Biochemistry (Moscow), Vol. 69, No. 2, 2004, pp. 201-207. Translated from Biokhimiya, Vol. 69, No. 2, 2004, pp. 251-259.
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Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM03-058, October 15, 2003.

Oxidation of Benzidine and Its Derivatives by Thyroid Peroxidase

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Received February 28, 2003
Revision received April 17, 2003

Abstract—Human thyroid peroxidase (hTPO) catalyzes a one-electron oxidation of benzidine derivatives by hydrogen peroxide through classical Chance mechanism. The complete reduction of peroxidase oxidation products by ascorbic acid with the regeneration of primary aminobiphenyls was observed only in the case of 3,3′,5,5′-tetramethylbenzidine (TMB). The kinetic characteristics (k_{cat} and K_m) of benzidine (BD), 3,3′-dimethylbenzidine (*o*-tolidine), 3,3′-dimethoxybenzidine (*o*-dianisidine), and TMB oxidation at 25°C in 0.05 M phosphate-citrate buffer, pH 5.5, catalyzed by hTPO and horseradish peroxidase (HPR) were determined. The effective K_m values for aminobiphenyls oxidation by both peroxidases raise with the increase of number of methyl and methoxy substituents in the benzidine molecule. Efficiency of aminobiphenyls oxidation catalyzed by either hTPO or HRP increases with the number of substituents in 3, 3′, 5, and 5′ positions of the benzidine molecule, which is in accordance with redox potential values for the substrates studied. The efficiency of HRP in the oxidation of benzidine derivatives expressed as k_{cat}/K_m was about two orders of magnitude higher as compared with hTPO. Straight correlation between the carcinogenicity of aminobiphenyls and genotoxicity of their peroxidation products was shown by the electrophoresis detecting the formation of covalent DNA cross-linking.

Key words: human thyroid peroxidase, horseradish peroxidase, tetramethylbenzidine, *o*-tolidine, *o*-dianisidine, benzidine, peroxidase oxidation, kinetic characteristics, DNA damage

Human thyroid peroxidase (hTPO) is a membrane-bound glycoprotein that is a key enzyme of thyroid hormone biosynthesis in thyroid gland cells [1]. The microsomal fraction of thyreocytes is a main source of purified thyroid peroxidase [2]. It is a common belief that hTPO exists in two forms: a dimer with molecular weight ~220-230 kD and a monomer with molecular weight ~105-110 kD [3, 4]. The membrane form of the enzyme is a dimer [3, 4] formed by means of disulfide bonds, which dissociates into monomers when affected by the reducers [5] or proteolytic enzymes [2]. As its prosthetic group, the hTPO contains ferroprotoporphyrin IX (according to [1]), or dihydroxylated heme (according to [6]) likewise lactoperoxidase [7]. There is a supposed presence of four N-glycosylation sites on the surface of extracellular part of hTPO molecule [3]. In the case of hTPO (similarly to the majority of animal and plant peroxidases) the ligands of iron ion in the heme molecule are proximal His494 and distal His239 [1, 8].

Problem of hTPO substrate specificity as well as quantitative estimation of enzyme catalytic activity during the xenobiotics and natural substrates oxidation remains unsolved due to the difficulties in accessing the object of our research. In thyroid gland the hTPO (EC 1.11.1.8) catalyzes the oxidation of iodide ion by hydrogen peroxide, iodination of tyrosine residues in thyroglobulin molecule, and also the conjugation reaction of mono- and diiodine derivatives of tyrosine, forming 3,3′,5-triiodo-L-thyronine (T3) and 3,3′,5,5′-tetraiodo-L-thyronine (T4, thyroxine) [1, 2]. Thyroid peroxidase exhibits the ability to oxidize different phenolic compounds as well as thiourea derivatives [1] and ABTS [9] in the presence of H_2O_2, what points to a wide substrate specificity of this enzyme. Hence, from the practical point of view it seems to be important to investigate the role of hTPO in the metabolic transformation of xenobiotics, especially those, which are known to be potential carcinogens. Taking into account the physiological

Abbreviations: hTPO) human thyroid peroxidase; HRP) horseradish peroxidase; ABTS) 2,2′-azinobis(3-ethylbenzothiazoline-(6)-sulfonic acid); TMB) 3,3′,5,5′-tetramethylbenzidine; *o*-TD) 3,3′-dimethylbenzidine (*o*-tolidine); *o*-DA) 3,3′-dimethoxybenzidine (*o*-dianisidine); BD) benzidine; DMF) dimethylformamide.

