzed oxidation of pairs TMB—4,4′-dioxydiphenylsulfone (OPS) and TMB—poly(disulfide-4,4′-dioxydiphenylsulfone) (poly(DSOPS)) was associated with a strong activation of TMB conversion in aqueous medium and in reversed micelles of aerosol OT (AOT) in heptane [21]. Moreover, the oxidation of such pairs as TMB—ANP and TMB—poly(ANPDS) depended on the medium and varied from a deep inhibition to a strong activation of its oxidation in reversed AOT micelles in heptane [21].

We think that the conversion of amines during their coupled peroxidase-catalyzed oxidation with various phenols can be reasonably explained by the nonenzymatic reaction discovered by N. M. Emanuel et al. [23, 24] during the liquid phase oxidation of hydrocarbons inhibited by amine—phenol mixtures:

$$PhO^{\cdot} + AmNH_2 \Longleftrightarrow PhOH + AmNH^{\cdot}. \qquad (1)$$

If the reaction goes from left to right, the oxidation of amine is accelerated and phenol is regenerated (as in the pairs of AAP—phenols [11-13] and of luminol—phenols [3, 14-16]); if the reaction goes from right to left, the oxidation of phenol is accelerated and amine is regenerated (as in the pairs of TMB with gallic acid [17], ANP [20, 21], DNR [22], and their polydisulfide derivatives [17-22]).

The third type of activation of the peroxidase-dependent oxidation occurs during the interaction of the enzyme with synthetic polyelectrolytes that significantly changes catalytic properties of HP and values of K_m and k_{cat} and results in displacement of the pH optimum of the biocatalyst activity. Oxidation rates of luminol and *p*-iodophenol during the coupled peroxidase-catalyzed oxidation increase proportionally to concentration of poly-N-ethyl 4-vinylpyridinium bromide [25]. It is most generally suggested that the electrostatic field of a polymeric molecule influences the HP conformation favorably for the catalysis changing pK_a of ionogenic groups in the active site of the enzyme. Note that the interaction of synthetic polyelectrolytes with enzymes is often associated with a suppression of their catalytic functions depending on the character of the enzyme and the polyelectrolyte charge. In this case a complexity of the enzyme activation mechanism is, first of all, explained by the structure of proteins, which are polyampholytes, with a nonhomogeneous distribution of charged groups in the macromolecule [26].

A careful analysis of the activation of peroxidase-catalyzed oxidation of aromatic amines shows that its mechanisms are not quite clear in all three cases.

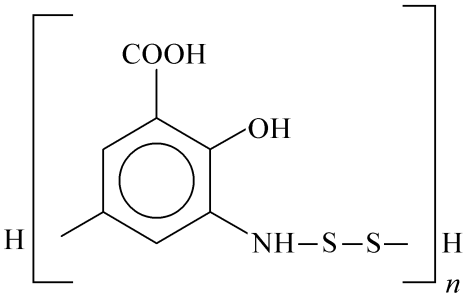
The purpose of the present work was to study the activation kinetics of the peroxidase-catalyzed oxidation of TMB with poly(salicylic acid 5-aminodisulfide (poly(SAADS)) which, on one hand, is a polyphenol and contains ten OH groups in a molecule and, on the other hand, is a typical polyelectrolyte. We intended to find how the antioxidant properties of poly(SAADS) molecule and its influence as a polyelectrolyte on peroxidase should affect the oxidation of TMB in the pH range from 6.2 to 7.4. The purpose of this work was also to compare the effects of poly(SAADS) and of an analog of its monomeric link, 5-aminosalicylic acid (5-ASA), on the oxidation of TMB.

MATERIALS AND METHODS

Reagents. The acidic isoform of horseradish peroxidase, type A, with optical purity index *RZ* 2.75 was from Biolar (Latvia). The enzyme concentration was determined spectrophotometrically with the molar absorption coefficient (ε) at the absorption maximum of the Soret band (403 nm) which was 102,000 $M^{-1}\cdot cm^{-1}$ [27]. Diluted hydrogen peroxide was used as a solvent, and the H_2O_2 concentration was determined spectrophotometrically, with $\varepsilon_{230} = 72.4\ M^{-1}\cdot cm^{-1}$ [28].

