Effect of hemoglobin on the NO-donor ability of μ^2-*S*-bis(pyrimidine-2-thiolato)tetranitrosyldiiron

L. A. Syrtsova, N. A. Sanina, A. F. Shestakov, N. I. Shkondina, T. N. Rudneva, N. S. Emel´yanova, A. I. Kotel´nikov, and S. M. Aldoshin*

Institute of Problems of Chemical Physics, Russian Academy of Sciences, 1 prosp. Akad. Semenova, 142432 Chernogolovka, Moscow Region, Russian Federation. E-mail: syrtsova@icp.ac.ru

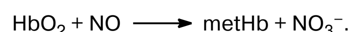
The hydrolysis of the iron nitrosyl complex $[Fe_2(\mu_2\text{-}SC_4H_3N_2)_2(NO)_4]$ ($C_4H_3N_2S^-$ is pyrimidine-2-thiolate) in the presence of hemoglobin (Hb) is accompanied by the NO release into a solution. In the absence of Hb, the starting complex is oxidized by nitric oxide that is released into a solution, which leads to further transformations of NO, nitric oxide being present in the solution only partially. The effective rate constant for the decomposition of the complex is high and depends on its concentration. On the one hand, in the presence of Hb, NO molecules rapidly and irreversibly bind to Hb to form HbNO, which is the intermediate in the nitric oxide metabolism. On the other hand, the reversible binding of the iron nitrosyl complex to the surface functional groups of Hb leads to a decrease in its concentration in a solution and deceleration of the formation of NO. Therefore, Hb can ensure the complete and more prolonged assimilation of NO.

Key words: nitric oxide, iron sulfur nitrosyl complexes, hemoglobin, methemoglobin, kinetic measurements.

The iron thiolate nitrosyl complexes $[Fe_2(SR)_2(NO)_4]$, like *S*-nitrosothiolates[1] and diazeniumdiolates,[2,3] are hydrolyzed in protic media to form nitric oxide[4] and belong to a new class of NO donors holding promise for the pharmacology.[5—10] Previously, we have found that iron sulfur nitrosyl complexes are decomposed with the NO release in protic media without additional activation,[4,11] the rate constants for this reaction being dependent on the molecular structures of the complexes. In the present study, we investigated the new dinuclear iron tetranitrosyl complex with the pyrimidine-2-thiolate ligand $[Fe_2(\mu_2\text{-}SC_4H_3N_2)_2(NO)_4]$ (**1**) exhibiting high antitumor activity (86—88% of the growth retardation in human tumor cells, the ovarian cancer cell line SCOV3).[7] The pyrimidine-2-thiolate ligand $C_4H_3N_2S^-$ can be considered as the structural analog of antimetabolites (6-mercaptopurine, *etc.*), which are used for the synthesis of antitumor agents,[12—17] and NO is a key agent in the cancer genesis.[18]

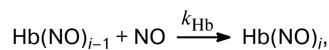
The direct determination of the NO concentration in a solution by the electrochemical method with the use of a sensor electrode showed that the maximum NO concentration after the hydrolysis of complex **1** in a protic medium is substantially lower than the stoichiometrically possible yield, the decomposition of complex **1** occurring rapidly.[19] By contrast, a larger amount of NO is released in the presence of hemoglobin (Hb) (one NO molecule per molecule of the complex), and the decomposition occurs more slowly. In our opinion, this fact is important both in terms of the insight into the mechanisms of reactions of heme proteins with new exogenic NO donors and from the point of view of drug design. Thus, the pharmacologically active agent (NO donor) should have the period of performance sufficient for the NO delivery to target sites.

The formation of $Hb(NO)_4$ in the reaction with Hb can serve as a convenient method for the estimation of the NO-donor ability of iron nitrosyl complexes.[4] Due to the high rate constant for the reaction of Hb with NO (k_{Hb}, $1.02 \cdot 10^8$ L mol^{-1} s^{-1}) and the high binding constant (K_{Hb}, $3 \cdot 10^{10}$ L mol^{-1}),[20] Hb serves as the effective NO trap. At the same time, the complex $Hb(NO)_4$ is an important intermediate in the metabolism of iron nitrosyl complexes. In arterial blood, HbO_2 reacts with NO to form the nitrate ion and methemoglobin (metHb) depending on the NO to O_2 ratio:



$$HbO_2 + NO \longrightarrow metHb + NO_3^-.$$

In venous blood, nitrosyl heme complexes containing different numbers of NO groups, up to $Hb(NO)_4$, are formed:



$$Hb(NO)_{i-1} + NO \xrightarrow{k_{Hb}} Hb(NO)_i,$$

$i = 1—4.$

At higher partial pressures of O_2, the complexes $Hb(NO)_i$ are decomposed with the release of NO molecules, which

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 12, pp. 2148—2159, December, 2010.

1066-5285/10/5912-2203 © 2010 Springer Science+Business Media, Inc.

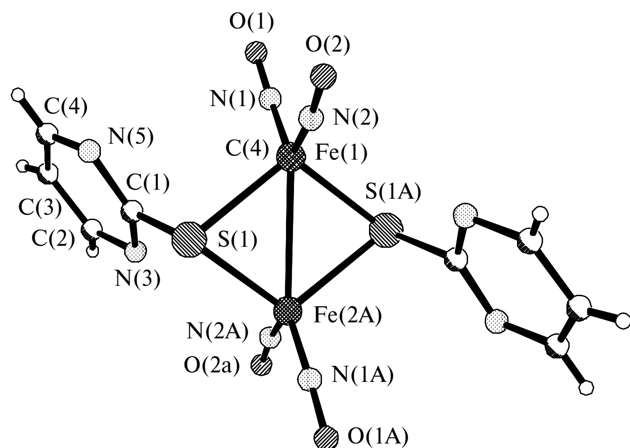
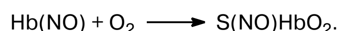


Fig. 1. Structure of complex **1**.[19]

cause the nitrosylation of the SH group in HbO_2 that is formed:[21—23]



$$\mathrm{Hb(NO)} + \mathrm{O_2} \longrightarrow \mathrm{S(NO)HbO_2}.$$

Ntrosothiols accumulate NO and, consequently, play an important role in the transport, storage, and metabolism of nitric oxide. It is suggested that $S(NO)HbO_2$ acts as the allosterically controlled NO buffer.[24] Therefore, Hb can serve as a chemical reservoir for NO, in which NO is accumulated in the form of $Hb(NO)_4$ or nitrosothiol before it is consumed.

The above-described reactions of Hb with NO under conditions of metabolism gave impetus to the present study. The aims were to investigate the *in vitro* reaction of complex **1** with Hb and to reveal the difference between the NO release from complex **1** in the absence and in the presence of Hb. Complex **1** has a dinuclear μ_2-S structure (Fig. 1), which we have described previously.[19]

Experimental

The commercial reagents $Na_2S_2O_3 \cdot 5H_2O$, KOH, and pyrimidine-2-thiol (Aldrich) were used for the synthesis of complex **1**. The complex $Na_2[Fe_2(\mu_2\text{-}S_2O_3)_2(NO)_4] \cdot 4H_2O$ was synthesized according to a known procedure.[25] Complex **1** was synthesized in the polycrystalline state as described earlier.[19] All operations (the preparation of solutions, the reactions, and the isolation of complex **1**) were carried out under a pure nitrogen atmosphere. Commercial bovine hemoglobin (MP Biomedicals, Germany), $Na_2HPO_4 \cdot 6H_2O$, and $NaH_2PO_4 \cdot H_2O$ (MP Biomedicals, Germany) were used in experiments; DMSO (reagent grade, Khimmed, Russia) was additionally purified by distillation at 10 Torr with collection of the fraction boiling at 73—75 °C according to a procedure described earlier.[26] Water was purified by successive distillation using a DE-4 distillation apparatus and a Bi/Duplex Pyrex aqua-purator (Germany).

The elemental analysis was performed on a Vario Micro cube CHNS/O-analyzer in the Analytical Center of Collective Use of the Institute of Problems of Chemical Physics of the Russian Academy of Sciences; the Fe content was determined according to a procedure described earlier.[27] Found (%): C, 20.00; H, 1.17; Fe, 24.47; N, 23.83; S, 13.96. $C_8H_6Fe_2N_8O_4S_2$. Calculated (%): C, 21.20; H, 1.34; Fe, 24.59; N, 24.68; S, 14.15.