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importance of hTPO, the need of systematic approach in studies dedicated to the catalytic mechanism of this enzyme and its substrate specificity is obvious. The comparison of hTPO with more investigated peroxidases, such as HRP, and also to heme- and iron-containing enzymes exhibiting pseudo-peroxidase activity is relevant in terms of revealing the evolutional links between thyroid peroxidase and other proteins, and extending the knowledge about its structure and function.

It has been shown that commercially used dyes based on benzidine and its derivatives can be metabolized in the human body releasing free aminobiphenyls [10]. At the same time, benzidine (BD) is a strong carcinogen causing cancer in bladder, liver, mammary gland, and also resulting in the development of carcinoma in thyroid gland in animals; *o*-tolidine (*o*-TD) and *o*-dianisidine (*o*-DA) have a limited carcinogenic activity; 3,3′,5,5′-tetramethylbenzidine (TMB) is not a carcinogen [10]. Results of model studies suggest that one of the metabolic oxidation pathways for xenobiotics from this group can be their oxidation by different biocatalysts according to peroxidase mechanism forming highly reactive products that in turn may lead to the damage of cellular macromolecules, and thereby induce chemical carcinogenesis [11]. For instance, cytochrome P450 LM_2 [12], cytochrome b_5 [12], hemoglobin [12], ferritin [13], and prostaglandin synthetase [14] can oxidize benzidine and its derivatives with various efficiency. Determination of the possible role of specific cellular peroxidases such as hTPO in aminobiphenyl biotransformation is of special interest.

By continuing our work started earlier in this direction [15, 16], our goal was to present the comparative kinetic characteristics of catalytic activity of purified human thyroid peroxidase and horseradish peroxidase in the oxidation of benzidine congeners with increasing carcinogenic activity, and also to study the reactive ability of their peroxidation products towards DNA.

MATERIALS AND METHODS

Reagents. Peroxidase substrates used in this study were TMB (Serva, Germany) and also *o*-TD, *o*-DA, and BD (Reakhim, Russia), additionally purified by sublimation. Dimethylformamide that was used for the preparation of stock solutions of aminobiphenyls was freshly distilled. Buffer solutions contained chemically pure salts, acids, and bases produced by Reakhim. Diluted hydrogen peroxide (Reakhim) was used as an oxidizer. Concentration of H_2O_2 was determined spectrophotometrically using molar coefficient of absorption $\varepsilon_{230} = 72.4\ M^{-1}\cdot cm^{-1}$ [17].

Enzymes. Horseradish peroxidase (EC 1.11.1.7) produced by Biolar (Latvia) with RZ = 2.7 was used for comparison with hTPO. The concentration of peroxidase was determined spectrophotometrically using molar coefficient of absorption at $\lambda = 403$ nm of $102{,}000\ M^{-1}\cdot cm^{-1}$ [18].

Purified hTPO was obtained from post-surgical and histologically classified samples of human thyroid gland (kindly provided by Prof. E. P. Demidchik) using earlier described techniques [19, 20]. Microsomal fraction of thyroid gland was obtained employing differential centrifugation in 0.05 M K-phosphate buffer containing 0.25 M sucrose (pH 7.4). A combination of methods was used to purify hTPO, including solubilization of hTPO by sodium cholate and Tween-20, fractioning in polyethylene glycol, gel filtration, and dialysis. The gel filtration carrier was Toyopearl HW-65 in 0.05 M Na-phosphate buffer (pH 7.5) containing 0.05% Tween-20 and 10% glycerol. Purity of the obtained hTPO fraction was estimated to be >90% using electrophoresis in 10% polyacrylamide gel in the presence of SDS [21].