TMB (Serva, Germany) and 5-aminosalicylic acid (5-ASA) (Reakhim, Russia) were used as reducing substrates. The UV spectrum of 5-ASA in 10% DMSO was characterized by absorption maximums at 240 and 300 nm with ε values of 3310 and 3470 $M^{-1}\cdot cm^{-1}$, respectively.

As a possible activator of the TMB oxidation, poly(SAADS) was used with the average molecular weight of ~2200 daltons prepared as described in [29] and kindly provided by Yu. P. Losev (School of Chemistry, Belorussian State University, Minsk). The UV spectrum of poly(SAADS) in 10% DMSO had absorption maximums at 241 and 286 nm with ε values of 49,200 and 94,200 $M^{-1}\cdot cm^{-1}$, respectively. The structure of the poly(SAADS) molecule which contained on average about ten monomeric units in accordance with calculations based on the molecular weight ratio is presented below:



The organic solvents DMF and DMSO were distilled before use. All salts and bases for buffer solutions were from Reakhim.

Initial solutions of phosphate and phosphate-citrate buffers, H_2O_2, and peroxidase were prepared with distilled water, solution of TMB was prepared with DMF, and 5-ASA and poly(SAADS) solutions were prepared

with DMSO. Final concentrations of the reagents in different experiments are shown in the figure captions and in the text.

Peroxidase-catalyzed oxidation of 5-ASA. To prepare reaction mixture with total volume of 1 ml, 0.7 ml of distilled water, 0.1 ml of 0.015 M phosphate-citrate buffer (pH 6.4), 0.05 ml of 0.04 M 5-ASA, 0.05 ml of 20 nM HP, and 0.05 ml of DMF were introduced into a tube. Before addition of H_2O_2 the mixtures were kept for 3 min at 20°C and then 0.05 ml of 0.04 M H_2O_2 was added, and this was considered to be the reaction starting point. The final concentrations of the reagents were as follows: 1 nM HP, 1 mM H_2O_2, 5% DMSO, 5% DMF, the concentration of 5-ASA was varied. The peroxidase-catalyzed oxidation of 5-ASA was followed spectrophotometrically by recording the reaction product absorption at 455 nm; the initial oxidation rates were calculated using the coefficient of 15,000 $M^{-1}\cdot cm^{-1}$ of the product molar oxidation at 455 nm [27].

Peroxidase-catalyzed oxidation of TMB and of TMB—5-ASA and TMB—poly(SAADS) pairs. To prepare the reaction mixture of the total volume of 1 ml, 0.7 ml of distilled water, 0.1 ml of 0.015 M phosphate-citrate buffer or 0.01 M phosphate buffer, 0.05 ml of 0.02 M TMB solution in DMF, 0.05 ml of DMSO, 0.05 ml of 20 nM HP, and 0.05 ml of 0.02 M H_2O_2, and also various concentrations of 5-ASA or of poly(SAADS) in DMSO were introduced into a tube. In the typical experiment 0.015 M phosphate-citrate or 0.01 M phosphate buffer with different pH values contained 5% DMF and 5% DMSO, 1 nM HP, 1 mM TMB, 1 mM H_2O_2, and various concentrations of 5-ASA or of poly(SAADS).

The reaction was initiated by addition of H_2O_2 and followed by accumulation of a TMB oxidation product with absorption maximum at 655 nm. The initial rates of the TMB oxidation were determined by initial linear parts of A_{655} kinetic dependences and the v_o value was calculated using the molar absorption coefficient (39,000 $M^{-1}\cdot cm^{-1}$) of the TMB oxidation product [27]. In the control experiments the peroxidase-catalyzed transformation of 5-ASA and of its polydisulfide no products with absorption at about 655 nm were recorded, thus, the spectrophotometric determination of the TMB oxidation product resulting during its oxidation with 5-ASA or with poly(SAADS) was correct.

