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Thiamine Inhibits Formation of Dityrosine, a Specific Marker of Oxidative Injury, in Reactions Catalyzed by Oxoferryl Forms of Hemoglobin

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Abstract—Effects of thiamine and its derivatives on inhibition of dityrosine formation were studied in reactions catalyzed by oxoferryl forms of hemoglobin. At high thiamine concentrations a complete inhibition of dityrosine formation was observed due to interaction of tyrosyl radicals with thiamine tricyclic and thiol forms. In neutral and alkaline media, tyrosyl radicals oxidized thiamine to thiochrome, oxodihydrothiochrome, and thiamine disulfide. In the absence of tyrosine, oxoferryl forms of hemoglobin manifested peroxidase activity towards thiamine and its phosphate esters by inducing their oxidation to disulfide compounds, thiochrome, oxodihydrothiochrome, and their phosphate esters, respectively, in neutral media. Thiamine and its phosphate esters were oxidized by both oxoferryl forms of hemoglobin, viz., ‡Hb(IV=O) (compound I with an additional radical on the globin) and Hb(IV=O) (compound II). Putative mechanisms of thiamine conversions under oxidative stress and the protective role of hydrophobic thiamine metabolites are discussed.

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Key words: thiamine, dityrosine, hemoglobin, oxoferryl forms, tyrosyl radicals, thiochrome, oxodihydrothiochrome

Similarly to 3-nitrotyrosine and other tyrosine oxidation products, dityrosine is a significant biomarker of oxidative and nitrosyl stress [1, 2]. Dityrosine content in various pathological states, e.g. cataract, atherosclerosis, inflammation, Alzheimer's disease, etc., is usually elevated. Dityrosine formation is induced by various factors, such as UV- or γ-irradiation, free radical oxygen species, nitrogen dioxide, peroxynitrite, and lipid peroxides [3-6].

In the presence of hydrogen peroxide, different peroxidases, e.g. myeloperoxidase, and also methemoglobin, metmyoglobin catalyze the oxidation of phenols and formation of dityrosine [4], which plays the significant role in protein aggregation [5, 6]. In alkaline media in the presence of peroxidase and hydrogen peroxide, neighboring tyrosine residues of proteins undergo enzymatic oxidation, resulting in oxidative crosslinking of phenolic rings and formation of dityrosine (Fig. 1) [7].

It has been reported [8] that horseradish peroxidase induces polymerization of peptides containing tyrosine residues. Myeloperoxidase of activated neutrophils and macrophages oxidizes L-tyrosine to give dityrosine as the major product [8]. Considering that dityrosine is resistant to acid hydrolysis and protease treatment and manifests intense fluorescence, its presence in biological media is a marker of oxidative stress [5].

Our studies showed that thiamine inhibits dityrosine formation in reactions catalyzed by oxoferryl forms of hemoglobin. Oxidation of thiamine by tyrosyl radicals yields thiochrome and oxodihydrothiochrome. In the presence of hydrogen peroxide, oxidation of thiamine by methemoglobin also yields thiochrome and oxodihydrothiochrome.

MATERIALS AND METHODS

Thiamine (T), thiamine monophosphate (TMP), thiamine diphosphate (TDP), and thiochrome (Tch)

Abbreviations: Hb(IV=O)) oxoferryl form of hemoglobin (compound II); ‡Hb(IV=O)) oxoferryl form of hemoglobin with a radical on globin (compound I); ODTch) oxodihydrothiochrome; T) thiamine; Tch) thiochrome; TDP) thiamine diphosphate; TMP) thiamine monophosphate; Tyr$^{\bullet}$) tyrosyl radical.

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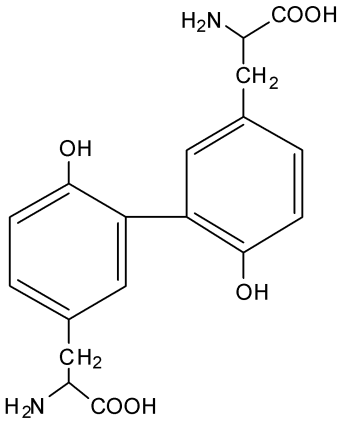

Fig. 1. Structural formula of dityrosine.

were from Sigma (USA); L- and D-tyrosine, D,L-arginine, D,L-histidine, D,L-valine, D,L-phenylalanine, L-serine, D-aspartic acid, D,L-threonine, ferricyanide, and hemin were from Fluka (USA). All other chemicals were purchased in Russia and Belarus' and were of analytical grade of purity.

Oxyhemoglobin was isolated from fresh donor blood as described previously [9]. Oxyhemoglobin concentration was determined using extinction coefficient ε_{412} = 125,000 $M^{-1}\cdot cm^{-1}$ or ε_{542} = 14,250 $M^{-1}\cdot cm^{-1}$ [9]. Methemoglobin was obtained by adding 10-20-fold molar excess of ferricyanide to oxyhemoglobin. Low molecular weight compounds were separated from the protein by gel filtration on Sephadex G-25. Methemoglobin concentration at pH 7.2 was determined using extinction coefficient ε_{630} = 3700 $M^{-1}\cdot cm^{-1}$ [10].

The oxoferryl form of hemoglobin was obtained by adding 100-1000-fold molar excess of hydrogen peroxide to methemoglobin. After adding the H_2O_2, the Soret band of methemoglobin with a maximum at 407 nm (ε_{407} = 190,000 $M^{-1}\cdot cm^{-1}$) was shifted towards 418 nm (ε_{418} = 110,000 $M^{-1}\cdot cm^{-1}$, pH 7.5). The concentrations of methemoglobin and the oxoferryl forms of hemoglobin were determined by decomposition of the absorption spectra of the mixture into individual spectra using the least squares method. The absorption spectra of methemoglobin and the oxoferryl form of hemoglobin were used as standards.

Dityrosine was prepared by adding 1 μM methemoglobin and 2 mM hydrogen peroxide to 2 mM tyrosine. The reaction was run in 0.05 M phosphate buffer, pH 7.5. Hydrogen peroxide was added after a 30-min incubation of the reaction mixture; the sample was incubated further at room temperature. Hydrogen peroxide concentration was determined spectrophotometrically using the extinction coefficient at 240 nm (ε_{240} = 39.4 $M^{-1}\cdot cm^{-1}$) [11].

Dityrosine formation was monitored spectrophotometrically (ε_{315} = 5000 $M^{-1}\cdot cm^{-1}$, pH 7.5) [12] and using a fluorescence method. Fluorescence was excited at 315 nm; fluorescence intensity was measured at 410 nm [13]. Tyrosine concentration was determined spectrophotometrically at 277 nm (ε_{277} = 1500 $M^{-1}\cdot cm^{-1}$ [14]) or by measuring fluorescence intensity at 303 nm (λ_{ex} = 280 nm) [15].