The activity of hTPO during the purification was determined according to the rate of iodide peroxidase oxidation using molar extinction coefficient of I_3^- ($\varepsilon_{353} = 26{,}000\ M^{-1}\cdot cm^{-1}$) [22]. The concentration of hTPO was determined spectrophotometrically using molar extinction coefficient $\varepsilon_{412} = 121{,}000\ M^{-1}\cdot cm^{-1}$ [19].

Aminobiphenyl peroxidase oxidation. Oxidation of benzidine congeners by hydrogen peroxide catalyzed by hTPO and HRP was performed at 25°C in thermostatted cuvettes using a KFK-3 photometer (Russia) and 0.05 M citrate-phosphate buffer, pH 5.5, containing 5% v/v DMF. The use of an organic solvent is motivated by the low solubility of benzidine derivatives in aqueous solutions. The total volume of the reaction mixture was 1.5 ml. The reaction was started by adding H_2O_2. Initial reaction rates were determined based on initial linear ranges of optical density growth curves using the following molar absorption coefficients of aminobiphenyl oxidation: TMB, $\varepsilon_{655} = 39{,}000\ M^{-1}\cdot cm^{-1}$ [23]; *o*-TD, $\varepsilon_{582} = 14{,}000\ M^{-1}\cdot cm^{-1}$ [24]; *o*-DA, $\varepsilon_{460} = 30{,}000\ M^{-1}\cdot cm^{-1}$ [25]; BD, $\varepsilon_{590} = 34{,}000\ M^{-1}\cdot cm^{-1}$ [13].

Genotoxicity of aminobiphenyl peroxidase biotransformation products was determined by an electrophoretic DNA cross-linking test [11]. Samples containing aminobiphenyls at different concentrations were analyzed using a non-denaturing electrophoresis technique in agarose gel [21]. Aminobiphenyls were oxidized by hydrogen peroxide catalyzed by hTPO in the presence of high molecular weight double helix DNA from bovine thymus (Serva) at room temperature in 0.05 M citrate-phosphate buffer, pH 5.5. Fifteen minutes after the initiating the reaction, the samples were mixed with 0.05 M Tris-borate buffer (pH 8.0) containing 50% glycerol and 0.025% bromophenol blue. Electrophoretic DNA separation was performed in 0.9% agarose gel in 0.05 M Tris-borate buffer (pH 8.0) containing 2 mM EDTA at 10 V/cm. Type I agarose (Serva) used in this study has low electroosmotic mobility. DNA in gels was visualized by ethidium bromide (Sigma) at the concentration of 0.4 μg/ml of agarose gel. Gel images were obtained using an Olympus C-2500L digital camera.

RESULTS AND DISCUSSION

Oxidation kinetics of benzidine congeners. It was determined that the initial oxidation rate of *o*-TD at 25°C is directly proportional to hTPO concentration in the range between 12 and 69 nM. As we demonstrated earlier, the optimal pH for hTPO in the reaction of aminobiphenyl oxidation is within acidic medium for all examined enzyme samples; however, the value of the pH optimum varies within the range 4.5-5.5 [15]. Figure 1 illustrates that maximal initial rates of oxidation product accumulation for all aminobiphenyls investigated by us in systems containing hydrogen peroxide and hTPO are observed at pH ~ 4.5. However, due to an earlier discovered low operational stability of thyroid peroxidase under these conditions [26], further experiments were performed at pH 5.5. It has been many times confirmed that the aminobiphenyl oxidation products are the most stable at pH 5-6 [14, 23, 24]. In neutral media, these studies are difficult also because of the hydrophobic behavior of aminobiphenyls, which requires a large amount of organic solvent dimethylformamide (DMF) to be introduced into the reaction medium. Because the pH optimum of the oxidation reaction of certain substrates (KI, ABTS) by thyroid peroxidase can shift with a change in hydrogen peroxide concentration [9], we studied the dependence of *o*-TD oxidation product accumulation rate on pH of the medium at different H_2O_2 concentrations. It was found that the pH optimum of *o*-TD oxidation product accumulation did not depend on the concentration of hydrogen peroxide in the range of 0.1-1 mM.

