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NITRATION OF TYROSINE AND TYROSYL RESIDUES IN MYOGLOBIN BY THE ACTION OF VISIBLE LIGHT IN THE PRESENCE OF RIBOFLAVIN AND NITRITE**

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The nitration of tyrosyl residues in proteins is considered a type of post-translational modification of proteins, indicating disruptions in the metabolic and signaling functions of nitric oxide $^{\bullet}$NO and the development of oxidative nitrosative stress. We have examined the nonenzymatic pathway of protein nitration by the action of visible light in the presence of riboflavin and nitrite. Mass spectrometry was used to show that riboflavin-photosensitized redox processes involving nitrite and tyrosine or tyrosyl residues lead to nitration of residues Tyr-103 and Tyr-146 in the polypeptide chain of horse heart myoglobin. A possible role is discussed for riboflavin and other natural photosensitizers in the modification and damage of proteins when the body is exposed to intense visible light in the presence of nitrites in the blood.

Keywords: *tyrosine nitration, nitrotyrosine, riboflavin, myoglobin, dityrosine, reactive oxygen species.*

Introduction. Studies in molecular biology have shown that the human proteome, namely, the set of all the proteins of an individual with a total amount of more than one million proteoforms, is considerably greater than the number of genes coding protein sequences (about 20,000) [1, 2]. This is possible if one and the same gene is used in coding for many proteins. The greater variety of protein forms is achieved by mechanisms such as alternative splicing and post-translational modification [1, 3, 4]. More than 200 types of post-translational modification are now known, most often involving phosphorylation, acetylation, ubiquitination, glycosylation, and S-nitrosylation [3–5].

The nitration of protein tyrosyl residues is considered one of the types of post-translational modification [6] and a marker for oxidative nitrosative stress in an organism due to disruption of the metabolism and signaling function of nitric oxide $^{\bullet}$NO [7, 8]. The nitration is seen in a change in the pK_a value of the phenol group, which can lead to disruption of the folding of the polypeptide chains and destabilization of the protein tertiary structure [9].

A number of mechanisms have been proposed for the nitration of tyrosine and tyrosyl residues in proteins under physiological conditions with formation of peroxynitrite as the intermediate in the reaction of *NO and the superoxide anion [7, 8, 10] or in the oxidation of NO_2^- ions in the presence of H_2O_2/peroxidase [11–15]. The heme-containing proteins myoglobin (Mb) and hemoglobin (Hb) , in addition to their major function, can also display pseudoperoxidase activity and their oxoferryl forms catalyze the oxidation of phenols [11, 16], thiamine [17, 18], and sulfides [19] as well as the nitration of tyrosine (Tyr) [11, 16, 20, 21].

Myoglobin is a monomeric protein containing a single polypeptide chain and one heme. Its concentration, for example, in the heart is ~0.2–0.3 mM. Myoglobin in cardiomyocytes is autoxidized to give superoxide anions and hydrogen peroxide [22–25]. The reaction of metmyoglobin (metMb) with hydrogen peroxide H_2O_2 gives oxoferryl protein forms: $^{+\bullet}$Mb(IV=O) (compound I) and Mb(IV=O) (compound II), which are readily detected spectrometrically [11, 18, 19, 26].

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Under normal physiological conditions, these autoxidation reactions are very slow. However, if cardiomyocytes function under ischemic conditions (low pH, low organ oxygen pressure), the formation of reactive oxygen species (ROS) and oxoferryl forms of Mb is sharply enhanced. The formation of 3-nitrotyrosine presumably can take place during cardiac reperfusion in the absence of peroxynitrite. The oxoferryl forms of Mb oxidize nitrite to give nitrogen dioxide, which then reacts with Tyr to give 3-nitrotyrosine. The mechanism of these reactions holds great interest since nitrated proteins containing 3-nitrotyrosine residues are detected in many pathological states [27].

In addition to processes involving the autoxidation of heme-containing proteins, photochemical reactions can also serve as a source of hydrogen peroxide in organisms. Many endogenic and exogenic photosensitizers [28–30] such as flavins and protoporphyrins can trigger damage to biostructures. The photochemical reactions of sensitizers involving oxygen can, in general, be seen as electron transfer (Type I) or energy transfer processes (Type II) resulting in the formation of hydroperoxyl radicals $HO_2^{\bullet}$ and the superoxide anion $O_2^{\bullet-}$, which convert to H_2O_2 in disproportionation reactions, or singlet oxygen $^1O_2$ [29, 31]. Such reactions are possible for many dye molecules, some drugs, as well as vitamins and can be activated by light, for example, for methyl blue, rose Bengal, and riboflavin [32, 33]. The nitration of tyrosine and tyrosyl residues of proteins can also proceed nonenzymatically (without the participation of enzymes). This process requires the presence of tyrosine or tyrosyl residues and $^{\bullet}NO_2$ free radicals, which can arise in the recombination of 3-nitrotyrosine and 3-nitrotyrosyl [8]. Thus, any oxidation process including photochemical oxidation leading to the formation of $^{\bullet}NO_2$ and tyrosine/tyrosyl free radicals can contribute to the nitration of proteins.