For isolation and purification of dityrosine, the methemoglobin, tyrosine, hydrogen peroxide, and dityrosine solutions were loaded onto a Sephadex G-25 column (50 × 1.5 cm) equilibrated with 0.05 M phosphate buffer, pH 7.5. Prior to chromatography residual hydrogen peroxide was removed by adding 0.1 ml catalase (0.5 mg/ml) per 5 ml of the solution. Elution with a phosphate buffer gave three individual non-overlapping peaks corresponding to methemoglobin, tyrosine, and dityrosine, respectively.

Fluorescent products (thiochrome and oxodihydrothiochrome (ODTch)) of thiamine oxidation by hydrogen peroxide in the presence of methemoglobin were identified by ascending paper chromatography in *n*-butanol–ethanol–water mixture (2 : 1 : 1) [16]. At pH 7.5, thiochrome and oxodihydrothiochrome have absorption maxima at 365 and 340 nm, respectively; the corresponding fluorescence maxima are at 450 and 440 nm [16].

Thiochrome and oxodihydrothiochrome concentrations were measured by the fluorescence method. Taking into account considerable overlapping of the emission spectra of thiochrome and oxodihydrothiochrome, fluorescence spectrum of their mixture was decomposed into individual spectra using the least squares method. Commercial thiochrome (Sigma) and oxodihydrothiochrome [17] were used as standards. Thiamine disulfide concentration was determined after incubation with excess of glutathione and subsequent oxidation of thiamine formed [18].

Absorption spectra in the UV and visible regions were recorded on a Specord M40 spectrophotometer (Carl Zeiss, Germany); fluorescence spectra were measured on an SFL 1211a spectrofluorimeter (Belarus').

RESULTS

Thiamine inhibits dityrosine formation in a reaction catalyzed by oxoferryl forms of hemoglobin. Mixing of methemoglobin with hydrogen peroxide yields the oxoferryl form of hemoglobin, which can be easily detected spectrophotometrically [19, 20]. Previous studies demonstrated that the solution contains two oxoferryl forms of hemoglobin: an oxoferryl form with a radical on the protein globule and an oxoferryl hemoglobin cation. These forms have also been detected in human blood [21].

The oxoferryl forms of hemoglobin are characterized by the presence of additional maxima in the absorption

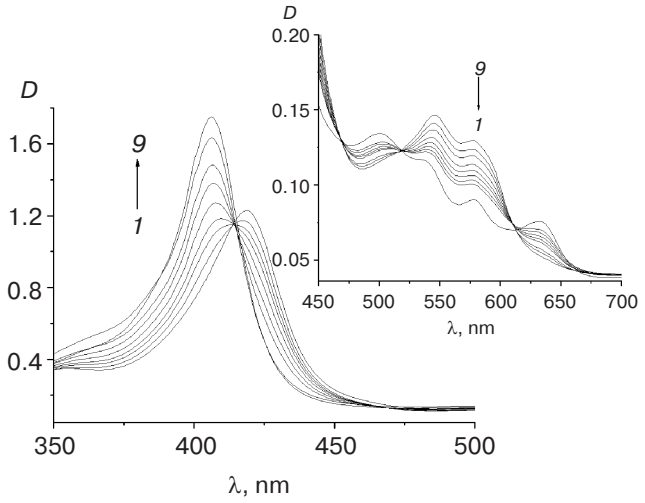

Fig. 2. Absorption spectra of free methemoglobin (*9*) and methemoglobin–hydrogen peroxide mixture after different incubation periods (*1-8*). In the latter case, the absorption spectra were recorded with 2.5-min intervals. Absorption spectrum *8* was recorded for the methemoglobin–hydrogen peroxide mixture after its 12-h preincubation. Concentrations: methemoglobin, $1.2 \cdot 10^{-5}$ M; hydrogen peroxide, 10^{-2} M; 0.05 M sodium phosphate buffer, pH 7.4.

spectra at 548 and 582 nm as well as by a decrease in absorption at 630 nm (Fig. 2). In the case of the oxoferryl form, the Soret band of methemoglobin with a maximum at 405 nm was shifted to 418 nm.

The absorption spectra were characterized by the presence of distinct isosbestic points suggesting the presence of two spectrally different forms of hemoglobin in equilibrium in the solution. At any time, the overall spectrum displayed the presence of two components with absorption bands at 407 nm (methemoglobin) and 418 nm (oxoferryl forms of hemoglobin) suggesting that the absorption spectra of oxoferryl hemoglobin with a radical on the protein globule $^{+}$Hb(IV=O) (compound I) and oxoferryl hemoglobin Hb(IV=O) (compound II) are similar.

Figure 2 shows that in the presence of 1000-fold excess of hydrogen peroxide, the oxoferryl form is reduced to methemoglobin without any protein denaturation (or aggregation) or hemichrome formation.

The formation of the oxoferryl form of hemoglobin occurs at a very fast rate and is complete within several minutes. The shape of the kinetic curve suggests that hydrogen peroxide-induced reduction of the oxoferryl form of hemoglobin proceeds in two stages—a fast stage (~20 min incubation) and a slow stage (Fig. 2, curves *7* and *8*; Fig. 3, curve *1*).

EPR studies established that the yields of myoglobin compound I are the highest at the high concentrations of the hemoprotein or in the presence of excess hydrogen peroxide. Myoglobin compound I is unstable and is rapidly (within ~30 min) decomposed at equimolar hemoprotein/hydrogen peroxide ratio [22].

The rate of methemoglobin formation was the highest during the first 15-20 min, but decreased in later periods (Figs. 2 and 3 (curve *1*)). We suppose that during this 15-20 min the unstable oxoferryl form with a radical on the protein globule is converted first into compound II and then into methemoglobin.

Incubation of tyrosine with methemoglobin and hydrogen peroxide yielded dityrosine that possessed intense fluorescence with maximum at 410 nm. The long-wavelength absorption maximum of dityrosine at neutral pH was at 315 nm. The oxoferryl form of hemoglobin oxidized tyrosine to tyrosyl radicals; their interaction yielded dityrosine (Fig. 1). The fluorescence characteristics of dityrosine differ significantly from those of tyrosine. In neutral aqueous solutions, tyrosine absorbs at 277 nm; its fluorescence has a maximum at 303 nm [15], which enables easy detection of small dityrosine concentrations in the presence of high tyrosine concentrations.

In the presence of tyrosine, the oxoferryl forms of hemoglobin were rapidly reduced to methemoglobin (Fig. 3). Under these conditions, the changes in the kinetics of methemoglobin formation induced by decomposition of oxoferryl forms of hemoglobin and the kinetics of dityrosine formation established by the fluorescence method were nearly symbatic (Figs. 3 and 4).