High-purity nitrogen was additionally purified by passing through a column with a chromium nickel catalyst. To transfer the working solutions (the buffer and DMSO) to a nitrogen atmosphere, they were pre-evacuated, and then nitrogen was passed through the vessels for 30 min with magnetic stirring of the solutions. Hereinafter, these solutions will be referred to as anaerobic. All vessels and quartz cells were sealed with rubber Septum stoppers (Sigma). Solutions were transferred from one vessel to another with the use of syringes with soldered needles or the solutions were cannulated under an excessive pressure of nitrogen with the use of two cannulas connected by Teflon capillaries. The excessive pressure was relieved through another needle connected to a Teflon capillary immersed into water. The cells and vessels with volumes of 4 and 5 mL, respectively, containing weighed samples of nitrosyl complexes or other reagents were purged with nitrogen through needles for 30 min.

The absorption spectra were recorded on a Specord M-40 spectrophotometer equipped with an interface enabling its link to a computer and a temperature-controlled cell holder.

Kinetics of the reaction of Hb with complex 1. A homogeneous solution of the protein was prepared from commercial bovine hemoglobin (MP Biomedicals) according to a procedure described earlier.[11] A phosphate buffer, pH 7.0 (2.8 mL, 0.05 mol L^{-1}), was placed into a 4-mL anaerobic test cell with an optical path length of 1 cm, and an Hb solution (0.1 mL) was added to the concentration of $2.2 \cdot 10^{-5}$ mol L^{-1}. Then the absorption spectrum was recorded. All subsequent experiments were carried out with the use of this buffer. Anaerobic anhydrous DMSO was added to nitrosyl complex **1** in a vessel filled with nitrogen in an amount such that solutions of **1** were obtained at concentrations of $1.5 \cdot 10^{-3}$, $6.0 \cdot 10^{-3}$, and $2.4 \cdot 10^{-2}$ mol L^{-1}. The solution was stirred for 3—5 min until complex **1** was completely dissolved, and aliquots (0.1 mL) of the solution were placed into the test cell containing Hb and the reference cell containing the anaerobic buffer (2.9 mL). The final concentrations of complex **1** were $5 \cdot 10^{-5}$, $2 \cdot 10^{-4}$, and $8 \cdot 10^{-4}$ mol L^{-1}; the concentration of DMSO was 3.3%. The first difference absorption spectrum was recorded within 0.5 min after the beginning of the reaction, and the subsequent spectra were recorded at 3 min intervals. The spectra were recorded until Hb was completely transformed into $Hb(NO)_4$ (the spectrum ceased to change).

Kinetics of the reaction of metHb with complex 1. Test and reference cells were used in the experiment. The reference cell contained the buffer (2.9 mL). The solution of complex **1** was prepared as described above. The test cell contained the buffer (2.58 mL) and metHb (0.32 mL, 0.32 mmol L^{-1}). The reaction was initiated analogously to the reaction with Hb by the addition of a solution of complex **1** (0.1 mL, 6 mmol L^{-1}) to the test and reference cells. The absorption spectra were recorded as described above for the reaction of Hb with complex **1**.

Electrochemical determination of the concentration of NO generated by complex **1**. An amiNO-700 sensor electrode of the inNO Nitric Oxide Measuring System (Innovative Instruments, Inc., USA) was used. The NO concentration was determined at 0.2 s

intervals during ~500 s. The electrochemical sensor was calibrated using the standard aqueous $NaNO_2$ solution (100 μmol L^{-1}), to which a mixture of aqueous 0.12 *M* KI (18 mL) and 1 *M* H_2SO_4 (2 mL) solutions was added. The experiments were performed in an anaerobic solution of the phosphate buffer (pH 7.0) containing DMSO (3.3%) and complex **1** (1.65—6.60 μmol L^{-1}) at 25 °C. For each experiment, an electrochemical cell and a fresh solution of complex **1** were separately prepared. The buffer solution (48.3 mL) was placed into a sealed electrochemical cell with a sensor electrode equipped with a Septum stopper to introduce a sample, and then nitrogen was bubbled through the solution with stirring for 1 h. The reaction was initiated by the addition of a solution of complex **1** prepared as follows: anaerobic anhydrous DMSO was added to complex **1** in a vessel filled with nitrogen in an amount such that the solution of **1** was obtained at a concentration of 1.65 mmol L^{-1}. The solution was stirred for 3—5 min until the complex was completely dissolved. A solution of complex **1** in DMSO (0.05, 0.10, or 0.20 mL) and DMSO (1.65, 1.60, or 1.50 mL, respectively) were placed into a cell at the initial moment. In the experiment with Hb, the buffer (46.3 mL) was placed into a cell, nitrogen was bubbled through the solution, and then a solution of Hb (2 mL, 0.5 mmol L^{-1}) was added. The reaction was initiated by the addition of a solution of complex **1** (0.2 mL, 1.65 mmol L^{-1}) and DMSO (1.5 mL).

The amounts of Hb, $Hb(NO)_4$, and metHb were estimated by spectrophotometry. The absorption spectra were decomposed into components with the use of the MATHCAD program as has been described earlier.[4]

Results and Discussion

All reactions with complex **1** were carried out under nitrogen because NO rapidly reacts with O_2 to give nitrogen oxides (the rate constant is $2 \cdot 10^6$ L^2 mol^{-2} s^{-1}).[28] To study the effect of Hb on the NO-donor ability of complex **1**, the rates of NO release were measured both in the absence and in the presence of Hb. For this purpose, an electrochemical sensor electrode was used, which made it possible to directly measure the current concentration of NO in solution. Figure 2 shows the kinetic curves measured in the absence of Hb (at different concentrations of complex **1**) and in the presence of Hb.

It is noteworthy that the maximum NO concentration in solutions in the experiments performed in the absence of Hb is substantially lower than the initial concentration of complex **1** and depends on the concentration of **1** in the reaction medium. The yield of NO based on the iron nitrosyl complex is lower than the number of NO molecules in complex **1**.

The steady-state NO concentration is achieved within 200—500 s depending on the concentration of complex **1**, and the ratio [NO] : [**1**] is approximately 0.1. It should be noted that the 10% yield of NO based on complex **1** is obtained already within 1.5 min. This indicates that the mechanism of the formation of NO is more complex than the monomolecular decomposition of the iron nitrosyl complex under study; otherwise, the relative amount of

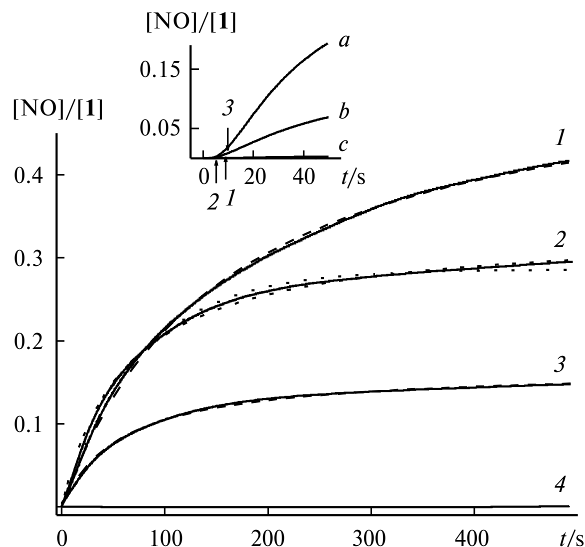


Fig. 2. Kinetic curves of the accumulation of NO in solution (the measurements were carried out with the use of a sensor electrode) in the course of the decomposition of complex **1** under anaerobic conditions at 25 °C within 30 s after the beginning of the reaction in the absence (*1—3*) and in the presence (*4*) of Hb (0.02 mmol L^{-1}). The dashed lines represent the approximation of the kinetic curves by a hyperbolic function. The points (for curve *2*) correspond to the approximation by the function $y = A[1 - \exp(-k_1 t)]$. A phosphate buffer (0.05 mol L^{-1}, pH 7.0) was used as the solvent; [**1**]/μmol L^{-1} = 1.65 (*1*), 3.3 (*2*), and 6.6 (*3*, *4*). The inset shows the same curves but measured in the interval t = 0—50 s; *a*, curve *2*; *b*, curve *1*; *c*, curve *3*; the induction periods (τ) for curves *1—3* are indicated by arrows.

NO that is released should be independent of the initial concentration of complex **1**.