The peroxidase-catalyzed oxidation of 5-ASA, TMB, and the oxidation of TMB—5-ASA and TMB—poly(SAADS) pairs was performed at 20°C in thermostatted cuvettes of a Specol-211 spectrophotometer (Carl Zeiss, Germany).

RESULTS AND DISCUSSION

The coupled peroxidase-catalyzed oxidation of TMB and 5-ASA. The dependence of the initial oxidation rate of 5-ASA on its initial concentration was studied in the presence of 1 nM HP and 1 mM H_2O_2 at 20°C in 0.015 M phosphate-citrate buffer, pH 6.4, containing 5% DMF and 5% DMSO. The dependence is described by the Michaelis—Menten equation, and its transformation in Lineweaver—Burk coordinates results in the kinetic characteristics of the reaction: k_{cat} = 1580 sec^{-1} and K_m = $0.95\cdot10^{-4}$ M, i.e., under these conditions 5-ASA had a rather high reactivity during the peroxidase-catalyzed oxidation.

Figure 1a shows a decrease in the initial rate of the peroxidase-catalyzed oxidation of TMB (1 mM) with an increase in the concentration of 5-ASA added to the system. The kinetic curves of accumulation of the TMB oxidation product in the presence of 5-ASA suggest the existence of induction periods with duration increasing with an increase in the concentration of 5-ASA up to about 10 min at $[5\text{-ASA}]_0$ ~ 0.5 mM. The presence of induction periods during the TMB-5-ASA pair oxidation suggests that the reaction

$$(TMB)^{+\cdot} + 5\text{-ASA} \rightarrow TMB + (5\text{-ASA})^{\cdot}$$

results in regeneration of the initial amine.

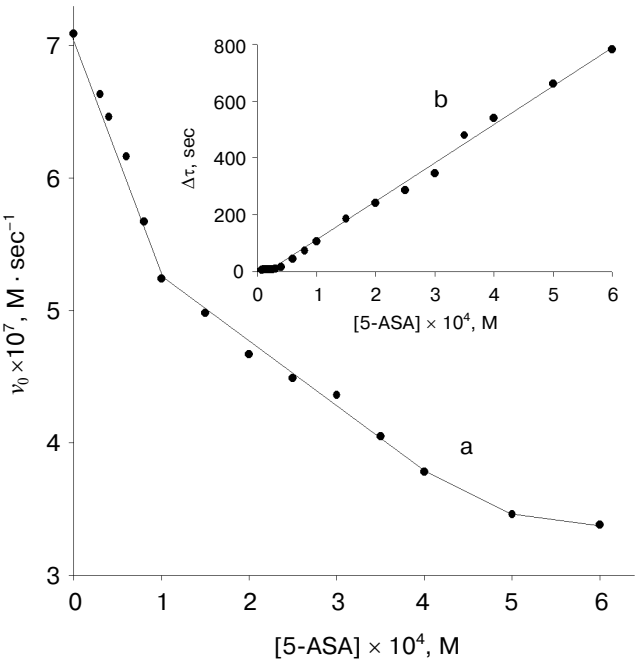


Fig. 1. Effect of 5-ASA concentration on the initial rate of peroxidase-catalyzed oxidation of 1 mM TMB (a) and the induction period Δτ in the accumulation of the reaction product (b) at 20°C in 0.015 M phosphate-citrate buffer (pH 6.4) supplemented with 5% DMSO, 5% DMF, 1 nM HP, and 1.0 mM H_2O_2.