Tyrosine and dityrosine were separated into individual components by gel filtration. Solution of methemoglobin, tyrosine, and hydrogen peroxide after preliminary incubation was loaded onto a Sephadex G-25 column

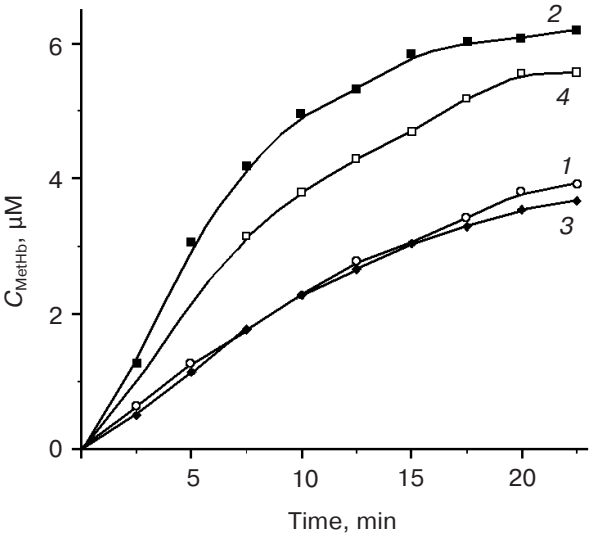

Fig. 3. Kinetics of methemoglobin formation induced by decomposition of oxoferryl forms of hemoglobin by hydrogen peroxide (*1*), D-tyrosine (*2*), D,L-histidine (*3*), and thiamine (*4*) in 0.05 M potassium phosphate buffer, pH 7.4. Concentrations: methemoglobin, $1.12 \cdot 10^{-5}$ M; D-tyrosine, D,L-histidine, and thiamine, $1.0 \cdot 10^{-3}$ M; hydrogen peroxide, $1.75 \cdot 10^{-2}$ M.

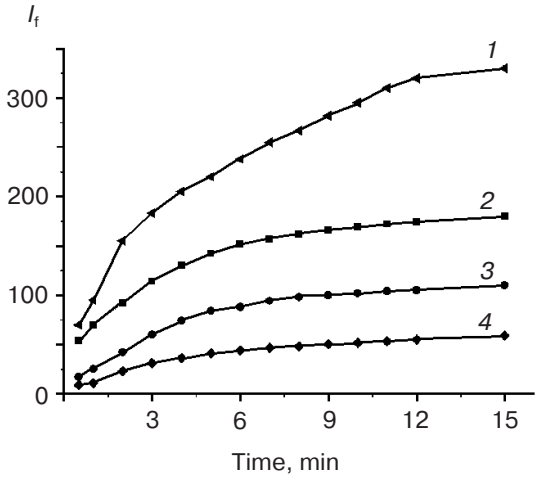

Fig. 4. Kinetics of dityrosine formation after incubation of D-tyrosine with oxoferryl forms of hemoglobin in the presence (*2-4*) and in the absence (*1*) of thiamine in 0.05 M potassium phosphate buffer, pH 7.4. Concentrations: D-tyrosine, 10^{-3} M; hemoglobin, 10^{-6} M; hydrogen peroxide, 10^{-3} M; thiamine, 10^{-3} (*2*), $2 \cdot 10^{-3}$ (*3*), and $5 \cdot 10^{-3}$ M (*4*).

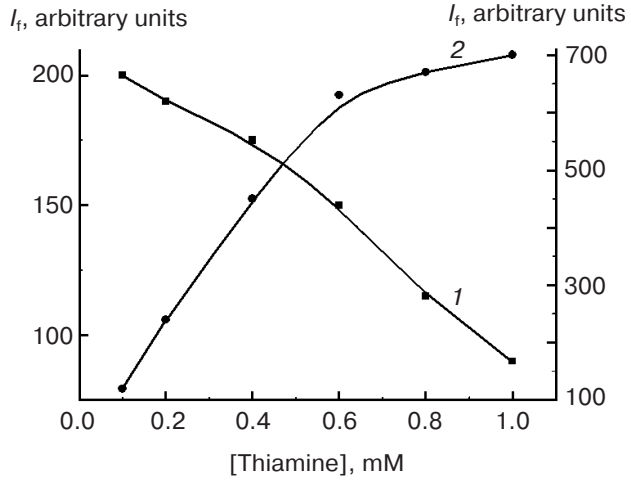

Fig. 5. Inhibition of dityrosine formation (*1*, left ordinate axis) and stimulation of formation of thiamine oxidation products (*2*, right ordinate axis) with increase in thiamine concentration in aqueous solutions containing methemoglobin, D-tyrosine, and hydrogen peroxide. Incubation time, 15 min. The 0.05 M potassium phosphate buffer, pH 7.5, contained methemoglobin (1 μM), tyrosine (1 mM), and hydrogen peroxide (1 mM).

equilibrated with 0.05 M phosphate buffer (pH 7.5). After elution with the same buffer, the samples left the column as three independent non-overlapping peaks corresponding to methemoglobin, tyrosine, and dityrosine. Separation of tyrosine and dityrosine was probably due to stronger binding of dextran to aromatic rings of dityrosine.

Tyrosine and dityrosine peaks were identified by their fluorescence. Dityrosine fluorescence was measured with λ_{ex} = 315 nm and λ_{em} = 410 nm. Thiochrome (thiamine oxidation product) fluorescence was measured with λ_{ex} = 365 nm and λ_{em} = 450 nm.

Figure 5 shows that thiamine inhibited dityrosine formation judging from the decrease in the fluorescence intensity of dityrosine in the presence of thiamine. At thiamine concentration equal to that of tyrosine, the yield of dityrosine was decreased nearly twofold (Fig. 5 and Table 1).

At high thiamine concentrations, dityrosine was not formed, but the concentrations of thiamine disulfide, thiochrome, and oxodihydrothiochrome in solution increased (data from paper chromatography). The long-wavelength bands in the absorption spectra of thiochrome and oxodihydrothiochrome were recorded at 365 and 340 nm, respectively.

We suggest that the tricyclic form of thiamine (whose content increased with an increase in pH) was oxidized to thiochrome by tyrosyl radicals. Thiochrome is not the final product of thiamine oxidation. Tyrosyl radicals further oxidized thiochrome to oxodihydrothiochrome (Fig. 6, a and b). The thiol form of thiamine was oxidized to thiamine disulfide by tyrosyl radicals (Fig. 6c).

The formation of thiamine disulfide, thiochrome, and oxodihydrothiochrome in methemoglobin-, thiamine-, and hydrogen peroxide-containing solutions took place in alkaline media. It is noteworthy that the fluorescence of oxidized thiamine products was dominated by thiochrome. The oxodihydrothiochrome content did not exceed 5% of the total thiochrome content (the reaction mixture contained thiamine, methemoglobin, tyrosine, and hydrogen peroxide); the oxodihydrothiochrome content was low and is not listed in Table 1.