It is interesting that NO was not detected in the solution after the addition of Hb (see Fig. 2, curve *4*). This is indicative of the high rate of the virtually irreversible binding of NO molecules, which are formed by the decomposition of the iron nitrosyl complex, to Hb. Due to the high rate constant for the reaction of hemoglobin with NO, the quasi-steady-state condition for [NO] should be met, *i.e.*, the rate of the formation of NO in the system should be equal to the rate of its consumption in the reaction with Hb*

$$k_{Hb}[NO](4[Hb] + 3[Hb(NO)] + 2[Hb(NO)_2] + [Hb(NO)_3]),$$

which, in turn, is equal to the rate of the accumulation of nitrosylation products of Hb

$$\frac{d}{dt}([Hb(NO)] + 2[Hb(NO)_2] + 3[Hb(NO)_3] + 4[Hb(NO)_4]).$$

Taking into account the mass balance for Hb, the rate of the formation of NO is related to the rate of the accu-

* This condition is written taking into account that Hb contains four heme Fe complexes exhibiting equal reactivity toward NO.

mulation of nitrosylation products of Hb by the following equation:

$$\frac{d[NO]}{dt} \approx k_{Hb}[NO]\{4([Hb]_0 - [Hb(NO)_4]) - [Hb(NO)] - 2[Hb(NO)_2] - 3[Hb(NO)_3]\} = \frac{d}{dt}([Hb]_0 - [Hb] + [Hb(NO)_2] + 2[Hb(NO)_3] + 3[Hb(NO)_4]),$$

where $[Hb]_0$ is the initial concentration of hemoglobin.

These equations can be written in a more simple form with the use of the concentrations of the free ([hem]) and nitrosyl ([hem(NO)]) hemes in the system:

$$\frac{d[NO]}{dt} \approx k_{Hb}[NO][hem] = \frac{d[hem(NO)]}{dt}.$$

Since the nitrosyl hemes in Hb have identical spectroscopic characteristics, the system is always spectroscopically equivalent to a mixture of the starting hemoglobin and fully nitrosylated $Hb(NO)_4$. Hence, the kinetic curves of the accumulation of $Hb(NO)_4$ provide direct information on the rate of the formation of NO:

$$\frac{d[NO]}{dt} \approx 4k_{Hb}[NO][Hb] = \frac{d[Hb(NO)_4]}{dt}. \quad (1)$$

The NO-donor ability of complex **1** in the presence of Hb was determined by spectrophotometry, which allows the detection of the amount of the complex $Hb(NO)_4$ produced in this reaction.[4] It is known[4] that the absorption spectra of Hb and $Hb(NO)_4$ strongly overlap with each other and have similar extinction coefficients for the absorption maxima. Therefore, in spite of the difference in the positions of these maxima, the absorption spectra were represented as the sum of the contributions of Hb and $Hb(NO)_4$ (see Ref. 4), and then the spectra were decomposed into components to obtain the correct evaluation of the amount of $Hb(NO)_4$ that is formed.

As can be seen from the spectrum of a freshly prepared solution of complex **1**, the change in the absorbance at λ = 556 nm for the absorption maximum of Hb is 0.028 after 1 h (Fig. 3), which is 3% of the absorption of $Hb(NO)_4$ and Hb (Fig. 4). Hence, to study the kinetics of the accumulation of $Hb(NO)_4$ in the presence of complex **1** under anaerobic conditions at 25 °C, we recorded the time-resolved difference absorption spectra (see Fig. 4, *a*) using a cell containing the reaction system and a reference cell containing complex **1** at the same concentration. The concentration of complex **1** in the reaction was an order of magnitude higher than the concentration of Hb to ensure the complete nitrosylation of Hb. The difference absorption spectra (see Fig. 4, *a*) show a decrease in the absorbance near the absorption spectrum maximum of Hb at λ = 556 nm and an increase in the absorbance at λ = 545

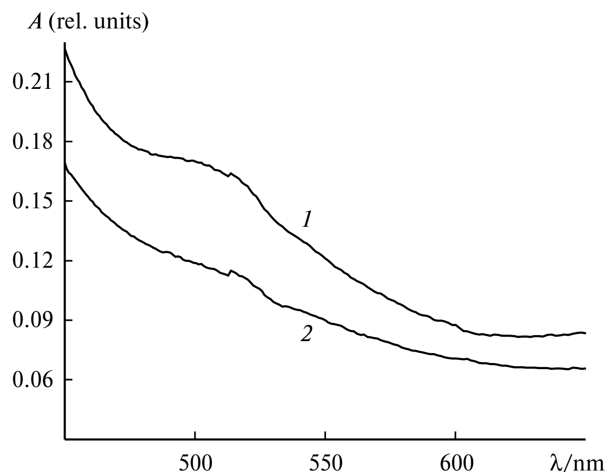


Fig. 3. Absorption spectra of complex **1** (0.2 mmol L^{-1}) measured immediately after the dissolution under anaerobic conditions in a phosphate buffer (0.05 mol L^{-1}, pH 7.0) containing DMSO (3.3%) (*1*) and within 1 h after the incubation at 25 °C (*2*).

and 575 nm, which is indicative of the formation of $Hb(NO)_4$.[4,11] These spectra have three isosbestic points at λ = 551, 570, and 595 nm. This indicates that only Hb and $Hb(NO)_4$ contribute to the absorption spectrum, as in the case of the reactions of this class of iron nitrosyl complexes with Hb, which we have studied earlier.[4]

After the decomposition of the absorption spectra into components (the absorption of Hb and $Hb(NO)_4$) by the least-squares method,[4] the time dependence of the accumulation of $Hb(NO)_4$ was found (Fig. 5). This curve is adequately approximated by the function $y = A[1 - \exp(-k_1t)]$, where k_1 is the effective first-order rate constant and A is the final concentration of $Hb(NO)_4$. The constant k_1 is $(3.7\pm0.4)\cdot10^{-4}$ s^{-1}. Hence, the initial rate of the formation of NO under these conditions can be evaluated as ~$(33.3\pm3)\cdot10^{-3}$ μmol L^{-1} s^{-1}.

Therefore, there is a substantial difference in the kinetics of the formation of NO during the decomposition of complex **1** under different conditions. At low concentrations of the Fe complex in the absence of Hb, 10% of NO per molecule of complex **1** are released already within 1.5 min (see Fig. 2). The relative yield of NO is no higher than 40%, and the yield decreases as the concentration of complex **1** in the reaction mixture increases. By contrast, at high concentrations of the Fe complex in the presence of Hb, at least 45% of NO per molecule of complex **1** are released, the 10% yield of NO (*i.e.*, the formation of 5 μmol L^{-1} of $Hb(NO)_4$) being achieved only after 10 min (see Fig. 5). This indicates that the formation of NO does not occur according to the simplest kinetic scheme involving the monomolecular decomposition of the starting complex **1** followed by its binding to Hb.

To explain the difference in the data obtained in the two test systems for the determination of the NO-donor

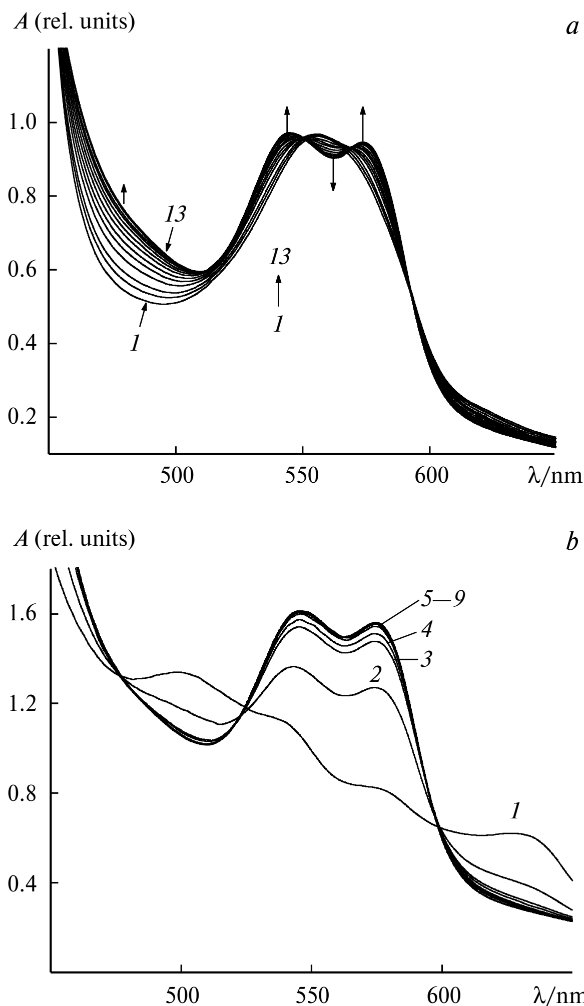


Fig. 4. Changes in the difference absorption spectra for the reaction of complex **1** (0.2 mmol L^{-1}) with Hb (0.022 mmol L^{-1}) (*a*) and metHb (0.02 mmol L^{-1}) (*b*): *a*, the spectra recorded within 1 (*1*), 3 (*2*), 5 (*3*), 10 (*4*), 15 (*5*), 20 (*6*), 25 (*7*), 30 (*8*), 35 (*9*), 40 (*10*), 43 (*11*), 49 (*12*), and 53 min (13) after the beginning of the reaction; *b*, the spectrum of the starting metHb (0.032 mmol L^{-1}) (*1*) and the spectra recorded within 0.5 (*2*), 3 (*3*), 5 (*4*), 8 (*5*), 11 (*6*), 14 (*7*), 17 (*8*), and 20 min (*9*) after the beginning of the reaction. A phosphate buffer (0.05 mol L^{-1}, pH 7.0) containing DMSO (3.3%) was used as the solvent; 25 °C.

ability, it was interesting to study the reaction of Hb with the oxidized form of complex **1**. For this purpose, we used metHb, which can act as the oxidant with respect to complex **1** because its standard redox potential is 0.085 V (see Ref. 29).