Figure 2 presents in double reciprocal coordinates dependences of the initial rate of the TMB oxidation on its concentration in the absence of 5-ASA (curve *1*) and in the presence of its increasing concentrations (curves *2-4*): the dependence type one-to-one shows a mixed type of inhibition of the amine oxidation [30], and this can be explained by a partial competition between TMB and 5-ASA for binding in the hydrophobic channel on the heme distal side [31, 32] and by competitive reactions of TMB and 5-ASA with active forms of peroxidase, compounds I (E_1) and II (E_2), on one hand, and by the deactivating effect on HP of the peroxidase-catalyzed oxidation of 5-ASA products that decreases the catalytic function of the enzyme, on the other hand. 5-ASA is an aminophenol and as a result of the peroxidase-catalyzed oxidation can deactivate the enzyme, as has been shown in our laboratory for the peroxidase-catalyzed oxidation of *meta*-aminophenol.

Thus, the peroxidase-catalyzed oxidation of TMB in the presence of 5-ASA shows mixed-type inhibition, which reflects a complicated manner of this oxidation.

Peroxidase-catalyzed oxidation of TMB in the presence of poly(SAADS). The dependence of the initial oxidation rate of TMB on its initial concentration was studied at 20°C in 0.01 M phosphate buffer, pH 6.4, containing 5% DMF and 5% DMSO in both the absence of poly(SAADS) and its presence (in the concentrations of 1.4-7.0 μM). In all cases the dependences were described by the Michaelis—Menten equation, and their transformation in Lineweaver—Burk coordinates is presented in Fig. 3a. This figure shows that poly(SAADS) significantly activates the peroxidase-catalyzed oxidation of TMB with a symbatic increase in values of the catalytic constant value and K_m with an increase in the activator concentration (Fig. 3b). Based on data shown in Fig. 3b, the catalytic constant k^a_{cat} of the oxidation activated and the Michaelis constant K^a_m are presented by empiric equations as follows:

$$k^a_{cat} = k_{cat}\,(1 + \alpha[A]_0)\ \text{sec}^{-1}, \qquad (2)$$

$$K^a_m = K_m\,(1 + \alpha[A]_0)\ \text{M}, \qquad (3)$$

where $[A]_0$ is the initial concentration of the activator (poly(SAADS)), α is the coefficient (the degree) of the oxidation activation which presents an increase in the kinetic parameters at the activator concentration of 1 M. The coefficient α values calculated from Eqs. (2) and (3) are $1.61\cdot10^5$ and $1.602\cdot10^5$ M^{-1}, respectively. Note that the activation concentration (1.4-7.0 μM) is three orders of magnitude lower than the TMB concentration and 1400-1700 times higher than the HP concentration (1.0 nM). The dependences in Fig. 3b are strictly linear and have no tendency for saturation, and a further increase in the concentration of poly(SAADS) is impossible because of limited solubility of the activator in the reaction mixture.

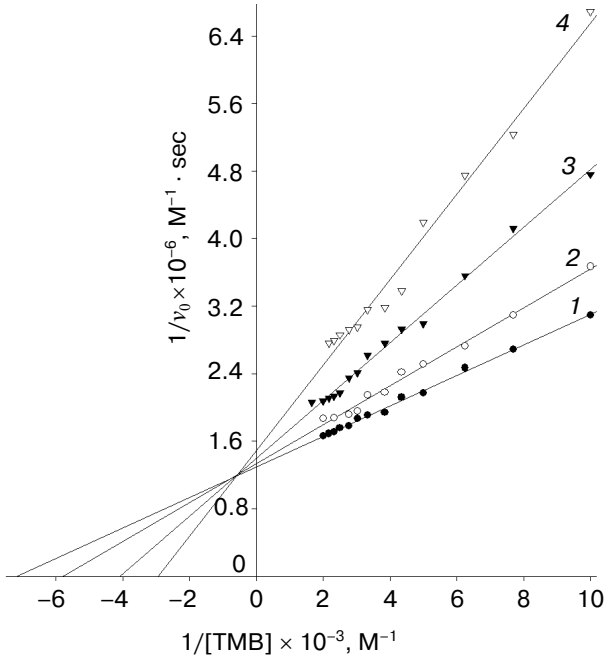


Fig. 2. Dependences in double reciprocal coordinates of initial rate of peroxidase-catalyzed oxidation of TMB on its initial concentration in the absence (*1*) and in the presence of 5-ASA (*2-4*) at 20°C in 0.015 M phosphate-citrate buffer (pH 6.4) supplemented with 5% DMSO, 5% DMF, 1 nM HP, and 1.0 mM H_2O_2. The 5-ASA concentrations: 0.1 (*2*), 0.3 (*3*), and 0.5 mM (*4*).