In the present work, the oxidation reactions photosensitized by riboflavin (RF) involving ROS and reactive nitrogen species (RNS) are considered to be responsible for the observed nitration. Riboflavin (also known as vitamin B_2), which has an absorption spectrum with maxima at 374 and 445 nm, is an efficient natural photosensitizer [33, 34]. Upon the photoexcitation of the RF molecule (Fig. 1), intercombinational system conversion (ISC) gives triplet $^3RF^{\bullet}$ in high quantum yield ($\Phi_{ISC} \sim 0.7$) [33–35]. These triplets can rapidly react with dissolved oxygen to give singlet oxygen $^1O_2$ (Type II photosensitization):

$$^3RF^{\bullet} + {}^3O_2 \rightarrow RF + {}^1O_2,\ k = 0.98 \cdot 10^9\ M^{-1} \cdot s^{-1}$$

or with phenol-containing molecules such as tyrosine [36] leading to their oxidation

$$^3RF^{\bullet} + Tyr \rightarrow RF^{\bullet} + Tyr^{\bullet},\ k = 1.4 \cdot 10^9\ M^{-1} \cdot s^{-1}\ .$$

The subsequent reaction of molecular oxygen with the semiquinone form of riboflavin $RF^{\bullet}$ leads to the superoxide anion $O_2^{\bullet-}$.

Riboflavin-sensitized photochemical reactions also proceed *in vivo*. The contents of RF in the skin and ocular fluid are 7.9 and 4.5 μM, respectively [35] and the action of sunlight can initiate riboflavin-photosensitized oxidative processes in the skin and ocular structural components, leading to pathology [34].

In the present work, we studied the riboflavin-photosensitized oxidation of tyrosine and tyrosyl residues of Mb by the action of visible light in the presence of nitrite, identified 3-nitrotyrosyl residues in the polypeptide chain of this macromolecule, and evaluated the contributions of the riboflavin-photosensitized oxidative processes to protein nitration.

Materials and Methods. We used horse heart myoglobin and L,D-tyrosine from Sigma-Aldrich (USA) and 99.5%-pure riboflavin from Chem-Impex (USA). The samples of sodium nitrite, sodium azide, and hydrogen peroxide were produced in Russia and Belarus. The absorption spectra were taken on an Agilent Cary-100 spectrometer (manufactured in the USA). The fluorescence spectra were taken on a Solar CM2203 spectrofluorimeter produced in Belarus. The hydrogen peroxide concentration in solution was determined spectrophotometrically using the molar absorption coefficient at 240 nm ($\varepsilon_{240} = 39.4\ M^{-1} \cdot cm^{-1}$) [37]. The formation of dityrosine was determined by absorption spectroscopy using the molar absorption coefficient $\varepsilon_{315} = 5000\ M^{-1} \cdot cm^{-1}$ (pH 7.5) [38, 39] as well as by a method measuring the fluorescence at 410 nm with excitation at $\lambda_{exc} = 315$ nm [39, 40].

The concentration of 3-nitrotyrosine was determined spectrophotometrically [16]. In acid media, the concentration of 3-nitrotyrosine was determined relative to the absorption at 360 nm using the molar absorption coefficient $\varepsilon_{360} = 2790\ M^{-1} \cdot cm^{-1}$. In alkaline media at pH 9.0, the absorption was measured at 428 nm, $\varepsilon = 4200\ M^{-1} \cdot cm^{-1}$. The concentration of metMb in neutral or slightly acidic media was determined relative to the absorption at 408 and 630 nm ($\varepsilon_{408} = 188{,}000\ M^{-1} \cdot cm^{-1}$, $\varepsilon_{630} = 3900\ M^{-1} \cdot cm^{-1}$) and in alkaline media at pH 9.5 relative to the absorption of the Soret band at 411 nm, ($\varepsilon_{411} = 119{,}000\ M^{-1} \cdot cm^{-1}$) [41]. The concentration of the oxoferryl forms of Mb were determined relative