To study the reaction of complex **1** with metHb under anaerobic conditions at 25 °C, we recorded the time-resolved difference absorption spectra (see Fig. 4, *b*) using a cell containing the reaction system and a reference cell containing complex **1** at the same concentration. Then the absorption spectra were decomposed into the absorption spectra of Hb, $Hb(NO)_4$, and metHb. It was found

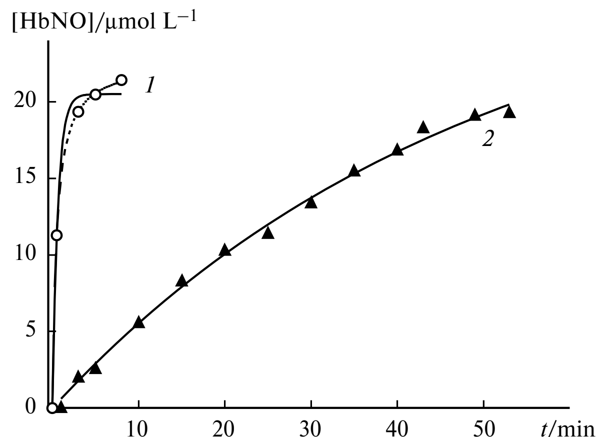


Fig. 5. Kinetic curves of the formation of HbNO in the reaction of complex **1** (0.2 mmol L^{-1}) with metHb (0.022 mmol L^{-1}) based on the experimental data presented in Fig. 4, *b* (*1*), and in the reaction of complex **1** (0.2 mmol L^{-1}) with Hb (0.022 mmol L^{-1}) based on the experimental data presented in Fig. 4, *a* (*2*). The points correspond to the experimental data, the solid lines represent the approximation of the kinetic curves by the function $y = A[1 - \exp(-k_1 t)]$, the dashed line represents the approximation of the kinetic curve by the hyperbolic function $[HbNO]_t = Bkt/(1 + kt)$.

that the reaction affords $Hb(NO)_4$ (absorption maxima at λ = 545 and 575 nm) rather than $metHb(NO)_4$ and that the intermediate Hb is not accumulated (its concentration is within experimental error, 0.5 μmol L^{-1}). The kinetic curve of the formation of $Hb(NO)_4$ is well described by the hyperbolic function (see Fig. 5):

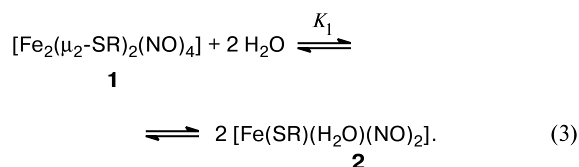
$$[Hb(NO)_4]_t = Bkt/(1 + kt), \quad (2)$$

where the constant k is $(32.9\pm0.6)\cdot10^{-3}$ s^{-1}, and B = = 22.6±0.08 μmol L^{-1} is almost equal to the starting concentration of metHb. Therefore, the initial rate of the formation of $Hb(NO)_4$ is (0.74±0.02) μmol L^{-1} s^{-1}. Taking into account the estimate of the the maximum possible concentration of Hb (<0.5 μmol L^{-1}) and the large constant k_{Hb}, this initial rate is provided at a relatively low NO concentration (~0.004 μmol L^{-1}). This suggests that the quasi-steady-state condition for NO (see Eq. (1)) is met in the experiments with metHb as well. Hence, the rate of the formation of $Hb(NO)_4$ is equal to the rate of NO release, and its initial value (~0.7 μmol L^{-1} s^{-1}) is very high.

The kinetic curves for the formation of NO (see Fig. 2) clearly show short induction periods (τ) of a few second (see the inset in Fig. 2). For other related iron nitrosyl complexes, the induction periods are longer.[30] The appearance of induction periods provides direct evidence that NO is formed not directly from the starting complex (**1**) but from the transformation products of **1** in an aqueous solution.

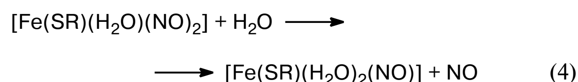
It seems reasonable that the rapid dissociation of dinuclear complex **1** into two mononuclear complexes

$[Fe(SR)(H_2O)(NO)_2]$ (**2**) in aqueous solutions is responsible for the induction period:



It should be assumed that molecules of the medium (for example, of water) are involved in this reaction. These molecules occupy coordination sites instead of the bridging thiolate ligands that are eliminated. Since the induction periods are substantially shorter than the time of the transformation of complex **1** accompanied by the NO release, the dissociation (3) at times larger than τ can be considered as the equilibrium reaction.

It should be noted that the direct investigation by high-resolution electrospray ionization mass spectrometry of aqueous solutions of the related complex with the benzenethiol bridging ligand revealed[31] the presence of substantial amounts of mononuclear dinitrosyl complexes. The quantum chemical calculations for the complex $[Fe_2(\mu_2\text{-}SPh)_2(NO)_4]$ provide direct evidence for the high stability of the dinuclear complex because the bond energy of the NO ligand after the dissociation according to Eq. (3) decreases by 3.7 kcal mol^{-1}. At the same time, the results of calculations[31] showed that the elimination of the nitrosyl ligand from the dinitrosyl complex requires much lower energy (lower by more than 10 kcal mol^{-1}), which can be compensated by the binding of a solvent (water) molecule instead of the NO group. Hence, the simplest decomposition reaction of the mononuclear complex



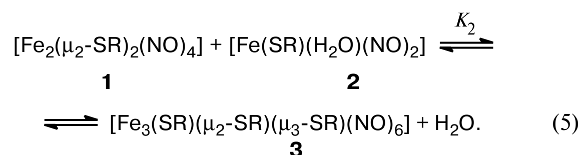
is endothermic.

An in-depth consideration of the kinetic curves given in Fig. 2 also shows that this reaction is not the major pathway of the formation of NO. Actually, as can be seen from the kinetic curves, the initial relative rates (the rate constant k') of the formation of NO are

$$k' = \frac{1}{a_0}\frac{d[NO]}{dt}\bigg|_{t\to 0},$$

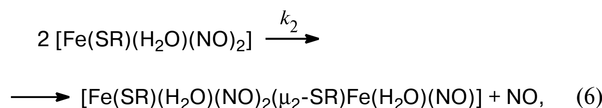
where a_0 is the initial concentration of complex **1**, strongly depend on a_0, the dependence being nonmonotonic. For the first-order reaction, the k' value should be constant, which is independent evidence that the starting dinuclear complex **1** does not serve as a source of NO. When taking into account the equilibrium (3) between the dinuclear and mononuclear complexes, it is seen that the fraction of the latter consistently decreases, which does not provide an explanation for the fact that the initial region of curve *2* (a_0 = 3.3 μmol L^{-1}) is located above the initial region of curve *1* (a_0 = 1.65 μmol L^{-1}) in Fig. 2.

To explain the characteristic features of the kinetic curves for the formation of complex **1**, it seems important that other related dinuclear iron nitrosyl complexes undergo both the dissociation and association to give mononuclear and polynuclear complexes, respectively.[32] Hence, in our opinion, both the dissociation and association of complex **1** can occur in aqueous solutions. Mononuclear complex **2** contains the weakly coordinated aqua ligand, and the thiolate ligands can act both as μ_2- and μ_3-bridges. Consequently, the primary association of complexes **1** and **2** giving the trinuclear complex $[Fe_2(\mu_2\text{-}SR)(NO)_4(\mu_3\text{-}SR)Fe(NO)_2]$ (**3**) seems to be the most probable reaction:



The preliminary quantum chemical modeling showed that complex **3** exists as the stable species, and the different Fe—S bond lengths with the μ_3-S atoms differ only slightly. Because of insufficient experimental data, we will consider only this association reaction.

The formation of NO in the reaction of two mononuclear complexes can be represented in a similar way:



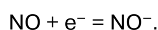
resulting in that the terminal ligand SR of one of the complexes becomes bridging and displaces the NO molecule into the solution. We hypothesize that this reaction is responsible for the formation of NO. As will be shown below, this hypothesis provides explanation for the main features of the kinetics of the NO release (see Fig. 2).