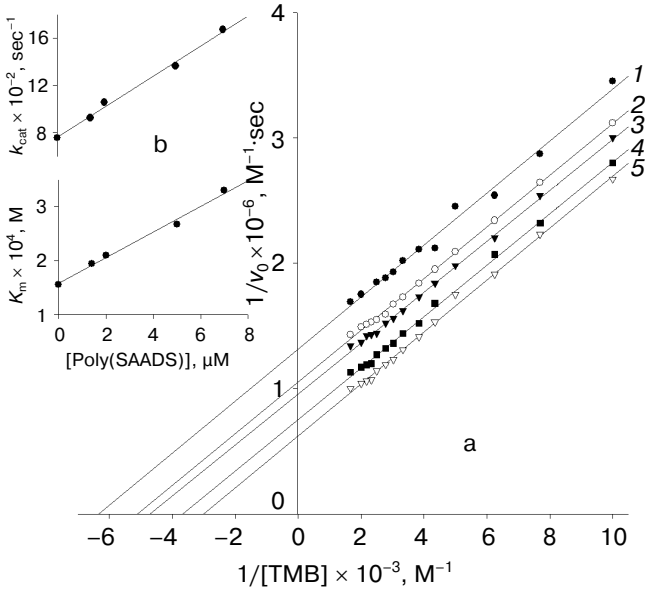


Fig. 3. Dependences in double reciprocal coordinates of the initial rate of the peroxidase-catalyzed oxidation of TMB on its initial concentration (a) in the absence (*1*) and in the presence of poly(SAADS) (1.4 (*2*), 2.0 (*3*), 5.0 (*4*), and 7.0 μM (*5*)) and the effect of poly(SAADS) on the kinetic parameters of the reaction (b): 0.01 M phosphate buffer (pH 6.4) supplemented with 5% DMSO, 5% DMF, 1 nM HP, and 1.0 mM H_2O_2 (20°C).

At constant concentration of hydrogen peroxide (1.0 mM) the initial rate of the peroxidase-catalyzed oxidation of TMB in the presence of the activator (A) is described by Eq. (4):

$$v_0 = \frac{k_{cat}(1 + \alpha[A]_0)\,[H_2O_2]_0[TMB]_0\,[HP]_0}{K_m(1 + \alpha[A]_0) + [TMB]_0}, \qquad (4)$$

which adequately characterizes the activation of the peroxidase-catalyzed TMB oxidation and at $[A]_0 = 0$ is transformed to the usual Michaelis—Menten equation. It follows from the Table 1 that the HP efficiency during the TMB oxidation expressed in k_{cat}/K_m terms is virtually unchanged in the presence of the activator.

Table 1. Kinetic parameters of the peroxidase-catalyzed TMB oxidation at 20°C in the presence of different concentrations of poly(SAADS) in 0.01 M phosphate buffer (pH 6.4) supplemented with 5% DMSO, 5% DMF, 1 mM H_2O_2, and 1 nM HP

Poly(SAADS), μM	k_{cat}, sec^{-1}	$K_m \times 10^4$, M	$(k_{cat}/K_m) \times 10^{-6}$, $M^{-1}\cdot sec^{-1}$
0	757	1.56	4.85
1.4	920	1.93	4.76
2.0	1030	2.12	4.86
5.0	1322	2.67	4.95
7.0	1612	3.33	4.84

Because poly(SAADS) is a typical polyelectrolyte (the pK_a value of a close analog of its monomeric link, salicylic acid, is 3.0), it is especially important to know how pH of the reaction mixture influences the character and quantitative parameters of activation of the peroxidase-catalyzed oxidation of the TMB—poly(SAADS) pair. Therefore, we studied the kinetic characteristics of TMB oxidation in the presence of poly(SAADS) in the pH range of 6.2-7.4 (Table 2). It is important that at all pH values during the peroxidase-catalyzed TMB oxidation the k_{cat} and K_m values are changing symbatically. The k^a_{cat} and K^a_m dependences on the poly(SAADS) concentration are described by Eqs. (2) and (3), respectively, and from them the coefficient α value of $2.44\cdot10^5$ M has been calculated, i.e., the activation of the TMB oxidation significantly increased with an increase in the pH value from 6.4 to 7.0.