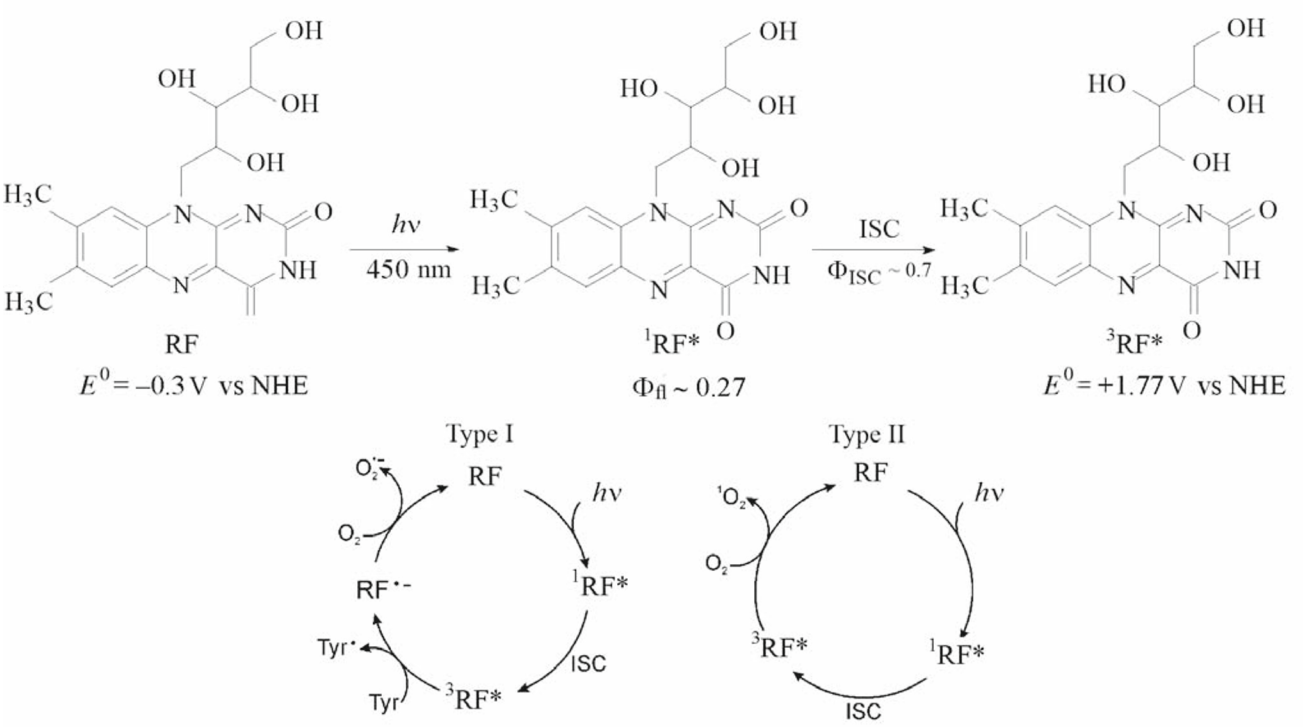

Fig. 1. Scheme for the photoinduced processes involving the riboflavin molecule, adapted from the work of Cardoso et al. [35]: the structure of the RF molecule and transitions to the excited singlet $^1RF^{\bullet}$ and triplet $^3RF^{\bullet}$ states, ISC = intercombinational system conversion (ISC), Φ_{ISC} and Φ_{fl} are the quantum yields of the intercombinational system conversion and fluorescence, E^0 is the standard electrode potential; mechanisms for Type I and Type II photosensitization involving the RF molecule.

to the absorption at 421 nm (ε_{421} = 111,000 $M^{-1}\cdot cm^{-1}$) [42]. The visible light source was a DRK-120 mercury lamp with a ZhS-11 light filter separating out the longwavelength region with λ > 420 nm (transmission coefficient T_{420} = 50%).

The nitration of the tyrosyl residues of Mb in the photochemical reaction was carried out by irradiation of solutions of 20 μM Mb in a mixture of 50 μM RF, 1 mM tyrosine, and 1 mM nitrite in a phosphate buffer with pH 6.5 using the visible light from the mercury lamp in the air. The irradiation time was 60 min. The nitration of Mb in a dark reaction was carried out by incubating 20 μM metMb with 1 mM hydrogen peroxide and 1 mM nitrite for 6 h in the dark.

The protein samples after nitration were subjected to tryptic hydrolysis. The mass spectra of the resultant peptides were recorded after separation on a 2.1 × 100-mm Hypesill Gold C18 reversed phase column (1.9 μm) using the Agilent 1290 chromatographic system equipped with a quadrupole time-of-flight Agilent Q-TOF 6550 detector in the positive ion mode (ESI^+).

Results and Discussion. *Riboflavin-photosensitized formation of dityrosine and 3-nitrotyrosine.* Let us examine the oxidationreduction processes involving the tyrosyl free radical and $^{\bullet}NO_2$ initiated by the irradiation of aqueous solutions of tyrosine and nitrite by visible light in the air in the presence of RF as the photosensitizer. Photoexcitation of aqueous solutions of 0.2 mM tyrosine in the presence of 0.02 mM RF leads to the appearance of a new peak in the absorption spectrum with maximum at 315 nm corresponding to the absorption of dityrosine formed during the photolysis. The formation of dityrosine in the irradiated solution is also indicated by the appearance of a fluorescence band at 410 nm with maximum in the excitation spectrum at 315 nm (Fig. 2). The chromatogram of the photolysis products shows a peak with *m/z* = 361.131 amu corresponding to dityrosine in addition to the peak with *m/z* = 181.074 amu for the starting tyrosine.

The tyrosine concentration decreases with increasing photoirradiation of an aqueous mixture of RF and tyrosine. The tyrosine fluorescence intensity at 305 nm decreases upon photoexcitation with λ_{exc} = 280 nm, while the dityrosine emission intensity at 410 nm increases (Fig. 2). Thus, the irradiation of an aqueous solution of 0.2 mM tyrosine in the presence of 0.02 mM RF for 10 and 20 min leads to a decrease in the tyrosine concentration by 35 and 57%, respectively, and increase in the concentration of the photolysis products.