According to the Henderson–Hasselbalch equation, the concentration of the thiol form of thiamine at pH 7.5 was 2.0% of the total thiamine content. According to our calculations, at pH 7.5 the tricyclic form of thiamine also constituted ~2% of the total thiamine content in the solution.

Table 1. Yields of dityrosine, thiamine disulfide, and thiochrome (μM) after incubation of, respectively, tyrosine (Tyr) or thiamine (T) (and also a thiamine–tyrosine mixture) with methemoglobin and hydrogen peroxide

Composition of the solution	Dityrosine	Thiochrome	Thiamine disulfide
[Hb(III)] + Tyr + H_2O_2	2.50	–	–
[Hb(III)] + Tyr + T + H_2O_2	1.0	0.72	0.81
[Hb(III)] + T + H_2O_2		0.12	0.14

Note: Concentrations: methemoglobin, 1 μM; tyrosine, thiamine, and hydrogen peroxide, 1 mM. Incubation time, 15 min.

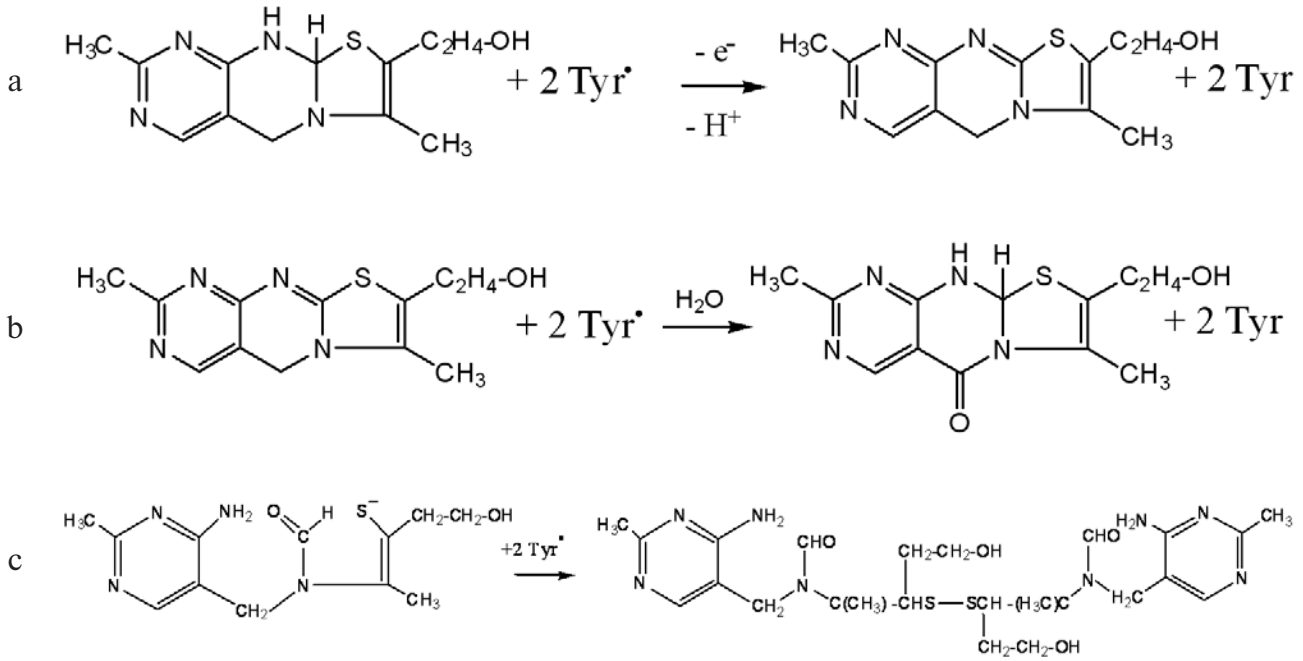

Fig. 6. Oxidation of the tricyclic form of thiamine to thiochrome (a), of thiochrome to oxodihydrothiochrome (b), and oxidation of the thiol form of thiamine to thiamine disulfide (c) induced by tyrosine radicals.

The concentration ratio of the reaction products in concurrently proceeding reactions correlates with the ratio of their rate constants:

$$\frac{k_{\text{Tyr–Tyr}}}{k_{\text{Tch}}} = \frac{[\text{Tyr}^{\bullet}][\text{T}_{\text{tric}}]}{[\text{Tyr}^{\bullet}][\text{Tyr}^{\bullet}]} = \frac{[\text{Tyr–Tyr}]}{[\text{Tch}]}. \qquad (1)$$

The concentrations of dityrosine, thiochrome, and thiamine disulfide are listed in Table 1. The rate constant of the reaction between tyrosine radicals ($k_{\text{Tyr–Tyr}}$) is $4.5 \cdot 10^8$ $M^{-1} \cdot sec^{-1}$ [23]. According to our estimates, the value of rate constant for the interaction between tyrosine radicals and the tricyclic form of thiamine is ~$3.0 \cdot 10^4$ $M^{-1} \cdot sec^{-1}$.

Oxoferryl forms of hemoglobin have peroxidase activity and are concomitant with oxidation of thiamine and its derivatives. Addition of thiamine to the oxoferryl form of hemoglobin increased the rate of its reduction to methemoglobin (Fig. 3). The decomposition of the oxoferryl form of hemoglobin was accompanied by oxidation of thiamine and its derivatives (Table 2). Thiamine derivatives able to induce effective reduction of the oxoferryl form of hemoglobin to methemoglobin were characterized by the highest yields of the fluorescent products.

The absorption spectra of the thiamine–methemoglobin–hydrogen peroxide mixture manifested an additional absorption band at 370 nm, which corresponded to thiochrome. However, the yield of thiochrome was not high and the measurement of its concentration by spec-

Table 2. Formation of fluorescent products after incubation of thiamine and its derivatives with methemoglobin and hydrogen peroxide

Thiamine and its derivatives	R_1	R_2	R_3	Yield of fluorescent products, %
Thiamine	CH_3	NH_2	C_2H_4OH	100
TMP	CH_3	NH_2	$C_2H_4OPO_4$	29.5
TDP	CH_3	NH_2	$C_2H_4O(PO_4)_2$	20.8
2'-Ethylthiamine	C_2H_5	NH_2	C_2H_4OH	130

Note: Concentrations: methemoglobin, 1 μM; thiamine and its derivatives, 1 mM; hydrogen peroxide, 1 mM. Yield of thiamine fluorescent products was taken as 100%.