Table 2 presents kinetic parameters of the peroxidase-catalyzed TMB oxidation at various pH values in both the absence and presence of poly(SAADS) in the same concentration of 7 μM. The catalytic constant of the TMB oxidation without the activator was maximal at pH 6.2 (1115 sec^{-1}) and was monotonically decreasing with the increase in pH and became about fourfold lower at pH 7.4 (268 sec^{-1}). Changes in the K_m value were complicated, with the minimum at pH 6.8 (Fig. 4, dependence *2*). Usually the HP activity during TMB oxidation was maximal in the pH range of 5.0-6.5 because the isoelectric point p*I* of the acidic form of HP is 5.0 [32]. At all pH values the efficiencies of HP in terms of k_{cat}/K_m were close in both the activity of the activator and in the presence of poly(SAADS).

The dependence of the activation coefficient α on pH (Fig. 4, dependence *1*) seems to be the most interesting: α increases linearly with increasing pH to 7.0 and decreases with a further increase in pH. The peroxidase-catalyzed oxidation of TMB in the pair with poly(SAADS) is maximally activated in neutral medium and depends on the enzyme interaction with the activator as with a polyelectrolyte with dissociation confirmed by

Table 2. Kinetic parameters of the peroxidase-catalyzed TMB oxidation at 20°C in the absence (A) and in the presence of 7 μM poly(SAADS) (B) at different pH values in 0.01 M phosphate buffer supplemented with 5% DMSO, 5% DMF, 1 nM HP, and 1 mM H_2O_2

pH	k_{cat}, sec^{-1}		$\alpha \times 10^5$, (k_{cat})	$K_m \times 10^4$, M		$\alpha \times 10^5$, (K_m)	$(k_{cat}/K_m) \times 10^{-6}$, $M^{-1}\cdot sec^{-1}$	
	A	B		A	B		A	B
6.2	1115	2070	1.22	1.75	3.27	1.24	6.37	6.33
6.4	785	1672	1.61	1.47	3.18	1.66	5.34	5.26
6.6	621	1472	1.96	1.17	2.83	2.03	5.31	5.20
6.8	465	1212	2.29	0.95	2.40	2.18	4.89	5.05
7.0	303	820	2.44	1.35	3.65	2.43	2.24	2.25
7.2	277	581	1.57	1.30	2.74	1.58	2.13	2.12
7.4	268	511	1.32	1.25	2.45	1.33	2.14	2.09