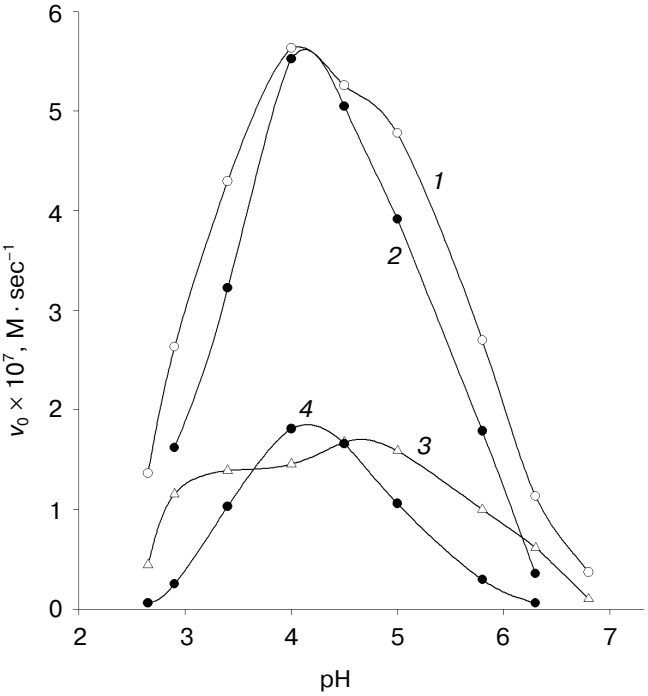

Fig. 1. pH-dependence of initial rate of TMB (*1*), *o*-TD (*2*), *o*-DA (*3*), and BD (*4*) peroxidation. Conditions: 30°C, 0.05 M citrate-phosphate buffer containing 5% DMF, 23.1 nM hTPO, 1 mM H_2O_2.

Spectral characteristics of peroxidation products of aminobiphenyls. Oxidation of aminobiphenyls mediated by hTPO was performed at pH 5.5, which is an optimal value for peroxidase oxidation of these compounds catalyzed by horseradish peroxidase and some other heme proteins [12, 24], which is important for the comparison of spectral characteristics of the reaction products that are dependent on the pH of the medium [13]. Analysis of absorption spectra for oxidation products of BD, *o*-TD, *o*-DA, and TMB mediated by purified hTPO reveals that a subsequent transformation of all these aminobiphenyls occurs, forming reaction products similar to those known for HRP [23], ferritin [13], and prostaglandin synthetase [14]. The spectral characteristics of the reaction products are presented in Table 1.

As seen from the data in Table 1, three absorption maxima for methyl-substituted benzidine derivatives oxidation products were detected spectrophotometrically in

Table 1. Spectral characteristics of oxidation products of benzidine congeners catalyzed by purified hTPO in 0.05 M citrate-phosphate buffer, pH 5.5, at 20°C

Substrate	Aminobiphenyl, λ_{max}, nm	Oxidation products		
		intermediate products, λ_{max1}, nm	final product, λ_{max}, nm	intermediate products, λ_{max2}, nm
BD	284	360	~458	602
o-TD	284	368	~448	635
o-DA	305	347*	466	543*
TMB	288	379	460	655

* Products of deep oxidation of *o*-dianisidine.

the hTPO-catalyzed reaction, which correspond to the intermediate reaction products of one electron peroxidase oxidation mechanism according to the earlier suggested scheme [14, 23]. The formation of charge transfer complexes was demonstrated for *o*-TD and TMB; the complexes are equimolar compounds of the original aminobiphenyl and a cation radical formed during the one electron oxidation reaction. Charge transfer complexes formed during the oxidation *o*-TD and TMB have two absorption maxima (368-379 and 636-661 nm). Diimines are formed in the aminobiphenyl peroxidation reaction, being the products of two subsequent one-electron oxidation acts, and are characterized by absorption maxima at 450-460 nm. It should be pointed out that the absorption maximum at ~600 nm (corresponding to the charge transfer complex) becomes hardly distinguishable already after a few minutes from the start of BD peroxidation reaction, whereas absorption increase is observed at wavelengths of ~458 and 360 nm. As described earlier for HRP and prostaglandin synthetase [14, 23], the absorption maximum at 458 nm in the hTPO reaction corresponds to the diimine, which can be reduced by ascorbate to the original substrate; the wide maximum at 300-400 nm probably corresponds to the polymeric products of BD peroxidase oxidation, which are not subject to reduction. A slightly different picture is observed in the case of *o*-DA oxidation mediated by hTPO, where no absorption maxima corresponding to the charge transfer complex was detected at pH 5.5. It can be assumed that (similarly to the HRP case) the creation of quinodiimine takes place mainly in the peroxidase active site without the release of an intermediate cation radical into the medium [27, 28]. The absorption spectrum of the *o*-DA oxidation products suggests the formation of bis-azodiphenyl, the absorption maximum of which is found within the 453-475 nm range, overlapping with the quinodiimine (maximum at 452, 514 nm) [27, 28]. Similar absorption spectra were found earlier for *o*-DA oxidation products mediated by horseradish peroxidase (pH 5.5) [24].