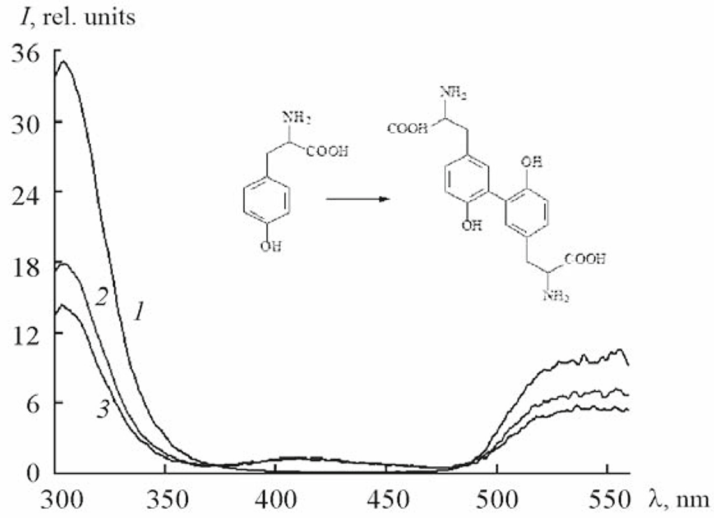

Fig. 2. Fluorescence spectra of aqueous solutions of 0.2 mM tyrosine in a mixture with 0.02 mM RF after visible light irradiation for 0 (1), 10 (2), and 20 min (3); λ_{exc} = 280 nm; the fluorescence bands with maxima at 305, 410, and 550 nm belong to tyrosine, dityrosine, and RF, respectively; the solutions were irradiated with light from a DRK-120 mercury lamp using a ZhS-11 light filter.

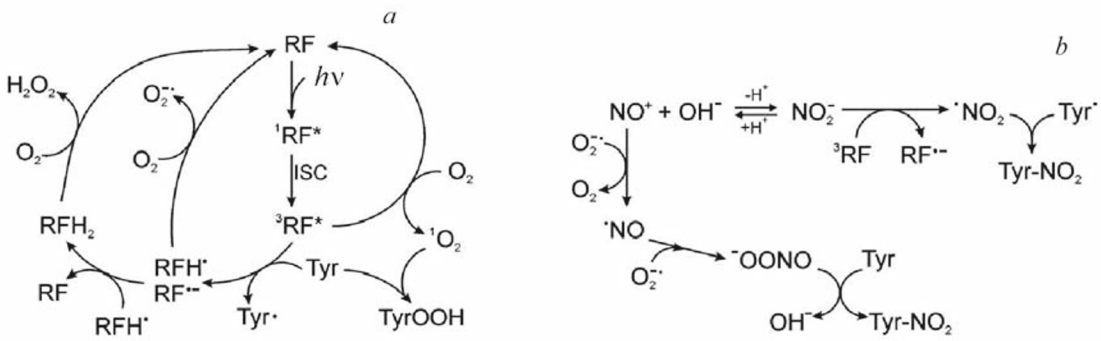

Fig. 3. Proposed scheme for the formation of tyrosine free radicals (a), peroxynitrite and $^\bullet NO_2$ (b) upon the action of visible light on aqueous solutions of tyrosine, nitrite, and riboflavin under aerobic conditions.

The irradiation was carried out in the air and, under the indicated experimental conditions. The concentration of dissolved oxygen in the solution was ~0.25 mM, i.e., RF can act as both Type I and Type II photosensitizers leading to the formation of the products of tyrosine oxidation both upon direct reaction with tyrosine and indirectly through the generation of singlet oxygen $^1O_2$. In addition to singlet oxygen, the photoirradiation of RF can also initiate reactions involving such ROS as H_2O_2, $O_2^{\bullet-}$, $HO_2^\bullet$, and $HO^\bullet$ (Fig. 3, Table 1).

Dimerization of tyrosyl radicals $Tyr^\bullet$ formed in the reaction with an RF molecule in the triplet state ^3RF is considered the major pathway for the formation of dityrosine (Fig. 3) [47]. The reduction redox potential for singlet oxygen $^1O_2$ is insufficient for the oxidation of tyrosine to $Tyr^\bullet$. The reaction of the tyrosine molecule with $^1O_2$ gives tyrosine hydroperoxide TyrOOH as an intermediate with subsequent formation of a series of stable oxidation products [48]. An additional process leading to the formation of tyrosine hydroperoxides is the recombination of the tyrosine radical $Tyr^\bullet$ with the superoxide anion $O_2^{\bullet-}$ [49, 50]. Thus, dityrosine possessing strong fluorescence as well as tyrosine hydroperoxide and the products of its subsequent transformation are the major products of the photolysis of tyrosine.