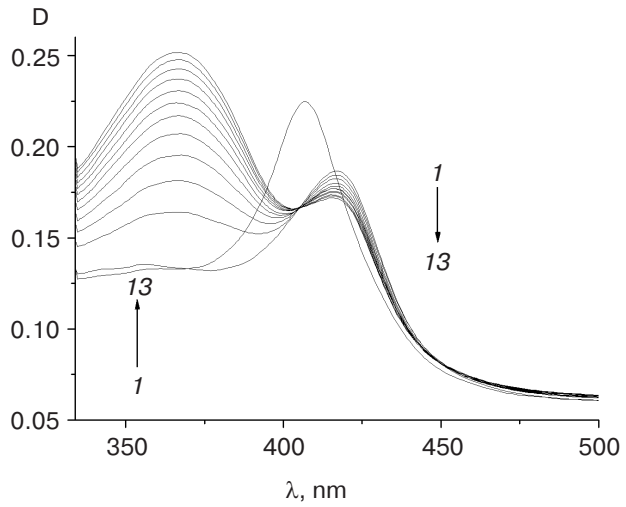

Fig. 7. Time-dependent changes in the absorption spectra of methemoglobin, thiamine, L-tyrosine, and hydrogen peroxide solutions. The interval between recordings of absorption spectra was 2 min. Concentrations: methemoglobin, 1 μM; thiamine, 1 mM; tyrosine, 1 mM; hydrogen peroxide, 1 mM; 0.05 M potassium phosphate buffer, pH 7.5.

trophotometric methods was difficult. But thiochrome formation can be easily followed by fluorescence methods. The fluorescence intensity and, as a consequence, the yields of fluorescent products increased with an increase in the concentration of thiamine or its derivatives in the solution.

Thiamine phosphate esters were oxidized by oxoferryl forms of hemoglobin at lower rates than thiamine. The yields of the fluorescent products after incubation of solutions containing methemoglobin, thiamine (or its derivatives), and hydrogen peroxide were also low (Table 2).

In our opinion, this might be due to lower content of the tricyclic form of thiamine phosphate esters in neutral media. The concentration of the tricyclic form of thiamine increased symbatically with thiol concentration. The pK_a values for the thiol forms of thiamine, TMP, and TDP were 9.2, 9.5 and 9.7, respectively. Separation of fluorescent thiamine oxidation products by ascending paper chromatography demonstrated that thiochrome and oxodihydrothiochrome were responsible for the fluorescence. Similar thiamine oxidation products were formed after incubation of thiamine with oxoferryl forms of hemoglobin in the presence of tyrosine. The yield of thiamine oxidation products markedly increased under these conditions (Figs. 7 and 8).

Tyrosine accelerates thiamine oxidation catalyzed by oxoferryl forms of hemoglobin. The yield of thiamine oxidation products increased after addition of L- or D-tyrosine to solutions containing thiamine and the oxoferryl form of hemoglobin (Fig. 7). In the presence of high (≥1 mM) L-tyrosine concentrations, thiochrome yield increased several-fold in comparison with L-tyrosine-free solutions as a result of oxidation of thiamine by the oxoferryl forms of hemoglobin (Fig. 8).

As can be seen from Fig. 7, in the presence of L-tyrosine the formation of thiochrome can be easily monitored spectrophotometrically. The fluorescence intensities of thiamine oxidation products increased linearly with increase in the L-tyrosine concentration in the incubation medium. At 2 mM L-tyrosine, the yield of the fluorescent thiamine oxidation products (predominantly thiochrome) increased by one order of magnitude in comparison with that in the absence of tyrosine (Fig. 8). However, after 20-min incubation of a solution containing methemoglobin, thiamine, and hydrogen peroxide and subsequent addition of tyrosine to the incubation mixture, the increase in the yields of the fluorescent products was insignificant (Table 3). Previous studies based on the use of the stop-flow technique revealed that myoglobin compound I reacts with tyrosine and other low molecular weight compounds with rate constants exceeding those for compound II by 10-50-fold. Under these conditions, myoglobin compound I is converted into the less reactive compound II as a result of its interaction with low molecular weight ligands [4]. The addition of tyrosine to a solution of methemoglobin, thiamine, and hydrogen peroxide after a 20-min preincubation gave only 1.0 μM thiochrome (cf. 0.6 μM in the absence of tyrosine) (Table 3). Consequently, tyrosine added to the solution after 20-min preincubation increased thiochrome yields by 0.4 μM. If tyrosine was added early in the reaction, the

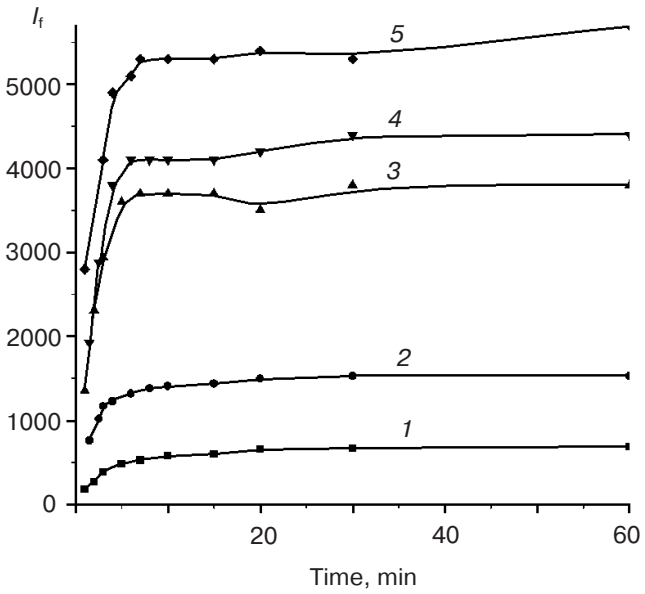

Fig. 8. Thiamine-induced oxidation of the oxoferryl form of hemoglobin to thiochrome in the absence (*1*) and in the presence of L-tyrosine (*2-5*). Concentrations: methemoglobin, 10^{-5} M; thiamine, 10^{-3} M; hydrogen peroxide, $5\cdot10^{-3}$ M; L-tyrosine, 10^{-4} (*2*), $5\cdot10^{-4}$ (*3*), 10^{-3} (*4*), and $2\cdot10^{-3}$ M (*5*). MetHb concentration was calculated per monomer basis.

Table 3. Dependence of thiochrome yields on the time of tyrosine addition to the incubation mixture containing methemoglobin, thiamine, and hydrogen peroxide

Time of incubation of solution A before addition of tyrosine	Yield of thiochrome (μM) 20 min after addition of L-Tyr to solution A	Yield of thiochrome (μM) after 20-min incubation of solution A
[A + L-Tyr] (all reaction components were mixed simultaneously)	5.5	–
A → 5' + L-Tyr	2.5	–
A → 10' + L-Tyr	2.0	–
A → 15' + L-Tyr	1.5	–
A → 20' + L-Tyr	1.0	–
A → 20'	–	0.6

Note: Concentrations: methemoglobin, 10 μM; thiamine and L-tyrosine, 1 mM; hydrogen peroxide, 2 mM; 0.05 M phosphate buffer, pH 7.5. A = Hb(III) + T + H_2O_2, the aqueous solution containing methemoglobin, thiamine, and hydrogen peroxide.

increment in thiochrome content was 4.9 μM (Table 3) suggesting that after 20 min the rate of generation of tyrosyl radicals responsible for oxidation of thiamine to thiochrome was sharply decreased. This finding prompts the conclusion that after 20-min incubation of the methemoglobin–thiamine–hydrogen peroxide mixture, the experimental solution predominantly contains a less reactive hemoglobin compound II.