It is known that the transformation of certain products of aminobiphenyl peroxidase oxidation into the initial substrates can be observed in the presence of reducing agents [14, 29]. About 15 min after the reaction was started by addition of hydrogen peroxide ascorbate was introduced into the reaction mixture until the final concentration was 0.33 mM. The complete reduction of the peroxidase reaction products to the original substrate was observed only in the case of TMB, but not BD, *o*-TD, and *o*-DA. This feature can be connected to the fact that the TMB molecule, unlike other benzidine congeners, contains methyl groups in the most reactive 3, 3′, 5, and 5′ positions. Hence, the intermediate products of TMB oxidation are not prone to polymerization and easily accept electrons in the presence of reducing agents [23]. Incomplete reduction of *o*-TD, *o*-DA, and BD peroxidase oxidation products after the addition of ascorbate can be explained by less expressed steric hindrance for these compounds compared to TMB, which is due to the complete absence of the substituents in 3, 3′, 5, and 5′ positions or to their decreased number. As the result, BD, *o*-TD, and *o*-DA oxidation products are able to interact with each other with formation of polymeric compounds, losing their ability for reversible reduction by ascorbate with the regeneration of the initial aminobiphenyls.

In our opinion, another reason for incomplete reduction of *o*-TD, *o*-DA, and BD oxidation products can be the low reactive ability of cation radicals of these substrates towards ascorbate compared to the activity of $(TMB)^{\cdot+}$ cation radical.

The results of spectrophotometric analysis show the ability of hTPO to oxidize benzidine congeners in acidic medium via the one-electron peroxidase mechanism, earlier illustrated for HRP [23] and prostaglandin synthetase [14].

Comparison of catalytic activity of human thyroid peroxidase and horseradish peroxidase. The dependence of the initial rate of accumulation of reaction products on the concentration of substrates (BD, *o*-TD, *o*-DA, TMB, and H_2O_2) was determined under strictly identical conditions, following Michaelis–Menten kinetics both in the case of HRP and hTPO, from where the kinetic characteristics at 25°C, pH 5.5, were obtained according to the Lineweaver–Burk technique.

As seen in Table 2, the effective K_m characterizing the peroxidase oxidation of the investigated aromatic amines is lower in the case of hTPO than in the case of HRP, except for benzidine. Along with the increasing number of substituents in the benzidine molecule, a tendency for growth of the effective K_m values is observed for hTPO an HRP. Table 2 presents the kinetic characteristics for the peroxidation of benzidine derivatives mediated by certain heme- and iron-containing biocatalysts [12, 13]. It can be seen that for cytochromes b_5 and P450 LM_2 the same tendency in the change in effective K_m values is preserved for the aminobiphenyl pseudo-peroxidase oxidation process, similarly to hTPO and HRP. However, in the case of hemoglobin and ferritin the reverse dependence is observed, and the K_m values change in the series BD > *o*-TD > TMB, what suggests significant differences in the structures of the active sites of these biocatalysts compared to the investigated peroxidases and cytochromes.