TABLE 1. Oxidation–Reduction Potentials $E^{o'}$ at pH 7 for Reactions of Aromatic Amino Acids with RF, ROS and RNS [43]

Reduction reaction	$E^{o'}$ vs. NHE at 298 K, pH 7
$O_2 + e^- \rightarrow O_2^{\bullet-}$	–0.18*
$^1O_2 + e^- \rightarrow O_2^{\bullet-}$	+0.81*
$O_2^{\bullet-} + e^- + 2H^+ \rightarrow H_2O_2$	+0.91
$HO_2^{\bullet} + e^- + 2H^+ \rightarrow H_2O_2$	+1.05
$H_2O_2 + e^- + H^+ \rightarrow HO^{\bullet} + H_2O$	+0.39
$H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O$	+1.35
$HO^{\bullet} + e^- + H^+ \rightarrow H_2O$	+2.31
$^{\bullet}NO_2 + e– \rightarrow NO_2^-$	+1.04 [44, 45]
$NO^+ + e^- \rightarrow {}^{\bullet}NO$	+1.21 [44]
$Tyr^{\bullet} + e^- + H^+ \rightarrow Tyr$	+0.93 [46]
$Trp^{\bullet} + e^- + H^+ \rightarrow Trp$	+1.015 [46]
$RF + e^- \rightarrow RF^{\bullet-}$	–0.3 [35]
$^3RF + e^- \rightarrow RF^{\bullet-}$	+1.77 [35]

*Taking account of solubility in water.

TABLE 2. Effect of Added NaN_3 Azide to an Aqueous Solution of Tyrosine and 0.02 M RF on the Formation of Tyrosine Photolysis Products

Starting tyrosine concentration, μM	Tyrosine concentration upon photolysis in the presence of azide, μM		Contribution of $^1O_2$ to the formation of tyrosine photoproducts, %	
	$[N_3^-] = 0$ mM	$[N_3^-] = 1$ mM	experimental	calculated
10	8.6	0.8	91	95
50	21.0	6.0	71	78
100	25.6	10.4	59	64

Note: The solutions were irradiated with light from a DRK-20 mercury lamp for 20 min using a ZhS-11 light filter.

The participation of singlet oxygen in the photolysis of tyrosine was confirmed by experiments on the quenching of singlet oxygen using azide. Sodium azide is known as an efficient quencher of $^1O_2$ with quenching rate constant $(1.5–2)\cdot10^9$ $M^{-1}\cdot s^{-1}$ [31, 51]. At high concentrations of azide (≥1.0 mM) in the irradiated solutions containing RF and tyrosine in low concentrations (≤0.1 mM), the yield of the photolysis products is reduced by a factor of ~10–15 (Table 2). The effect of the added quencher (1 mM azide) on the decrease in the yield of tyrosine photoproducts becomes less significant with increasing concentration of tyrosine in the irradiated mixture. This result shows that the direct oxidation of tyrosine in the reaction with ^3RF makes the major contribution to the photoproduct formation with increasing concentration of tyrosine in the irradiated solution, while the contribution of reactions involving $^1O_2$ decreases. The values of the rate constants of the reactions of ^3RF with tyrosine ($k = 1.4\cdot10^9$ $M^{-1}\cdot s^{-1}$ [36]) and oxygen ($k = 0.98\cdot10^9$ $M^{-1}\cdot s^{-1}$ [35]) can be used to calculate the contribution of the photochemical processes involving $^1O_2$ to the formation of tyrosine photoproducts depending on the initial tyrosine concentration in solution (the quenching of the ^3RF state by azide in this case was not taken into account due to its insignificance [31]). Table 2 shows that, despite the correlation of the contributions of singlet oxygen to the photolysis

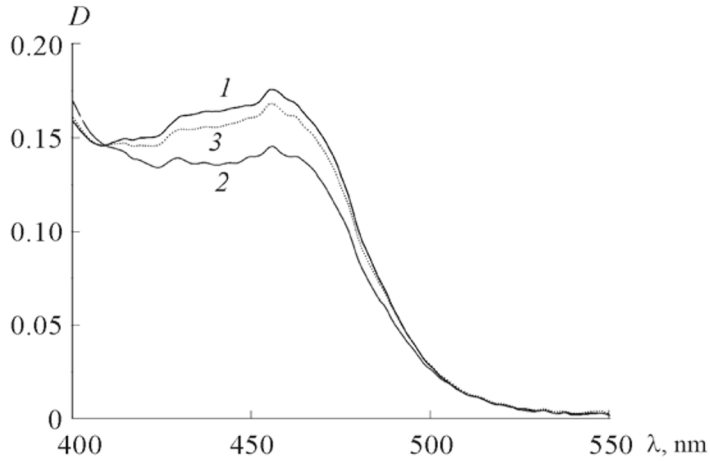

Fig. 4. Absorption spectra of aqueous solutions of 20 μM RF in a mixture with 1 mM Tyr in sealed cells upon photoexcitation: 1) no irradiation, 2) 30 min irradiation, 3) after passage of air into the cell with a solution, which had been irradiated for 30 min.

of tyrosine, the experimental values are somewhat lower than the calculated values, perhaps indicating the consumption of molecules of dissolved O_2 during the photoinduced oxidation processes.