The observed maximum NO concentrations during the decomposition of complex **1** are substantially lower than the stoichiometric values (see Fig. 2) corresponding to Eq. (6).

This means that the NO molecule is subjected to further transformations in a solution. In some cases,[19] the kinetic curves of the accumulation of NO have maxima, which is direct evidence that reactions consuming NO proceed.

Let us consider the nature of possible reactions resulting in the disappearance of NO in the system. Based on the fact that in some studies[33,34] nitrous oxide was detected in solutions of iron dinitrosyl complexes, it can be concluded that N_2O is the final product of the transformation of NO. This is definitely indicative of the formation of the N—N bond. Two NO molecules do not form strong bonds with each other. At the same time, it is known that the

reaction of NO with NO^- proceeds with a high rate.[35] This reaction affords the hyponitrite radical anion $ONNO^-$, which is a strong oxidizer and which undergoes further transformations to give nitrous oxide.[36] The competitive pathway for the formation of N_2O by the recombination of two nitroxyls HNO is less probable because the protonation of NO^- is spin-forbidden. In both cases, the reduction of NO molecules is the most probable first elementary event of the reaction, which then gives nitrous oxide:

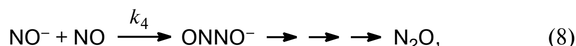


$NO + e^- = NO^-$.

The formation of the NO^- anion from the NO molecule requires the presence of a reducing agent. The standard redox potential of NO is approximately –0.8 (±0.2) V (see Ref. 37). Apparently, the iron nitrosyl complex LFe (L = $Fe(SR)(H_2O)(NO)_2(\mu_2\text{-}SR)(H_2O)(NO)$) by itself serves as a reducing agent after the dissociation of the Fe—NO bond, resulting in the occupation of the vacant coordination site thus arised by a water molecule. The reducing properties of this complex are stronger than those of the starting complex due to an increase in the electron density on the Fe atom as a result of both the loss of the NO ligand bearing a partial negative charge[38] and the coordination of the donor water molecule. Therefore, there are the following two reactions, in which NO is consumed: the primary reaction (the electron transfer from the complex to NO giving the complex LFe_{ox})

$$LFe + NO \xrightarrow{k_3} LFe_{ox} + NO^- \quad (7)$$

and the secondary reaction



$$NO^- + NO \xrightarrow{k_4} ONNO^- \rightarrow \rightarrow \rightarrow N_2O, \quad (8)$$

which occurs only in the case of deeper degrees of the transformation of the complex, when a sufficient amount of the reduced form NO^- is accumulated in reaction (7).

In the initial steps of the decomposition of the complex, reaction (8) can be ignored. As a result, we obtain the following kinetic equation for NO:

$$\frac{d[NO]}{dt} = k_2x^2 - k_3b[NO], \quad (9)$$

where x is the concentration of complex **2** and b = [LFe]; according to the stoichiometry of reactions (6) and (7), [LFe] = [NO]. Since we consider the kinetics of the formation of NO at moderate degrees of the conversion of the complex and ignore the perturbing effect of secondary reactions (reaction (8)), the time dependence of a can also be ignored. In this case, the kinetic equation takes a particularly simple form*

$$\frac{d[NO]}{dt} = k_2(x_0)^2 - k_3[NO]^2, \quad (10)$$

where x_0 is the initial concentration of complex **2**. Equation (10) has the following solution:

$$\frac{[NO]}{a_0} = Y\frac{\exp(2k't/Y) - 1}{\exp(2k't/Y) + 1}, \quad (11)$$

where

$$Y = \frac{x_0}{a_0}\sqrt{\frac{k_2}{k_3}},\ k' = \frac{(x_0)^2k_2}{a_0}.$$

It should be noted that, due to the high rate of reaction (8), it can be assumed that the concentration of the NO^- anion rapidly reaches the quasi-steady-state value $d[NO^-]/dt \approx 0$. Taking into account Eqs (7) and (8), we obtain the following equation:

$$-k_4[NO][NO^-] = -k_3[NO]b,$$

i.e., the rate of the consumption of NO in reaction (7) is equal to that in reaction (8). In terms of the above approximations, this relationship adequately accounts for the contribution of reaction (8) to the kinetic equation for [NO]:

$$\frac{d[NO]}{dt} = k_2(x_0)^2 - 2k_3[NO]^2,$$

which takes the same mathematical form as Eq. (10). Hence, it can be assumed that the coefficient of the square term $[NO]^2$ in this equation is the effective rate constant characterizing the consumption of NO both in the primary and secondary reactions. From this we obtain the dependence of k_3 on the concentration of the Fe complex under consideration.

Based on the above approximations, this kinetic dependence is applicable to the description of the experimental data only at short times of the conversion. Hence, let us expand the exponential term in Eq. (11) in a series and take into account only the linear terms for t. This procedure gives the hyperbolic dependence for the description of the kinetics of the formation of NO:

$$\frac{[NO]}{a_0} = Y\frac{k't}{Y + k't}. \quad (12)$$

Equation (12) adequately describes, within experimental error, the experimental kinetic curves in the regions outside the induction periods (see Fig. 2). The maximum yields Y and the highest relative initial rates k' are listed in Table 1. For comparison, this table gives the parameters of the kinetic curve described by Eq. (11) and the simple exponential approximation

$$\frac{[NO]}{a_0} = Y[1 - \exp(-k't/Y)]. \quad (13)$$

It can easily be seen that the ratio of functions (11) and (13) is 1 at short and very long times, and the maximum

* It should be noted that if the percentage of mononuclear complexes in the starting mixture is low, their consumption in the reaction resulting in the NO release is partially compensated by the dissociation of dinuclear complex **1**, and, consequently, the rate of the NO release remains constant over long periods of time.

Table 1. Different approximations of the kinetic curves for the formation of NO in the absence of Hb at different initial concentrations (a_0) of complex **1** (see Fig. 2)

a_0/μmol L^{-1}	Eq. (11)		Eq. (12)			Eq. (13)	
	Y	$k' \cdot 10^3$/s^{-1}	Y	$k' \cdot 10^3$/s^{-1}	k_3/L mol^{-1} s^{-1}	Y	$k' \cdot 10^3$/s^{-1}
1.65	0.405	2.39	0.548	3.47	7.0	0.406	2.44
3.3	0.282	2.99	0.334	5.43	14.8	0.274	3.38
6.6	0.140	1.95	0.186	3.19	14.0	0.133	1.63

discrepancy (~15%) is observed in the intermediate region $k't \sim Y$. Hence, the exponential approximations rather adequately describe the experimental data, and their parameters differ only slightly (see Table 1). However, as can be seen from Fig. 2, the use of Eq. (13) instead of the hyperbolic function (12) leads to systematic errors in the description.

The bimolecular constant $k_3 = k'/(a_0Y^2)$ is unambiguously determined by the parameters k', a_0, and Y^2. As can be seen from Table 1, the parameter k_3 varies, but the character of these changes is consistent with the proposed kinetic model. Thus, the parameter k_3 increases with increasing concentration of the complex by a factor of 2 and then remains constant. To find out whether the parameter k_2 retains the assumed constant value, it is necessary to find the dependence of the degree of dissociation of complex **1** (x_0/a_0) on its concentration in the solution. The latter is determined by equilibria (3) and (5). We assume that these equilibria are also rapidly established. Then the concentrations of mononuclear complex **2** (x_0) and trinuclear complex **3** (y_0) with account for the mass balance for Fe can be determined from the system of equations

$$x_0^2 = K_1(a_0 - x_0/2 - 3y_0/2),$$

$$y_0 = K_2x(a_0 - x_0/2 - 3y_0/2).$$

From this system, we obtain the equation for the estimation of x_0:

$$(3K_2/2)x_0^3 + x_0^2 + (K_1/2)x_0 - K_1a_0 = 0, \quad (14)$$

in which the constant water concentration is included in the constants K_1 and K_2, and, consequently, K_1 and K_2^{-1} have the same dimensionality (mol L^{-1}).