The K_m values determined for hTPO from the dependences of v_0 of aminobiphenyl peroxidase oxidation reactions on H_2O_2 concentrations were 484 μM for BD, 709 μM for *o*-TD, 805 μM for *o*-DA, and 716 μM for TMB. These values are 4-14 times higher than the K_m values determined from dependences of v_0 on reducing substrates concentrations. Hence, regardless of 1.3-1.8 time increase in k_{cat} values, k_{cat}/K_m values determined based on the dependences of v_0 on H_2O_2 concentrations and expressed in $M^{-1}\cdot sec^{-1}$ were $2.21\cdot10^4$ for BD, $6.04\cdot10^4$ for

Table 2. Kinetic parameters of peroxidation of benzidine congeners catalyzed by certain heme proteins and ferritin

Substrate	Parameters determined from dependences of ν_0 on aminobiphenyl concentrations					
	Enzyme	pH	k_{cat}, sec^{-1}	$K_m \times 10^4$, M	k_{cat}/K_m, $M^{-1} \cdot sec^{-1}$	Reference
BD	hTPO	5.5	6.0	0.49	124×10^3	this work
	HRP	5.5	1051.0	0.12	8600×10^3	this work
	hemoglobin	6.0	163.8	1.43	1145×10^3	[12]
	cytochrome b_5	6.0	0.7	0.83	8.6×10^3	[12]
	cytochrome P450 LM_2	5.5	6.7	1.96	34.2×10^3	[12]
	ferritin	4.2	0.7	0.91	0.073×10^3	[13]
o-TD	hTPO	5.5	27.5	1.77	156×10^3	this work
	HRP	5.5	3410.2	2.03	$16\,800 \times 10^3$	this work
	hemoglobin	6.0	3.2	1.17	27.4×10^3	[12]
	cytochrome b_5	6.0	1.6	4.00	4.0×10^3	[12]
	ferritin	6.0	0.15	0.28	0.053×10^3	[13]
	ferritin	4.2	0.71	0.66	0.107×10^3	[13]
o-DA	hTPO	5.5	11.9	0.58	203×10^3	this work
	HRP	5.5	1661.5	1.16	$14\,300 \times 10^3$	this work
	hemoglobin	6.0	6.9	1.04	66.3×10^3	[12]
	cytochrome b_5	6.0	0.86	1.85	4.6×10^3	[12]
	cytochrome P450 LM_2	6.0	0.4	1.82	2.15×10^3	[12]
TMB	hTPO	5.5	34.6	1.66	209×10^3	this work
	HRP	5.5	5659.7	2.47	$22\,900 \times 10^3$	this work
	hemoglobin	6.0	14.1	1.02	138.2×10^3	[12]
	cytochrome b_5	6.0	0.57	2.00	2.9×10^3	[12]
	cytochrome P450 LM_2	6.0	2.4	9.09	2.6×10^3	[12]
	ferritin	6.0	0.5	0.17	0.3×10^3	[13]
	ferritin	4.2	3.3	0.12	2.8×10^3	[13]

o-TD, $2.25 \cdot 10^4$ for *o*-DA, $6.27 \cdot 10^4$ for TMB; these values are 2.6-9-times lower than the corresponding data for aminobiphenyl oxidation efficiency shown in Table 2. This phenomenon is often observed for substrate peroxidation and is explained by the unproductive H_2O_2 depletion due to the destruction of heme-containing catalysts during the reaction [30]. Therefore, the K_m values determined based on the dependences of v_0 on H_2O_2 concentrations do not completely correspond to the true values of Michaelis constants for aminobiphenyl oxidation by hydrogen peroxide. Unfortunately, detailed discussion of the features of aminobiphenyl binding with hTPO is not now possible because the fine structure of the active site and properties of this enzyme are not sufficiently explored.

Enzyme efficiency is usually estimated by comparing the catalytic constants determined from the dependences of v_0 on initial concentration of aminobiphenyl and also k_{cat}/K_m ratio [31]. Along with the increase in K_m value in aminobiphenyl oxidation reaction by both peroxidases, there is a growth in k_{cat} and k_{cat}/K_m values observed when electron-donating substituents are introduced in the benzidine molecule. Data in Table 2 demonstrate that k_{cat} values of *o*-DA peroxidase oxidation for hTPO and HRP are at the intermediate position compared to those determined for BD and *o*-TD. The k_{cat} values of TMB peroxidase oxidation by both enzymes exceed k_{cat} for BD by ~5.5 times.