Photoirradiation of the solutions of RF and tyrosine in sealed cells leads to a decrease in the concentration of dissolved oxygen and reversible decoloration of the solution due to the conversion of RF to the metastable semi-quinone form $RF^{\bullet}$ and leuco form RFH_2. The introduction of air into the cell and aeration of the irradiated solutions leads to reoxidation of the leuco form RFH_2 by oxygen and partial restoration of the RF absorption spectrum (Fig. 4).

Upon the oxidation of tyrosine to give $Tyr^{\bullet}$, the ^3RF molecule is converted into protonated $RFH^{\bullet}$ or deprotonated semi-quinone form $RF^{\bullet}$, which then can be oxidized by oxygen to give superoxide anion $O_2^{\bullet-}$ and $HO_2^{\bullet}$ or undergo disproportionation to give starting riboflavin RF and the reduced leuco form RFH_2

$$2RFH^{\bullet} \rightarrow RF + RFH_2 .$$

The reduced leuco form RFH_2 is oxidized upon reaction with oxygen to give the starting RF molecule with the formation of hydrogen peroxide (Fig. 3a).

Thus, under our experimental conditions, the riboflavin-photosensitized photolysis of tyrosine proceeds through two pathways: 1) the reaction of tyrosine with $^1O_2$ and 2) the oxidation of tyrosine by RF in the triplet state. The formation of tyrosyl radicals $Tyr^{\bullet}$ was found to proceed predominantly through the second mechanism (Fig. 3a).

The addition of nitrite to the irradiated incubation mixture leads to the formation of an additional tyrosine photolysis product, namely, 3-nitrotyrosine, which absorbs in the vicinity of 360 nm (Fig. 5). The concentration of 3-nitrotyrosine was found to be ~ 8 μM in the mixture containing 0.1 mM tyrosine, 1 mM NO_2^-, and 0.02 mM RF after 20 min photoirradiation. The maximum yield of 3-nitrotyrosine was observed at pH ~4.5–5.0.

Peroxynitrite ONOO [8, 52] or nitrogen dioxide $^{\bullet}NO$ [8] can act as the nitrating agent in the formation of 3-nitrotyrosine. In the former case, the nitration proceeds in the reaction of peroxynitrite with tyrosine, while, in the latter, nitrogen dioxide should react with the tyrosine radical $Tyr^{\bullet}$ [8].

The highly efficient diffusion-controlled reaction of nitric oxide $^{\bullet}NO$ with the superoxide anion $O_2^{\bullet-}$ is usually considered the source of peroxynitrite. As noted above, the riboflavin photosensitized photolysis of tyrosine gives superoxide anions (Fig. 3a), which can participate in this reaction.

A decrease in the solution pH leads to protonation of the nitrite ion with pK_a ~ 3.3 and the formation of unstable nitrous acid, which can form the nitrosonium ion NO^+ in acid media

$$HNO_2 \leftrightarrow NO^+ + OH^- .$$

The nitrosonium ion can be further reduced to nitric oxide $^{\bullet}NO$ upon reaction, for example, with superoxide anion. At low pH, this pathway for the formation of $^{\bullet}NO$ and then peroxynitrite can be the predominant mechanism in the nitration

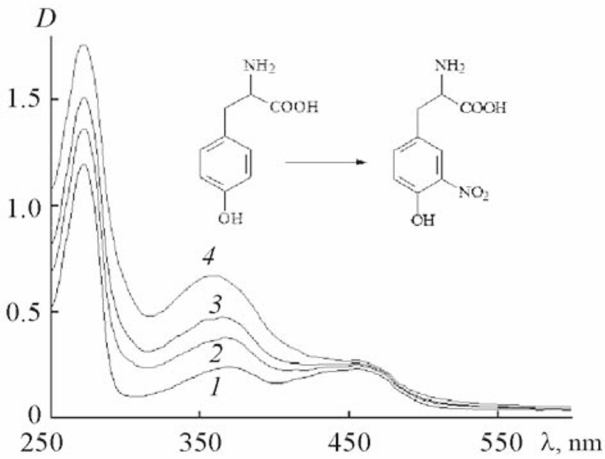

Fig. 5. Absorption spectra of aqueous solutions of 0.1 mM tyrosine and 1 mM nitrite in a mixture with 0.02 mM RF after irradiation with visible light from a mercury lamp using a ZhS-11 light filter for 0 (1), 10 (2), 20 (3), and 60 min (4).

of tyrosine but we may expect that its contribution is insignificant in media with neutral pH. Furthermore, the data on the reduction potential for $^{\bullet}NO_2$ (Table 1) indicate the possibility of the direct of oxidation of nitrite to give $^{\bullet}NO_2$ upon reaction with triplet RF. Fontana et al. [53] have shown the possibility of the riboflavin-photosensitized nitration of proteins involving this mechanism.