Similarly to tyrosine, after incubation with methemoglobin and hydrogen peroxide, tyramine increases the yields of thiamine oxidation products by one order of magnitude. D,L-Tryptophan also increases thiochrome yields, but to a lesser degree in comparison with tyrosine or tyramine. In contrast, L-cysteine inhibits thiamine oxidation by oxoferryl forms of hemoglobin. Other amino acids, e.g. D,L-arginine, D,L-histidine, L-proline, L-serine, D-aspartic acid, D,L-threonine, D,L-lysine, D,L-valine, D,L-phenylalanine, etc., have virtually no effect on the yields of thiamine oxidation products.

DISCUSSION

The reaction between methemoglobin and hydrogen peroxide yields an oxoferryl form of hemoglobin (compound I) and water molecule (reaction (2)) [22]. The second electron required for hydrogen peroxide reduction is donated by the porphyrin ring, which rapidly oxidizes one or several amino acid residues to give ‡Hb(IV=O), i.e. compound I [20, 22, 24]:

$$Hb(III) + H_2O_2 \rightarrow {}^{\ddagger}Hb(IV{=}O) + H_2O. \qquad (2)$$

EPR studies established that the oxoferryl form of hemoglobin (compound I) yields long-living tyrosyl radicals on the protein globule [22]. Phenylalanine 43 (CD1), histidine E7, and valine E11 form a part of the amino acid environment of the distal side of the heme [25].

Tyrosine 42 of the α-chain is in close proximity to the heme and therefore can donate electron to the porphyrin radical, giving rise to tyrosyl radical. Charge transfer takes place between tyrosyl radicals and other amino acid residues located on the protein globule [24]. Therefore, localization of the radical on the tyrosyl residue is theoretically possible, but can be realized only in early steps of the reaction.

Compound I can be schematically represented as an equilibrium mixture of two short-living interconvertible compounds with a radical initially localized on the porphyrin molecule or the tyrosyl residue. The formation of the oxoferryl form of hemoglobin (compound I) is complete within 1-1.5 min and is followed by its reduction to methemoglobin (Figs. 2 and 3).

In the presence of a large excess of hydrogen peroxide, the oxoferryl form of hemoglobin with a radical on the protein globule is formed in low yields and is rapidly decomposed, most probably as a result of a reverse reaction between the free-radical oxoferryl form of hemoglobin and hydrogen peroxide:

$$^{\ddagger}Hb(IV{=}O) + H_2O_2 \rightarrow Hb(III) + O_2. \qquad (3)$$

Moreover, a one-electron reaction of the oxoferryl form of hemoglobin with a radical on the protein globule (compound I) leads to hydrogen peroxide-induced reduction to compound II:

$$^{\ddagger}Hb(IV{=}O) + H_2O_2 \rightarrow Hb(IV{=}O) + HO_2^{\cdot}. \qquad (4)$$

The oxoferryl form of hemoglobin Hb(IV=O) (compound II) thus formed is slowly reduced to methemoglobin by hydrogen peroxide (slow stage):

$$Hb(IV{=}O) + H_2O_2 \rightarrow Hb(III) + HO_2^{\cdot}, \qquad (5)$$

$$Hb(IV{=}O) + HO_2^{\cdot} \rightarrow Hb(III) + O_2. \qquad (6)$$

Indeed, the shapes of the kinetic curves suggest that hydrogen peroxide-induced reduction of the oxoferryl form of hemoglobin to methemoglobin includes a fast and a slow stages (Figs. 2 and 3). Early in the reaction (≤15-20 min), the rate of methemoglobin formation is the highest, but it decreases gradually later.

Tyrosine oxidation to dityrosine is especially intensive during the first 20 min (Fig. 4) when the solution contains large amounts of the free-radical oxoferryl form of hemoglobin. The curve describing dityrosine yields (Fig. 4) is consistent with the kinetics of reduction of the oxoferryl form of hemoglobin to methemoglobin under the action of tyrosine (Fig. 3).

L-Tyrosine is known to react rapidly with the tyrosyl radicals of the protein or other radicals localized on the protein globule of the oxoferryl form of the hemoprotein (e.g. the rate constant for the reaction between the free-radical form of myoglobin and tyrosine is 600 $M^{-1}\cdot sec^{-1}$, whereas the corresponding value for the reaction between the oxoferryl cation and tyrosine is as low as 40 $M^{-1}\cdot sec^{-1}$) [4]. This process can schematically be presented as follows:

$$^{\ddagger}Hb(IV{=}O) + L\text{-}Tyr \rightarrow L\text{-}^{\bullet}Tyr + Hb(IV{=}O), \quad (7)$$

$$Hb(IV{=}O) + L\text{-}Tyr \rightarrow L\text{-}^{\bullet}Tyr + Hb(III), \quad (8)$$

where $^{\bullet}$Tyr is a tyrosine radical.

The fast reaction between tyrosine radicals yields dityrosine (the rate constant for this reaction is $4.5\cdot10^{8}$ $M^{-1}\cdot sec^{-1}$ [23]):

$$2\,^{\bullet}Tyr \rightarrow Tyr{-}Tyr. \quad (9)$$

The rate of dityrosine formation is the highest in methemoglobin- and hydrogen peroxide-containing solutions, especially during the first 15-20 min of incubation (Fig. 4, curve *1*). Preincubation of the methemoglobin–hydrogen peroxide mixture for 15-20 min and subsequent addition of tyrosine decelerates dityrosine formation (data not shown).

These findings suggest that under the given experimental conditions (15-20-min incubation), the solution predominantly contains a less reactive hemoglobin compound II, whereas the content of the oxoferryl form with a radical on the protein globule is insignificant. It was assumed that during the first 15-20 min the oxoferryl form of hemoglobin with a radical on the protein globule (compound I) is reduced to compound II by hydrogen peroxide (reaction (4)) [23] and tyrosine (reaction (7)). Tyrosine oxidation after 20-min preincubation proceeds at a slow rate and is induced by less reactive hemoglobin compound II (reaction (8)).

Thiamine inhibits dityrosine formation (Fig. 5). At high thiamine concentrations, dityrosine is not formed but the concentrations of thiochrome and oxodihydrothiochrome in the solution increases (Fig. 6).