Tendency for increase in k_{cat}/K_m value with the incorporation of methyl substituents into the benzidine molecule is not clearly expressed; in the case of hTPO

k_{cat}/K_m value is ~1.7 times higher for TMB compared to benzidine; in the case of HRP the value is ~2.7 times higher. It is interesting that hTPO efficiency in *o*-DA oxidation evaluated by k_{cat}/K_m value approaches this for the most reactive substrate, namely TMB. The data correlate with the values of substrate redox potentials, which increase in the order TMB < *o*-DA < BD and are equal to 0.27, 0.28, 0.31 V, respectively [32]. Thus, substrate redox potentials, and not only steric factors, can play a significant role for efficient peroxidase reaction. Analysis of data in Table 2 reveals that the reversed dependence of k_{cat}/K_m values on substituents number in the benzidine molecule is observed in the case of aminobiphenyl pseudo-peroxidase oxidation by cytochromes [12], compared to ferritin [13], hTPO, and HRP; the increase in K_m values and decrease of reaction efficiency are observed when the substituents are introduced in 3, 3′, 5, and 5′ positions of the benzidine molecule. This suggests that steric or other factors are crucial when the reaction is catalyzed by cytochromes and hemoglobin.

It has been shown that HRP exhibits very high efficiency in aminobiphenyl oxidation, while hTPO activity is much lower. Values of k_{cat} differ in the favor of HRP 175, 124, 140, and 163 times for BD, *o*-TD, *o*-DA, and TMB, accordingly, i.e., by more than 2 orders of magnitude (see Table 2). Ratios of k_{cat}/K_m also differ in the favor of horseradish peroxidase ~70 times for BD and *o*-DA and ~108 times for *o*-TD and TMB. These results correlate well with that obtained by us earlier regarding the catalytic activity of another preparation of purified hTPO in the TMB oxidation reaction [16]. Data in Table 2 allow comparing aminobiphenyl oxidation efficiency mediated by hTPO with the earlier determined parameters for certain biocatalysts of animal origin exhibiting pseudo-peroxidase activity. It can be seen that *o*-DA, *o*-TD, and TMB oxidation efficiency mediated by hTPO and hemoglobin are values of the same order, and in the case of BD the process catalyzed by hemoglobin is more efficient. Efficiency of pseudo-peroxidase oxidation of benzidine derivatives mediated by cytochromes P450 and b_5 is 40-95 times lower than hTPO efficiency under comparable conditions (Table 2).

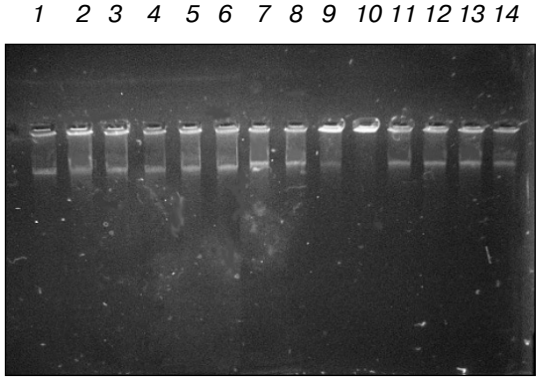

Fig. 2. Agarose gel electrophoresis of high molecular weight DNA from bovine thymus (~1 μg per lane) exposed to peroxidase reaction medium (0.05 M citrate-phosphate buffer, pH 5.5, 1 mM H_2O_2, 46 nM hTPO, 0.1 mg/ml DNA) for 20 min at room temperature: *1*) DNA; *2*) 1 mM BD, system without hTPO; *3*) 1 mM TMB, system without hTPO; *4*) system without aminobiphenyl; *5*) 1 mM BD, system without H_2O_2; *6*) 1 mM TMB, system without H_2O_2; *7-10*) increasing concentrations of BD (1 μM (*7*); 10 μM (*8*); 100 μM (*9*); 1 mM (*10*)); *11-14*) increasing concentrations of TMB (1 μM (*11*); 10 μM (*12*); 100 μM (*13*); 1 mM (*14*)).