Still another mechanism for the formation of nitrogen dioxide related to the discussion of subsequent results concerning protein nitration can involve the oxidation of nitrite in the presence of H_2O_2 and heme-containing proteins such as Mb, which display pseudoperoxidase activity. The data of Herold [11] and Kiline [16] indicate that H_2O_2 reacts with metMb to give the oxoferryl form $^{+\bullet}$Mb(IV=O) (compound I)

$$Mb(III) + H_2O_2 \rightarrow {}^{+\bullet}Mb(IV{=}O) + H_2O\,.$$

The oxoferryl forms of myoglobin, $^{+\bullet}$Mb(IV=O) (compound I) and Mb(IV=O) (compound II) oxidize nitrite to nitrogen dioxide in the following reactions:

$$^{+\bullet}Mb(IV{=}O) + NO_2^- \rightarrow Mb(IV{=}O) + {}^{\bullet}NO_2\,,$$

$$Mb(IV{=}O) + NO_2^- \rightarrow Mb(III) + {}^{\bullet}NO_2\,.$$

The processes involving the nitration of tyrosine in riboflavin-photosensitized reactions are shown schematically in Fig. 3b. The predominant pathway upon irradiation with visible light can be seen as a two-step mechanism entailing the oxidation of tyrosine molecules to give tyrosyl radicals $Tyr^{\bullet}$ and of nitrite molecules to give nitrogen dioxide $^{\bullet}NO_2$ and then the formation of 3-nitrotyrosine due to the reaction of nitrogen dioxide with tyrosyl radicals

$$^{\bullet}NO_2 + Tyr^{\bullet} \rightarrow Tyr\,NO_2\,.$$

An alternative pathway involving peroxynitrite as the nitrating agent gives an increasingly significant contribution to the nitration with decreasing solution pH since the probability of formation of the nitrosonium ion NO^+ involved in this process is low at neutral pH.

Riboflavin-photosensitized nitration of tyrosine in myoglobin. The nitration of tyrosyl residues in horse heart myoglobin was carried out by photoirradiation of a solution of 20 μM Mb, 1 mM $NaNO_2$, and 1 mM Tyr in the presence of 50 μM RF using light from a mercury lamp with $\lambda > 420$ nm and a ZhS-11 light filter for 60 min and chemically by incubation of 20 μM Mb, 1 mM $NaNO_2$, and 1 mM H_2O_2 for 6 h.

All the protein solutions were normalized relative to the content of the major protein to a concentration of 1 mg/mL. A sample of 10 μL 50 mM aqueous dithiothreitol was added to a solution containing 50 μg protein (20 μL). The mixture was stirred and maintained at constant 50°C for 30 min. Then, 10 μL 150 mM aqueous iodoacetamide was

Fig. 6. Sequence of the horse heart myoglobin primary structure and peptide fragments obtained after trypsin hydrolysis. The symbol **n** indicates the fragments with increased mass due to nitration.

added and the mixture was maintained in the dark at room temperature. The resultant mixture was diluted by adding 960 mL ammonium bicarbonate buffer (pH 8.0), stirred, and treated ultrasonically for 5 min. Then, 2 μL of a solution of 0.5 mg/mL trypsin in 50 mM acetic acid was added. The trypsinolysis was carried out for 16 h at 37°C. In order to separate the peptides, solid-phase extraction was carried out using a cartridge of SampliQ C18 (EC) containing 100 mg adsorbent. The cartridges were consecutively washed with 2 ml acetonitrile, 2 mL water, and 2 mL 0.2% formic acid. Then, the solution with the trypsinolysis products was inserted. The column was washed with 2 mL 0.2% formic acid. The peptides were eluted with 1 mL of a 3:2 (v/v) acetonitrile–water mixture containing 0.2% formic acid. The resultant solution was used for the HPLC/MS analysis.

HPLC/MS analysis. In order to separate the peptide mixture, we used a 2.1 × 50mm ZORBAX Extend-C18 column (1.8 μm). Mobile phase A was 0.2% aqueous formic acid and mobile phase B was 0.2% formic acid in acetonitrile. Elution gradient from 3 to 25% B for 35 min, gradient from 25 to 40% B for 20 min, gradient from 40 to 90% B for 15 min, and 90% B for 15 min. The flow rate of the mobile phase was 200 μL/min. The column thermostat temperature was 40°C. The input volume was 0.5 μL. Each sample was analyzed twice.

We used a Q-TOF 6550 mass spectrometric detector with an APESI electrospray ionization source. The ionization source parameters were 400°C sheath gas temperature, sheath gas flow 9 L/min. capillary voltage 2 kV, fragmentor voltage 360 V. The mass analyzer operating parameters: automatic MS/MS analysis, 275–1700 *m*/*z* range in the MS mode; 275–1700 *m*/*z* range in the MS/MS analysis mode, fragmentation energy: for $z = 2–3.1$ $(m/z) + 1$, for $z \geq 3–3.6$ $(m/z) – 4.8$; isotope model for peptides. In order to identify the modifications, the data were treated using the PEAKS Studio program package from Bioinformatics Solutions.

The myoglobin molecule has two tyrosyl residues Tyr-103 and Tyr-146 (or Y103 and Y146 in the singleletter notation), which are capable of undergoing nitration. Figure 6 and Table 3 show the existence of protein polypeptide chain fragments nitrated at both tyrosyl residues. Nitration of the tryptophan residues in myoglobin was not observed. In the riboflavin-photosensitized nitration of Mb, the modification of residue Tyr-103 occurs about ten times less efficiently than for Tyr-146 (Table 3) and the amount of Tyr-103–NO_2 is ~ 9% of the amount of Tyr-146–NO_2.