In the presence of thiamine used at concentrations equimolar to those of tyrosine, the yield of dityrosine decreases by ~50% (Fig. 5). In contrast, in the presence of tyrosine the yields of fluorescent thiamine oxidation products increase by approximately one order of magnitude (Fig. 5 and Table 1). Lower dityrosine yields can be attributed to competitive reactions between tyrosine radicals and minor forms of thiamine, viz., thiol and tricyclic forms (Fig. 6).

Many antioxidants, e.g. glutathione, S-nitrosoglutathione, and dinitrosyl iron complexes with glutathione, reduce oxoferrylmyoglobin formed upon interaction of *tert*-butyl hydroperoxide with metmyoglobin [26]. Thiamine and its derivatives also decompose the oxoferryl form of hemoglobin which is accompanied by oxidation of thiamine and its derivatives (Fig. 5 and Table 2).

Oxidation of thiamine to thiochrome is described by the set of equations (reactions (10)-(13)). Thiamine reacts with free radicals on the protein globule (porphyrin or tyrosyl radicals) [24]:

$$^{\ddagger}Hb(IV{=}O) + T \rightarrow {}^{\ddagger}T + Hb(IV{=}O), \quad (10)$$

$$^{\ddagger}Hb(IV{=}O) + {}^{\ddagger}T \rightarrow Tch + Hb(IV{=}O). \quad (11)$$

Indeed, the rate of thiochrome formation is the highest in the initial period of the reaction (Fig. 8, curve *1*) when the solution contains compound I.

Thiamine also reacts with the oxoferryl cation in compound II to give methemoglobin. This reaction is easily monitored spectrophotometrically; however, after 20-min incubation it proceeds at a low rate (Fig. 3):

$$Hb(IV{=}O) + T \rightarrow {}^{\ddagger}T + Hb(III) + H_2O, \quad (12)$$

$$Hb(IV{=}O) + {}^{\ddagger}T \rightarrow Hb(III) + Tch + H_2O. \quad (13)$$

Thiochrome is also formed in the course of a disproportionation reaction between free radicals of thiamine:

$$2\,{}^{\ddagger}T \rightarrow Tch + T. \quad (14)$$

A small amount of thiochrome is oxidized to dihydrothiochrome, presumably under the effect of free tyrosyl radicals localized on the protein globule. The addition of tyrosine to thiamine-, methemoglobin-, and hydrogen peroxide-containing solutions in early steps of the reaction sharply increases thiochrome yields (Figs. 7 and 8).

In our opinion, long-living free radicals localized on protein globules are more accessible for the free amino acid L-tyrosine than for thiamine. Reaction (7) takes place between the radicals localized on the protein globule and L-tyrosine.

The free L-tyrosine radicals thus formed oxidize thiamine to thiochrome:

$$L\text{-}^{\bullet}Tyr + T \rightarrow L\text{-}Tyr + {}^{\ddagger}T, \quad (15)$$

$$L\text{-}^{\bullet}Tyr + {}^{\ddagger}T \rightarrow L\text{-}Tyr + Tch, \quad (16)$$

where T is thiamine and ‡T is a thiamine radical. These data suggest that in neutral and alkaline media tyrosyl

radicals oxidize thiamine to thiochrome, oxodihydrothiochrome, and thiamine disulfide (Fig. 6).

A scheme for the interaction of oxoferryl forms of hemoglobin (compounds I and II) with tyrosine and thiamine is given in Fig. 9.

Addition of tyrosine after 15-20-min preincubation of the reaction mixture (thiamine, methemoglobin, hydrogen peroxide) only slightly increases thiochrome yields (Table 3). This finding led us to suggest that in 15-20 min of the experiment, the solution contains predominantly compound II, while the concentration of the oxoferryl form with a radical on the protein globule (compound I) is low. The value of the rate constant for the interaction of tyrosine with the compound II oxoferryl cation (reaction (7)) is much lower than that for the radicals localized on the protein globule (compound I) (reaction (6)) (40 and 600 $M^{-1}\cdot sec^{-1}$ [4], respectively).

These data clearly demonstrate that thiamine and its phosphate esters are oxidized by radicals located on tyrosyl residues of the protein (heme porphyrin) or via the interaction between thiamine and its phosphate esters with the oxoferryl cation of the heme. Dityrosine is also formed as a result of interaction between tyrosine and compounds I and II.

It is noteworthy that in dilute methemoglobin solutions (≤1 μM) tetrameric hemoglobin molecules dissociate into dimers. In 0.1 M sodium phosphate buffer, the value of the tetramer–dimer dissociation constant for oxyhemoglobin is $2.5\cdot10^{-6}$ M [27], suggesting that at ≤1 μM methemoglobin main part of the protein exists as the dissociated dimeric form.

The stability of tyrosyl radicals located on the protein globule and, as a consequence, their reactivity depends on their amino acid environment, which changes during dissociation of the tetrameric molecule. The yields of the oxidation products depend on H_2O_2 and hemoglobin concentrations. High (>2 mM) concentrations of hydrogen peroxide also inhibit dityrosine formation in reactions of oxoferryl forms of hemoglobin with tyrosine. High concentrations of hydrogen peroxide also inhibit methemoglobin-catalyzed oxidation of thiamine. In our opinion, the inhibiting effect of hydrogen peroxide on thiamine and tyrosine oxidation is due to decomposition of compounds I and II (reactions (3)-(6)). Moreover, high concentrations of hydrogen peroxide induce the degradation of hemin and the opening of the porphyrin ring with the concomitant disappearance of absorption in the Soret band [28]. Under our experimental conditions, methemoglobin (≥10 μM calculated per heme) was insensitive to the effect of hydrogen peroxide (Fig. 2). In contrast, in dilute solutions (1 μM) characterized by dissociation into dimeric forms the resistance of hemin was sharply decreased in the presence of hydrogen peroxide with simultaneous decrease in absorption in the Soret band (Fig. 7).