The solution of cubic equation (14) takes a simple form both at small and large values of a_0:

$$x_0 \sim 2a_0,\ a_0 << K_1;$$

$$x_0 \sim [2K_1a_0/(3K_2)]^{1/3},\ a_0 >> K_1. \quad (15)$$

It is noteworthy that at low concentrations of complex **1**, the latter almost completely dissociates ($x_0/a_0 \sim 2$), and then the degree of dissociation decreases with increasing a_0 according to the function $(a_0)^{-2/3}$. The intermediate region between the limiting cases is observed at $a_0 \sim [K_1/(12K_2)]^{1/2}$. As can be determined from Table 1, $(x_0/a_0)\sqrt{k_2} \equiv \sqrt{k_3}$ is 1.45, 1.28, and 0.70 L$^{1/2}$ mmol$^{-1/2}$ s$^{-1/2}$ at a_0 = 1.65, 3.3, and 6.6 mmol^{-1} L^{-1}, respectively. The ratio x_0/a_0 is approximately constant at $a_0 \le 3.3$ μmol^{-1} L^{-1} and substantially decreases at $a_0 \ge 3.3$ μmol^{-1} L^{-1}. Therefore, based on the asymptotic estimates (Eq. (15)), it can be assumed that $x_0/a_0 \approx 2$ at $a_0 < 3.3$ μmol^{-1} L^{-1} and $x_0/a_0 \approx (a_0)^{-2/3}$ at $a_0 > 3.3$ μmol L^{-1}. The bimolecular constant k_2 can be evaluated as ~ 0.5 L mmol^{-1} s^{-1}, and it can be shown that the value $x_0/a_0\sqrt{k_2}$ = 0.81 L$^{1/2}$ mmol$^{-1/2}$ s$^{-1/2}$ calculated from this constant at a_0 = 6.6 mmol^{-1} L^{-1} is in good agreement with the value of 0.70 L$^{1/2}$ mmol$^{-1/2}$ s$^{-1/2}$ determined from the experimental data. A more pronounced decrease in the ratio x_0/a_0 compared to the proposed scheme of two equilibria can be attributed to the subsequent association reactions, which lead to an additional decrease in the fraction of mononuclear complexes in solution.

It should be noted that, as follows from the proposed kinetic model, the relative initial rate nonmonotonically changes, $k' \approx 4k_2a_0$ increases at small a_0 and

$$k' \approx k_2\sqrt[3]{\frac{1}{a_0}\left(\frac{2K_1}{3K_2}\right)^2} \quad (16)$$

decreases at large a_0. This was observed experimentally. According to this estimate, k' decreases by approximately a factor of four as the concentration of complex **1** increases from 3.3 to 200 μmol L^{-1} (the concentration of complex **1**, which was used in the experiments with Hb). This gives the initial rate of the formation of NO equal to 0.27 μmol L^{-1} s^{-1}. By contrast, the fourfold initial rate of the accumulation of $Hb(NO)_4$ in the experiments with Hb is substantially smaller (0.033 μmol L^{-1} s^{-1}).

Experiments performed at other concentrations of complex **1** (50 and 800 μmol L^{-1}) in the presence of Hb (Figs 6 and 7, respectively) revealed the factors responsible for this difference. At concentrations of complex **1** equal to 50 and 200 μmol L^{-1}, the accumulation of $Hb(NO)_4$ occurs slowly at comparable rates; however, at [**1**] = = 800 μmol L^{-1} the rate of this reaction sharply increases due to which it is difficult to experimentally measure the initial rate. Therefore, it should be assumed that in the presence of Hb, there are additional factors responsible for a decrease in the current concentration of complex **1**. The

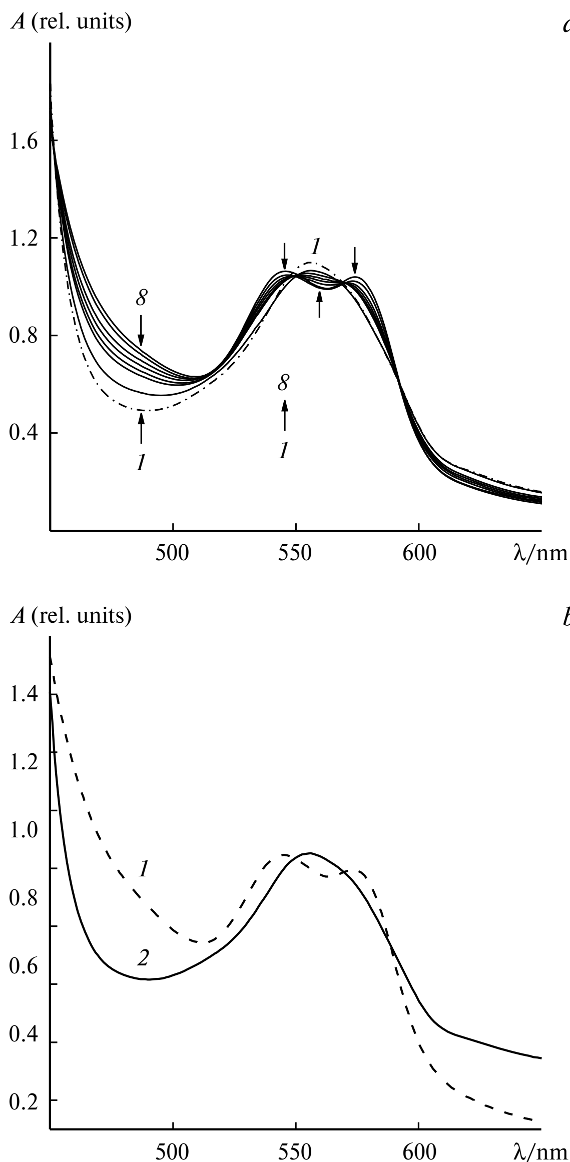


Fig. 6. Difference absorption spectra for the reaction of complex **1** at different concentrations with Hb (0.022 mmol L^{-1}): *a*, [**1**] = = 0.05 mmol L^{-1}; the spectrum of Hb (*1*) and the spectra recorded within 0.3 (*2*), 1.8 (*3*), 2.8 (*4*), 4.3 (*5*), 6.3 (*6*), 8.8 (*7*), and 11.8 h (*8*) after the beginning of the reaction; *b*, [**1**] = 0.08 mmol L^{-1}; the spectrum recorded within 0.5 min after the beginning of the reaction (*1*) and the spectrum of Hb (*2*). The reaction conditions are given in the caption to Fig. 4.

most probable factor is the binding of the iron nitrosyl complex to the functional groups on the surface of the Hb globule. This property of iron nitrosyl complexes has been mentioned in our previous studies.[39,40] Evidently, the binding will influence the NO-donor ability of iron nitrosyl complexes. Hence, in the presence of hemoglobin, NO would be expected to be formed in two reactions, in which the starting complexes that are present in the solution and the new complexes that are formed as a result of the binding of the initial complexes to Hb are decomposed. To distinguish between these pathways, it is necessary to perform delicate experiments. For example, it can be expected that these reactions will differ in the activation energy. In this case, studies of the temperature dependences of the kinetic parameters will allow definite conclusions to be drawn. Anyway, the immobilization of nitrosyl complexes on a protein globule should lead to a decrease in the rate of their association. Hence, it is necessary to know to which degree the formation of NO occurs in the presence of Hb in solution, because the association of the dissolved complexes in competitive reactions can give the undesired product $[Fe_4S_3(NO)_7]^-$ having the pronounced cytotoxic activity.[41] Based on our assumption about the bimolecular character of the elementary event giving NO in a solution, the following qualitative conclusion can be drawn. In the case of the rather high binding constant of the complexes to Hb, the concentration of the complexes in a solution sharply decreases and, correspondingly, the rate of NO release decreases. However, the complexes bound to Hb can serve as a buffer capable of maintaining an approximately constant concentration of the starting dinitrosyl complexes during their consumption as a result of the NO release. In this case, the formation of NO from the dissolved complexes is also described by the effective first-order kinetic curve, as in the case of the NO release from the immobilized complexes. Let us consider whether the kinetic characteristics of the formation of NO in the presence of Hb can be adequately described if the NO-donor ability of the complexes bound to Hb is ignored.

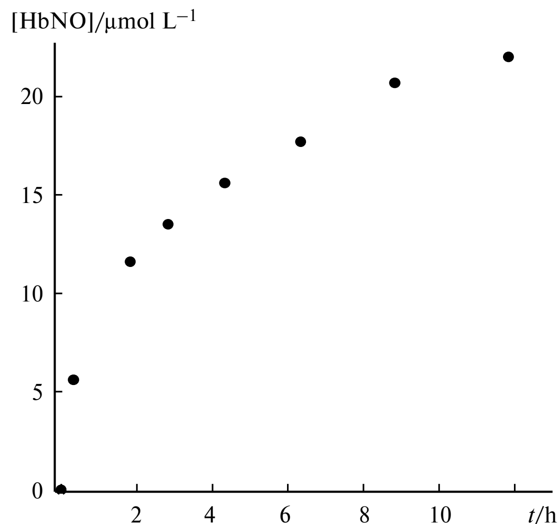


Fig. 7. Kinetics of the formation of HbNO in the reaction of complex **1** (0.05 mmol L^{-1}) with Hb (0.022 mmol L^{-1}) based on the data presented in Fig. 6, *a*. The points correspond to the experimental data.