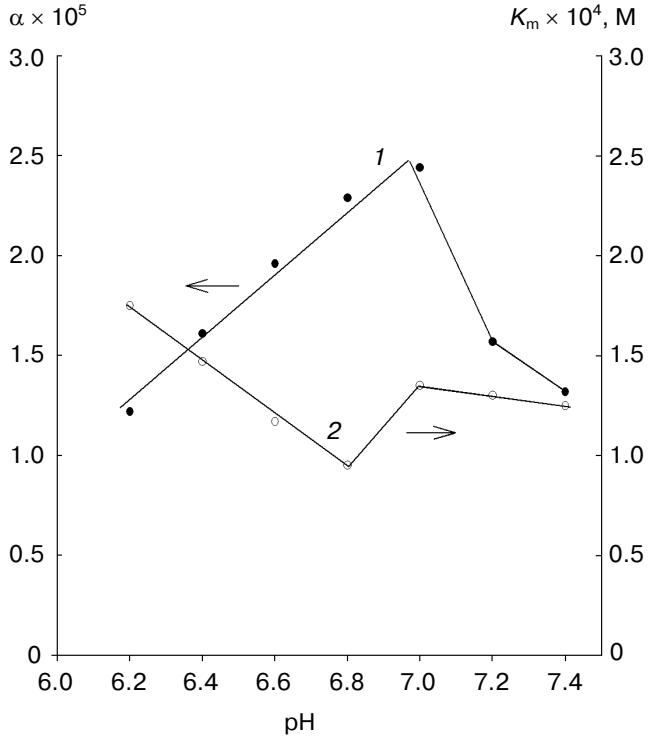


Fig. 4. Dependences of the activation coefficient α of the peroxidase-catalyzed oxidation of TMB with poly(SAADS) (*1*) and of the Michaelis constant K_m during the peroxidase-catalyzed oxidation of TMB without the activator (*2*) on pH of the reaction mixture (20°C, 0.01 M phosphate buffer (pH 6.4) supplemented with 5% DMSO, 5% DMF, 1 nM HP, 1.0 mM H_2O_2, and 7.0 μM poly(SAADS)).

the coefficient α dependence on pH (Fig. 4, the dependence *1*). Obviously, the dissociation degree of poly(SAADS) increases with an increase in pH from 6.2 to 7.4 and the activator polyanions interact with catalytically important sites of HP responsible for the enzyme activation (remember that the [poly(SAADS)]/[HP] ratio under the experiment conditions is 1400-1700). The optimal for catalysis interaction of HP with poly(SAADS) occurs at pH 7.0, and the catalytic activity of the HP—poly(SAADS) complex significantly decreases at pH values higher than this value.

Comparison of the effects of poly(SAADS) and of the analog of its monomeric unit, 5-ASA, on the peroxidase-catalyzed oxidation of the same substrate (TMB) under the same conditions (Figs. 1-4, Tables 1 and 2) shows one-to-one that the monomer completely loses its antioxidant properties in the structure of poly(SAADS). In fact, the amino group in the polydisulfide structure loses its antiradical activity and is changed to the group of –NH–S–S– (see the structure of poly(SAADS)) and the antiradical properties of OH^- groups seem not to be displayed because they are screened during the interaction of the polyanion $[poly(SAADS)]^{n-}$ with positively charged sites of HP. The complete loss of the inhibitory properties by the polydisulfide of 5-ASA also shows that under the experiment conditions the reaction

$$(TMB)^{+\cdot} + poly(SAADS) \rightarrow TMB + poly(SAADS)^{\cdot}$$

does not occur.

Thus, the coupled peroxidase-catalyzed oxidation of the TMB—5-ASA pair occurs by the mixed inhibition type. The polydisulfide of 5-ASA completely loses its inhibitory properties and at different pH values (6.2-7.4) interacts with HP as a polyanion that significantly activates the enzyme and is displayed as a multiple enhancement of the TMB conversion. Polydisulfide of 4,4′-dioxydiphenylsulfone also activated the peroxidase-catalyzed oxidation of this substrate but in this case the monomer also activated the oxidation of TMB [21]. The results of our work [21] allow us to ascribe in this case the activation of the peroxidase-catalyzed oxidation of TMB to the following reactions:

$$TMB + (OPS)^{\cdot} \rightarrow (TMB)^{+\cdot} + OPS,$$

$$TMB + (DSOPS)^{\cdot} \rightarrow (TMB)^{+\cdot} + poly(DSOPS).$$

During studies on peroxidase-catalyzed co-oxidation of aromatic amines with substituted phenols and with their polymeric derivatives it is necessary to consider the possibility of exchange reactions of the substrate radicals and phenol compounds and also the interaction of polymeric phenols as polymeric polyelectrolytes with peroxidase. In particular, in some cases not one of these possibilities can occur separately, but the two together, as found during the peroxidase-catalyzed oxidation of the TMB—poly(gallic acid disulphide) [18, 19] which is well-proved to be a polyelectrolyte [33].

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