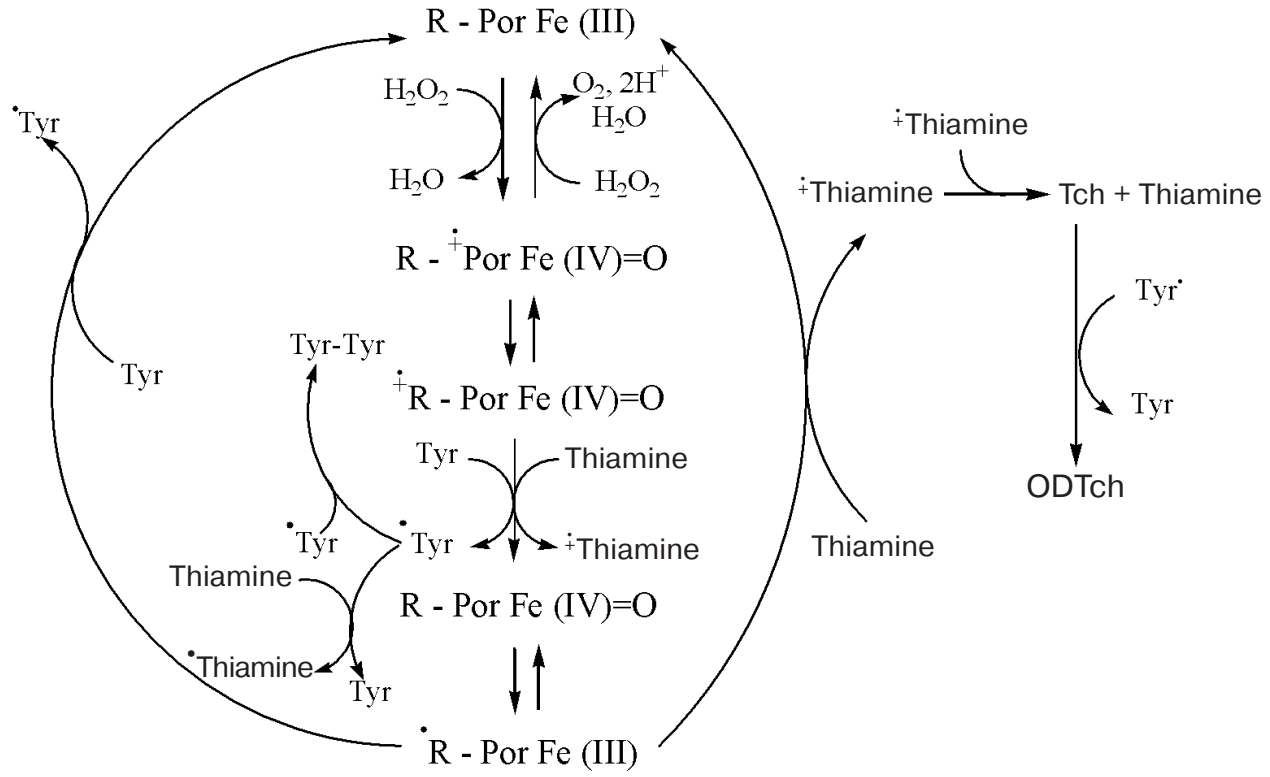

Fig. 9. Reactions between oxoferryl forms of hemoglobin (compounds I and II) with thiamine in the presence of tyrosine. Tch, thiochrome; ODTch, oxodihydrothiochrome; $^{+\bullet}$T, thiamine free radical; $^{\bullet}$Tyr, L- or D-tyrosine free radical; Tyr–Tyr, dityrosine.

TDP is a cofactor for key enzymes of energy metabolism, e.g. pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. The former catalyzes the oxidative decarboxylation of pyruvate formed from glucose via the Embden–Meyerhof cycle. TDP is a cofactor of transketolase, one of the key enzymes of the pentose phosphate cycle [29]. In addition to TDP, human tissues always contain non-coenzyme derivatives of thiamine. The relative percent content of thiamine and its phosphate esters is as follows: thiamine (10-12%), TMP (10-15%), TDP (70-75%), TTP (2-3%) [30]. In addition to thiamine phosphates, animal tissues always contain thiamine disulfide and thiochrome [17].

Apart from data on major mechanisms of TDP-catalyzed reactions, there is an increasing body of evidence on high biological activity of non-coenzymatic thiamine metabolites. Recent studies showed that the thiol form of thiamine stimulates nitric oxide release from S-nitrosoglutathione, resulting in vascular relaxation [18].

Thiamine prevents neuronal injuries under conditions of oxidative stress [31]. Moreover, thiamines (especially benzoylthiamine) prevent vascular injuries in patients with diabetes mellitus and nephropathies, diminishes the content of toxic products of nonenzymatic glycosylation of proteins, etc. [32, 33].

Protective properties of thiamine are not confined to its coenzymatic activity or effect on the metabolism of α-keto acids and carbonyl compounds. Thiamine and its phosphate esters suppress the formation of protein glycosylation products *in vitro*. In this case, inhibition of nonenzymatic glycosylation of thiamine proteins occurs in the absence of enzymes [16]. Moreover, thiamine exerts non-coenzymatic, neuroprotective, and cardioprotective effects by inactivating nitrogen dioxide and other reactive nitrogen species. The protective effect of thiamine is realized via formation of hydrophobic thiamine metabolites, e.g. thiochrome and oxodihydrothiochrome. Nervous and cardiac tissues contain the highest amounts of stored thiamine [29].

The interaction with peroxynitrites is one of the most plausible mechanisms underlying protective effects of thiamine. Peroxynitrite effectively induces nitrosylation of tyrosine residues in various enzymes and thus causes their inactivation [2]. The reaction of thiamine with peroxynitrite is accompanied by its degradation and inhibition of nitration of tyrosyl residues of proteins. Oxidative conversions of thiamine yield thiochrome and oxodihydrothiochrome, which appeared to be far more efficient peroxynitrite scavengers than thiamine [16].

Our studies demonstrated that the oxoferryl forms of hemoglobin and tyrosyl radicals effectively oxidize thiamine and its phosphate esters to thiochrome, oxodihydrothiochrome, and thiamine disulfide and their phosphate esters. It can thus be concluded that under oxidative stress content of thiamine decreases due to its transformation in inactive species. Thiamine deficit and oxidative stress provoke neuronal death. Nervous tissue injuries are characterized by augmented synthesis of nitric oxide, tyrosine nitration to form 3-nitrotyrosine under thiamine deficiency [34]. Tyrosine nitration represents a well-coordinated two-step mechanism, which involves capture of a tyrosine hydrogen atom in the presence of oxidants in the first step of the reaction and interaction of tyrosyl radicals with nitrogen dioxide in its second step:

$$^{\bullet}\mathrm{Tyr} + \mathrm{NO_2} \rightarrow \mathrm{NO_2Tyr}. \qquad (17)$$

Decrease in tyrosyl radicals also takes place in a parallel reaction of dityrosine formation (9). Thiamine interacts with tyrosyl radicals (reactions (15) and (16)) and diminishes release of 3-nitrotyrosine and dityrosine (Figs. 5 and 6).

Earlier, it was found that thiamine and especially its hydrophobic derivatives (thiochrome and oxodihydrothiochrome) act as peroxynitrite traps [16] and induce the degradation of toxic oxoferryl forms of hemoproteins [35]. Probably hydrophobic thiamine metabolites fulfill an important function under oxidative and nitrosyl stress by protecting nervous tissue, inhibiting NO-dependent tyrosine nitration, formation of dityrosine and inter-protein tyrosine–tyrosine crosslinks.

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