The results of research on the accumulation of $Hb(NO)_4$ at different concentrations of complex **1** correspond to different modes of the reaction: $a_0 < a_c$ and $a_0 > a_c$, where

a_c is the critical concentration of complex **1** (200 μmol L^{-1} < < a_c < 800 μmol L^{-1}) sufficient for the occupation of all binding sites. Complex **1** is in the equilibrium with mononuclear complexes **2**, and the latter are most suitable for the binding to Hb because they contain a weakly coordinated ligand (a solvent molecule). This conclusion is consistent with the observation that the concentration of complex **1** (50 μmol L^{-1}) is already sufficient for the complete nitrosylation of Hb at a concentration of 22 μmol L^{-1}. In this case, the release of approximately two NO molecules from complex **1** and only one NO molecule from complex **2** are required.

The kinetics of the formation of $Hb(NO)_4$ at a low concentration of **1** cannot be adequately described by simple two-parameter functions (see Fig. 7), either exponential or hyperbolic. This fact can be qualitatively explained taking into account the main reaction resulting in the NO release (Eq. (6)), which makes it possible to perform the nitrosylation of only half of Hb molecules, whereas the complete nitrosylation requires further transformations of the iron complex that is formed in reaction (6). Evidently, the kinetic parameters of these transformations are different. Hence, let us use the simple estimate for the initial rate of the formation of NO assuming the linear character of the kinetic curve up to the first experimentally measured point. This estimation gives $19 \cdot 10^{-3}$ μmol L^{-1} s^{-1}, which is smaller than the concentration of complex **1** (200 μmol L^{-1}) by a factor of 1.75. These results are in qualitative agreement with the model under consideration. Since the amount of free (nonbonded) complex **1** is small, the latter exists completely in the dissociated state, and, consequently, the rate of the formation of NO is proportional to the concentration of **1** in the solution. However, the total amount of free complex **1** increases with increasing concentration of **1** (a_0) in the system but not proportionally to a_0. Consequently, the rate of the formation of NO increases by only a factor of 1.75 when the concentration of complex **1** increases by a factor of 4 (see Fig. 2).

For the quantitative description, let us assume that n functional groups are located on the surface of the Hb globule, and these groups undergo equilibrium binding to mononuclear complexes **2**. We will describe this process by the equation with the effective equilibrium constant K

$$z = K([\mathrm{Hb}] - z)(2a - nz)^n, \quad (17)$$

where z is the concentration of Hb containing all filled functional groups and a is the concentration of complex **1**. The coefficient 2 in Eq. (17) takes into account the fact that the highest possible concentration of complex **2** is two times larger than a. It can be shown that Eq. (17) has two types of solutions:

$$(2a - nz) \sim 0$$

at small a;

$$[\mathrm{Hb}] - z \sim 0$$

at large a; consequently,

$$(2a - nz) \sim \{2a/[K(n[\mathrm{Hb}] - 2a)]\}^{1/n} \quad (18)$$

at na < [Hb]/2;

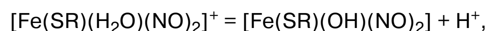
$$([\mathrm{Hb}] - z) \sim [\mathrm{Hb}]/[K(2a - n[\mathrm{Hb}])^n]$$

at na > [Hb]/2.

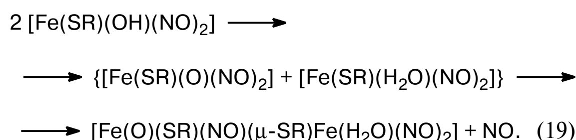
The numerical validation showed that Eq. (18) is a rather good approximation of the exact solution. At low concentrations of complex **1**, the initial rate of the formation of NO in the system is $k_2(2a - nz)^2$. The ratio of these rates at a = 200 and 50 μmol L^{-1} is consistent with the experimental ratio of 1.75 for n = 18. From this it can easily be determined that only 25% of complex **1** binds to Hb at a concentration of **1** equal to 800 μmol L^{-1}, and the concentration of complex **1** in solution is 597 μmol L^{-1}. With the use of the asymptotic estimation (Eq. (16)) for this concentration, we obtain the initial rate of the formation of NO equal to ~0.56 μmol L^{-1} s^{-1} and, correspondingly, the initial rate of the formation of $Hb(NO)_4$ is 0.14 μmol L^{-1} s^{-1}. It is difficult to experimentally estimate the latter value because the spectrum is recorded during 1 min and the absorption of Hb disappears within approximately 1 min after the mixing of the reagents. Hence, the initial rate of the formation of $Hb(NO)_4$ can be estimated at 0.35 μmol L^{-1} s^{-1}. Two estimates (theoretical and experimental) are in satisfactory agreement with each other.

In the experiments with metHb, a very high initial rate of the formation of NO is observed (see above), the kinetic curve of the accumulation of $Hb(NO)_4$ being also described by a hyperbolic function. This is direct evidence for the presence of a certain amount of complexes **1** in the solution, which exhibit different behavior in the presence of an oxidizing agent. The direct action of an oxidizing agent on the complexes bound to Hb requires further investigation.

We suggest the following interpretation of this result. First, metHb is a complementary oxidizing agent for the mononuclear complex and, in the presence of metHb, the latter is rapidly transformed into the oxidized form



which abstracts a proton in the presence of a base (a buffer). Second, in the oxidized state the neutral complexes undergo disproportionation, resulting in the NO release:



(19)

The deprotonated complexes are neutral and can undergo dimerization as a result of hydrogen bonding with

the participation of the coordinated hydroxyl ion. The intramolecular proton transfer between the hydroxy groups in this dimer facilitates the electron transfer between the ions of Fe-containing complexes, which is, in fact, the disproportionation reaction. An increase in the oxidation state of Fe by 2 in the complex $[Fe(SR)(O)(NO)_2]$ compared to the starting complex **1** leads to a decrease in its donor ability and a weakening of the Fe—NO bond. In addition, in the mixed-valence dinuclear complex $[Fe(SR)(O)(NO)_2]...[Fe(SR)(H_2O)(NO)_2]$, in which the oxo and aqua ligands are linked by a hydrogen bond, the vacant coordination site that is formed after the elimination of NO can be occupied as a result of the transformation of the terminal thiolate ligand into the bridging ligand. These facts, on the one hand, are responsible for the second-order kinetic curve and, on the other hand, cause an increase in the rate constant for reaction (19) compared to the rate constant for the formation of NO in the bimolecular reaction of the unoxidized complexes $[Fe(SR)(H_2O)(NO)_2]$.

* * *

In summary, we made an attempt to explain the effect of Hb on the kinetics of the formation of NO. In the absence of Hb, nitric oxide is accumulated in a solution as a result of the second-order reaction accompanied by the reduction of NO, in our opinion, by the iron nitrosyl complex, resulting in a decrease in the yield of NO. In the presence of Hb, the rapid and irreversible binding of NO molecules leads to such a substantial decrease in the NO concentration that further transformations of NO can be ignored. In addition, the reversible binding of iron nitrosyl complexes to the surface functional groups of Hb leads to a reduction in their concentration in a solution and a decrease in the rate of the formation of NO. Therefore, Hb has a dual effect. On the one hand, the accumulation of nitric oxide in the form of $Hb(NO)_4$ ensures its complete assimilation in further steps in the NO metabolism. On the other hand, Hb is a carrier (reservoir) of iron nitrosyl complexes stabilized on the surface of the Hb globule, and these complexes can directly act as NO donors after the desorption.

We thank B. L. Psikha for a deep and fruitful discussion.

This study was financially supported by the Division of Chemistry and Materials Science of the Russian Academy of Sciences (Program No. 9 "Medicinal and Biomolecular Chemistry").