Interaction of radical products of aminobiphenyl peroxidase oxidation with DNA. The possibility of DNA molecule damage by the peroxidation products of benzidine congeners was proved using an electrophoretic express-test. The technique allows separation of native DNA and its modified forms resulting from the interaction with peroxidation products of xenobiotics, causing the formation of intra- and inter-chain covalent links [11]. It was found that when high molecular weight double helix DNA from bovine thymus is exposed to medium containing BD peroxidase oxidation products, cross-linked DNA molecules can be detected that are irreversibly aggregated and have lost their electrophoretic mobility in the agarose gel under the analysis conditions (Fig. 2). Then, formation of DNA "links" during the hTPO catalyzed peroxidase reaction has dose-dependent nature; moreover, in the case of BD the links form even when DNA was added to the reaction mixture 15 min after the start of peroxidation of this substrate. Products of TMB oxidation carrying the methyl substituents in 3, 3′, 5, and 5′ positions do not exhibit genotoxicity regardless of the significantly higher oxidation efficiency of this substrate by thyroid peroxidase compared to BD. The data confirm the earlier proposed hypothesis [33] that higher carcinogen potential is exhibited by those benzidine congeners that are able to peroxidize modifications in *ortho*-position towards amino groups. According to this theory, TMB is not a carcinogen. Hence, with growing number of electron-donating substituents in 3, 3′, 5, and 5′ positions of benzidine molecule, the reactive ability of its peroxidase oxidation products towards DNA decreases and, as a consequence, also an extent of its covalent modification.

There are electrophilic products emerging in the course of peroxidation of aminobiphenyls, the main part being cation radicals, which are polyfunctional reagents potentially able to form DNA cross-links [11]. It can be assumed that the high heterofunctional potential of BD cation radicals (to a great extent along with *o*-TD and *o*-DA) is determined by several reasons: 1) the presence of an active electrophilic site at the nitrogen atom, whose activity is increased together with the decrease in delocalization ability of an uncoupled electron and the removal of steric hindrance due to the decreased number of electron-donating substituents in 3, 3′, 5, and 5′ positions of benzidine molecule; 2) the presence of free reaction sites

in *ortho*-position towards amino group [34]. These properties are a basis for the formation of BD–DNA adduct and cross-linking mechanism proposed by O'Brien [11].

The performed comparative study of oxidation of benzidine congeners by hydrogen peroxide mediated by human thyroid peroxidase and horseradish peroxidase, the comparison of quantitative characteristics of the reaction with that determined earlier for some other heme- and iron-containing biocatalysts [10, 11], and also the experiments dedicated to the investigation of the genotoxic action of peroxidation products of benzidine and its derivatives towards DNA allow the following conclusions.

First, the results of spectrophotometric analysis of the products prove the ability of hTPO to catalyze benzidine congener oxidation by hydrogen peroxide via a one-electron mechanism, i.e., Chance's classic scheme [35], proven earlier for HRP and prostaglandin synthetase [14, 23].

Second, hTPO is two orders of magnitude less effective in the oxidation of benzidine derivatives compared to HRP. With growing number of electron-donating substituents in 3, 3′, 5, and 5′ positions of the benzidine molecule, the oxidation efficiency of aminobiphenyls increases along with the increase in K_m values. The efficiency of aminobiphenyl oxidation mediated either by hTPO or HRP exhibits a correlation with the redox potential values of the investigated substrates.

Third, unlike peroxidase oxidation of TMB, the process of BD oxidation mediated by hTPO leads to the formation of reaction products able to react with each other non-enzymatically resulting in deeply oxidized and slightly soluble polymeric compounds or to attack reaction sites in the nitrogen bases of DNA and form cross-links. The revealed reverse dependence between the efficiency of aminobiphenyl biotransformation mediated by hTPO and their genotoxicity suggests that a key factor in the realization of carcinogenic potential of the investigated compounds is not the rate of oxidation reaction, but the structure and reactivity of the intermediate electrophilic products formed during the peroxidation towards DNA and other cell biopolymers.

The authors thank Prof. E. P. Demidchik for providing post-surgical samples of thyroid gland and Prof. D. I. Metelitsa and Dr. V. P. Kurchenko for valuable comments provided during discussions of the presented results.

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