In the case of the dark nitration in the presence of H_2O_2, the amount of nitrated residues Tyr-103–NO_2 is <2% of the amount of Tyr-146-NO_2. Thus, the probability of the nitration of the myoglobin Tyr-103 residue is greater in the photochemical reaction in the presence of RF.

The results for nitration of myoglobin tyrosyl residues in the dark reaction contradict the data of Herold [11], who found that the nitration of the tyrosyl residues in metMb either does not occur or proceeds only slightly even after prolonged incubation with hydrogen peroxide and nitrite. However, at high nitrite concentrations, a complex of the heme Fe(III) ion

TABLE 3. Relative Amount of Tyrosyl-Containing Myoglobin Peptides after Nitration

Tyrosine residue	Detected peptides	Protein nitration in the dark reaction with H_2O_2	Protein nitration in the photochemical reaction
Tyr-103	Y(+45.00)LEFISDAIIH	35	105
	Y(+45.00)LEFISDAI	–	–
Tyr-146	Y(+45.00)KELGFQG	2670	–
	NDIAAKY(+45.00)KELGFQG	–	1230

with peroxynitrite Mb(III)–N(O)OO can form as seen in a shift of the Soret band to 416 nm. This complex located near the Tyr-146 residue can efficiently nitrate heme in a competing reaction [20]. These results indicate that Tyr-146 predominantly undergoes the dark nitration reaction, while modification of Tyr-103 is less than 2%, which is in good accord with the results of Nicolis et al. [20]. We note that the fraction of modified Tyr-103 residues located far from the heme component upon nitration increases during the photochemical reaction (up to ~9% of the amount of Tyr-146–NO_2). This may imply that the tyrosyl residues near the site of formation of the nitrating agent (nitrogen dioxide or peroxynitrite) predominantly undergo nitration. Oxidation of nitrite in the reaction of triplet RF can also serve as the source of $^{\bullet}NO_2$ molecules in the photochemical reaction [53]. We attribute the enhanced nitration of the Tyr-103 residue far from the heme component to this process.

Both RF and its derivatives, namely, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) act as photosensitizers initiating the oxidation of lipids and proteins upon photoexcitation [34, 35]. There is a high content of flavins in the visual retina and, for rats: the content is ~46 pmole FAD, 18 pmole FMN, and 4.8 pmole RF per 1 mg protein [54]. Typical concentrations of RF in human skin and ocular fluid are 7.9 μmole/kg dry matter and 4.5 μM, respectively [35]. Skin and eyes are continually exposed to solar radiation and the photochemical processes occurring in these tissues are considered a risk factor causing an increase in a number of pathologies such as skin cancer, cataracts [55], and macular dystrophy [34, 35].

Crystallins are the major protein component of the eye lens and undergo nitration of the tyrosyl residues upon photoexcitation in the presence of RF [53], which can lead to pathological changes such as the formation of amyloid fibrils and the development of cataracts [55]. The nitrite concentration in blood plasma is on the micromolar level but can be higher in the case of chronic inflammatory processes [57]. The flavin-photosensitized nitration of tyrosine protein residues can presumably proceed under physiological conditions in the skin and eye cell structures. It is also important to note that the exchange of structural components of the eye (crystallin) and skin (elastin) is rather slow and the structural modification of proteins upon nitration and oxidative stress can accumulate over several years [53].

Conclusions. Mass spectrometry was used to show that visible light irradiation of aqueous solutions of riboflavin in a mixture with nitrite, tyrosine, and horse heart metmyoglobin leads to nitration of tyrosyl residues Tyr-103 and Tyr-146 in this protein. Nitration of tryptophan residues was not observed. The modification is found predominantly for residue Tyr-146, while the amount of nitrated Tyr-103–NO_2 residues is ~9% relative to the amount of Tyr-146–NO_2. The amount of nitrated Tyr-103–NO_2 residues is less than 2% of the nitrated Tyr-146–NO_2 residues in the dark nitration of myoglobin upon incubation of the protein with nitrite and hydrogen peroxide according to the mechanism described above [20] with formation of a complex of peroxynitrite with heme. Thus, the mechanisms for the modification of protein tyrosyl residues both in dark and photochemical nitration reactions are significantly different, which is seen in the differences in the probability of nitration of the myoglobin Tyr-103 residue. In experiments on the riboflavin-photosensitized formation of 3nitrotyrosine in solution with nitrite, we found that the photochemical nitration pathway can additionally entail formation of $^{\bullet}NO_2$ from nitrite in the reaction of the photosensitizer molecule in the triplet state ^{3}RF, as already proposed by Fontana et al. [53], or the initial formation of $^{\bullet}NO$ and then peroxynitrite in the reaction of the nitrosonium ion NO^+ with superoxide anions. The probability of formation of peroxynitrite through the latter pathway is significant only at low pH.

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