References

1. R. Butler, I. L. Megson, *Chem. Rev.*, 2002, **102**, 1155.
2. A. S. Dutton, J. M. Fukuto, K. N. Houk, *Inorg. Chem.*, 2004, **43**, 1039.
3. A. S. Dutton, Ch. P. Suhrida, K. M. Miranda, D. A. Wink, J. M. Fukuto, K. N. Houk, *Inorg. Chem.*, 2006, **45**, 2448.
4. N. A. Sanina, L. A. Syrtsova, N. I. Shkondina, T. N. Rudneva, E. S. Malkova, T. A. Bazanov, A. I. Kotel´nikov, S. M. Aldoshin, *Nitric Oxide: Biol. Chem.*, 2007, **16**, 181.
5. O. I. Pisarenko, V. S. Shulzhenko, I. K. Faingold, R. A. Kotel´nikova, V. S. Romanova, N. A. Sanina, A. I. Kotel´nikov, S. M. Aldoshin, *Cardiovascular Ther. Prevent.*, 2006, **5**, 88.
6. L. I. Serebryakova, O. I. Pisarenko, O. V. Tskitishvily, I. I. Faingold, R. A. Kotel´nikova, V. S. Romanova, N. A. Sanina, A. I. Kotel´nikov, S. M. Aldoshin, E. I. Chazov, *Cardiovascular Ther. Prevent.*, 2006, **5**, 339.
7. O. S. Zhukova, N. A. Sanina, L. V. Fetisova, G. K. Gerasimova, *Ros. Bioterapevt. Zh.* [*Russ. Biotherapeutic J.*], 2006, **5**, 14 (in Russian).
8. A. A. Timoshin, A. F. Vanin, Ts. R. Orlova, N. A. Sanina, E. K. Ruuge, S. M. Aldoshin, E. I. Chazov, *Nitric Oxide: Biol. Chem.*, 2007, **16**, 286.
9. A. A. Timoshin, T. R. Orlova, A. F. Vanin, N. A. Sanina, E. K. Ruge, S. M. Aldoshin, E. I. Chazov, *Ros. Khim. Zh. (Zh. Vseros. Khim. Obshch. im. D. I. Mendeleeva)* [*Mendeleev Chem. J.*], 2007, **LI**, No. 1, 88 (in Russian).
10. L. M. Borisova, I. Yu. Kubasova, M. P. Kiseleva, Z. S. Smirnova, N. A. Sanina, S. M. Aldoshin, T. N. Rudneva, *Ros. Bioterapevt. Zh.* [*Russ. Biotherapeutic J.*], 2007, **6**, 42 (in Russian).
11. N. A. Sanina, L. A. Syrtsova, N. I. Shkondina, E. S. Malkova, A. I. Kotel´nikov, S. M. Aldoshin, *Izv. Akad. Nauk, Ser. Khim.*, 2007, 732 [*Russ. Chem. Bull.*, *Int. Ed.*, 2007, **56**, 761].
12. J. A. Montgomery, *Med. Res. Rev.*, 1982, **2**, 271.
13. I. A. Latham, G. J. Leigh, C. J. Pickett, G. Huttner, I. Jibrill, J. Zubieta, *J. Chem. Soc.*, *Dalton Trans.*, 1986, 1181.
14. A. Amici, M. Emanuelli, G. Magni, N. Raffaelli, S. Ruggieri, *FEBS Lett.*, 1997, **419**, 263.
15. M. E. Lizarrada, R. Navarro, E. P. Urriolabeitia, *J. Org. Chem.*, 1997, **542**, 51.
16. S. Raic-Malic, D. Svedruzic, T. Gazivoda, A. Marunovic, A. Hergold-Brundic, A. Nagl, J. Balzarini, E. De Clercq, M. Mintas, *J. Med. Chem.*, 2000, **43**, 4806.
17. A. N. Fattakhova, N. A. Nigmatzyanova, A. V. Lontsova, V. S. Reznik, *Uch. Zap. Kazan. Gos. Univ.*, *Ser. Estestv. Nauki* [*Scientific Proceedings of Kazan State University, Ser. Nat. Sci.*], 2005, **147,** 201 (in Russian).
18. N. N. Blokhin, N. I. Perevodchikova, *Khimioterapiya opukholevykh zabolevanii* [*Chemotherapy of Tumor Diseases*], Meditsina, Moscow, 1984 (in Russian).
19. N. A. Sanina, G. V. Shilov, S. M. Aldoshin, A. F. Shestakov, L. A. Syrtsova, N. S. Ovanesyan, E. S. Chudinova, N. I. Shkondina, N. S. Emel´yanova, A. I. Kotel´nikov, *Izv. Akad. Nauk, Ser. Khim.*, 2009, 560 [*Russ. Chem. Bull.*, *Int. Ed.*, 2009, **58**, 572].
20. E. Antonini, M. Brunori, *North-Holland Research Monographs. Fronties of Biology*, Eds A. Neuberger, E. L. Tatum, North-Holland Publ. Co., Amsterdam—London, 1971, **21**, 276.
21. V. V. Zinchuk, *Usp. Fiziol. Nauk* [*Adv. Physiol. Sci.*], 2003, **34**, 33 (in Russian).
22. A. N. Osipov, G. G. Borisenko, Yu. A. Vladimirov, *Uspekhi Biol. Chem.*, 2007, **47**, 259 [*Biochemistry* (*Moscow*), *Special Issue*, *Biol. Chem. Rev.* (*Engl. Transl.*), 2007, **72**, 1491].
23. E. S. Kang, D. E. Miles, M. T. Tevlin, T. B. Cates, S. R. Acchiardo, *Am. J. Med. Sci.*, 2001, **321**, 113.

24. A. Wennmalm, G. Benthin, A. Edlund, L. Jungersten, N. Kieler-Jensen, S. Lundin, U. N. Westfelt, A. S. Petersson, F. Waagstein, *Circ. Res.*, 1993, **73**, 1121.
25. M. F. Perutz, *Nature*, 1996, **380**, 205.
26. N. A. Sanina, S. M. Aldoshin, T. N. Rudneva, N. I. Golovina, G. V. Shilov, Yu. M. Shul´ga, V. M. Martynenko, N. S. Ovanesyan, *Koord. Khim.*, 2005, **31**, 323 [*Russ. J. Coord. Chem.* (*Engl. Transl.*), 2005, **31**, 301].
27. A. Weissberger, E. Proskauer, J. A. Riddick, E. E. Toops, *Organic Solvents: Physical Properties and Methods of Purification*, Wiley, New York, 1955.
28. V. A. Klimova, in *Osnovnye mikrometody analiza organicheskikh soedinenii* [*Basic Methods of Organic Microanalysis*], Khimiya, Moscow, 1975, p. 21 (in Russian).
29. A. M. Miles, D. A. Wink, J. C. Cook, M. B. Grisham, *Methods Enzymol.*, 1996, **268**, 105.
30. N. A. Sanina, S. M. Aldoshin, O. S. Zhukova, N. P. Konovalova, in *Abstrs of the 1st European Chemistry Congress* (Budapest, Hungary, 27—31 August 2006), Budapest, 2006, p. 370.
31. N. A. Sanina, N. S. Emel´yanova, A. N. Chekhlov, A. F. Shestakov, I. V. Sulimenkov, S. M. Aldoshin, *Izv. Akad. Nauk, Ser. Khim.*, 2010, 1104 [*Russ. Chem. Bull.*, *Int. Ed.*, 2010, 1126].
32. N. A. Sanina, D. V. Korchagin, G. V. Shilov, A. V. Kulikov, A. F. Shestakov, I. V. Sulimenkov, S. M. Aldoshin, *Koord. Khim.,* 2010, **36**, 888 [*Russ. J. Coord. Chem.* (*Engl. Transl.*), 2010, **36**, 876].
33. Z. Huang, S. Shiva, D. B. Kim-Shapiro, R. P. Patel, L. A. Ringwood, C. E. Irby, K. T. Huang, C. Ho, N. Hogg, A. N. Schechter, M. T. Gladwin, *J. Clinic. Invest.*, 2005, **115**, 2099.
34. A. F. Vanin, *Biokhimiya*, 1998, **63**, 924 [*Biochemistry* (*Moscow*) (*Engl. Transl.*), 1998, **63**, 782].
35. K. A. Persall, F. T. Boner, *Inorg. Chem.*, 1982, **21**, 1978.
36. M. D. Bartberger, W. Liu, E. Ford, K. M. Miranda, C. Switzer, J. M. Fukuto, P. J. Farmer, D. A. Wink, K. N. Houk, *Proc. Nat. Acad. Sci. USA*, 2002, **99**, 10958.
37. S. V. Lymar, V. Shafirovich, G. A. Poskrebyshev, *Inorg. Chem.*, 2005, **44**, 5212.
38. G. A. Poskrebyshev, V. Shafirovich, S. V. Lymar, *J. Am. Chem. Soc.*, 2004, **126**, 891.
39. N. A. Sanina, L. A. Syrtsova, N. I. Shkondina, E. S. Malkova, A. I. Kotel´nikov, S. M. Aldoshin, *Izv. Akad. Nauk*, *Ser. Khim.*, 2007, 732 [*Russ. Chem. Bull.*, *Int. Ed.*, 2007, **56**, 761].
40. N. A. Sanina, L. A. Syrtsova, E. S. Chudinova, N. I. Shkondina, T. N. Rudneva, A. I. Kotel´nikov, S. M. Aldoshin, *Izv. Akad. Nauk, Ser. Khim.*, 2009, 554 [*Russ. Chem. Bull.*, *Int. Ed.*, 2009, **58**, 566].
41. A. Janczyk, A. Wolnicka-Glubisz, A. Chmura, M. Elas, Z. Matuszak, G. Stochel, K. Urbanska, *Nitric Oxide: Biol. Chem.*, 2004, **10**, 42.

Received October 26, 2010;
in revised form November 24, 2010