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Reduction of nitrates, the NO donors, by hemoglobin in the presence of cysteine

L. A. Syrtsova,★ A. I. Kotel´nikov, B. L. Psikha, E. S. Malkova, N. I. Shkondina, and E. I. Gainullina

Institute of Problems of Chemical Physics, Russian Academy of Sciences,
1 prosp. Akad. Semenova, 142432 Chernogolovka, Moscow Region, Russian Federation.
Fax: +7 (496) 515 5420. E-mail: syrtsova@icp.ac.ru

Hemoglobin (Hb) reduces 3,3-bis(nitroxymethyl)oxetane (NMO) only in the presence of cysteine (Cys) *via* intermediate cysteine thionitrate. The kinetics of the reaction of NMO with Cys and the kinetics and mechanism of formation of NO in the ternary system Hb—NMO—Cys were studied. The formation rate of Hb—NO in the ternary system is higher than that of Hb—NO in the reaction of Hb only with NO_2^- generated in the binary system NMO—Cys. The second-order rate constants of the main reaction steps in the system Hb—NMO—Cys were estimated by simulating the kinetics of the reactions with a system of equations taking into account equilibria between all components of the reaction mixture. Hemoglobin reduces cysteine thionitrate, the intermediate in the reaction of NMO with Cys, to NO.

Key words: hemoglobin, trinitroglycerol, thionitrate, vasodilation, cysteine, nitrite reductase.

Nitrogen monoxide (NO) is a unique biological regulator and acts as a mediator of intercellular interactions, a cytotoxic effector of the immune protection of an organism, a regulator of neurotransmission and immunity, and as an inhibitor of thrombocyte aggregation and their adhesion on walls of blood vessels.[1] Data on *in vivo* multifunctional biological activity of NO are used for the development of fundamentals of the design of new-generation drugs used in the therapy of cardiovascular and oncological diseases. The functions of NO are related primarily to regulation of the tone of blood vessels. The main mechanism of action of NO in this process involves coordination of NO by the heme of soluble guanylate cyclase. The activation of this important regulatory enzyme proceeds by the heme-dependent mechanism with accumulation of guanosine 3´,5´-cyclic monophosphate (cGMP). The latter activates cGMP-dependent protein kinase and Ca^{2+}-ATPase, which is, in turn, involved in dephosphorylation of myosin light chains, resulting in the release of Ca^{2+} from muscle cells and, finally, in vasodilation.[2,3]

Nitrates belong to the most well-known class of vasodilators, *viz*., NO donors. In the course of the biotransformation, nitrates are reduced to form NO_2^- and then NO. Three electrons are consumed for reduction of nitrates to NO: two electrons are involved in the transformation of the nitrate group into NO_2^-, and the third electron is used in the further transformation of NO_2^- into NO.[4] Since it is impossible to transfer three electrons in one elementary event, reduction of nitrates affords mainly NO_2^-. Living organisms contain a series of enzymatic and nonenzymatic reducing agents, which are involved in this reaction. For example, the nitrite ion is transformed into NO in the reaction with deoxyhemoglobin (Hb) and other heme-containing proteins as well as with xanthine oxidoreductase and various thiol-containing enzymes.[3] Extensive experimental data accumulated in the last five years provide evidence that NO_2^- present in blood at a concentration of 300 nmol L^{-1} (Ref. 5) is the greatest depot-form of NO in vessels and tissues. It is known that Hb is the most potent source of natural nitrite reductants (the concentration of nitrite in blood is 12 mmol L^{-1}; see Ref. 5). It is known[6] that Hb reduces trinitroglycerol (TNG) to NO_2^-. However, Hb reduces not all nitrates. Our experiments demonstrated that Hb does not reduce 3,3-bis(nitroxymethyl)oxetane (NMO). It was of interest to study this compound as a potential vasodilator, which can produce NO in the course of metabolism in the reaction with heme-containing proteins. In this connection, the aim of the present study was to examine the possibility of the involvement of Hb in reduction of nitrates.

Cysteine (Cys) was chosen as the reducing agent for the following reasons. This is an analog of the thiol cofactor of aldehyde dehydrogenase, which reduces nitrates (TNG) *in vivo* to NO_2^-.[7] In addition, the formation rate of NO_2^- from nitrates in the reaction with Cys correlates with their vasodilatory activity.[8] Finally, in the absence of Cys or other thiols, tolerance to nitrates is observed, which is undoubtedly indicative of their involvement in metabo-

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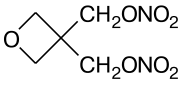
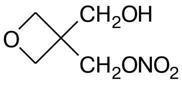
lism of nitrates.[9] Since NO_2^- can easily be reduced in an organism to NO, the nitrate—Cys system can serve as a test system for the assessment of the vasodilatory activity of nitrates. Although a number of enzymes reduce nitrates, thiols are necessary for the biotransformation pathway resulting in vasodilation. For example, there is evidence[10] that TNG and simple organic nitrates cannot activate soluble guanylate cyclase *in vitro* in the absence of thiols.

Experimental

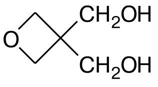
Materials. Commercial reagents cysteine (Serva, Germany), Sephadex G-25 (Pharmacia, Sweden), sodium dithionite (Merck, Germany), Silica gel 60 F_{254} TLC plates (Merck, Germany), sulfanilamide (SA, Sigma, USA), and *N*-(1-naphthyl)ethylenediamine (NEDA, MP Biomedicals, Germany) were used; DMSO (reagent grade, Khimmed, Russia) was additionally purified by distillation at 10 Torr, the fraction with b.p. 73—75 °C being collected. Water was purified by successive distillation in a D-4 distillator and a Pyrex-glass distillation apparatus Bi/Duplex (Germany). 3,3-Bis(nitroxymethyl)oxetane, 3,3-bis(hydroxymethyl)oxetane (MO), and 3-hydroxymethyl(3-nitroxymethyl)oxetane (NOO) were synthesized by A. M. Korolev at the Institute of Problems of Chemical Physics of the Russian Academy of Sciences according to a procedure described earlier.[11]



NMO



NOO



MO

Operations in a nitrogen atmosphere. All experiments described in the present study were carried out under nitrogen. High-purity nitrogen was additionally purified by passing through a column with a chromium—nickel catalyst at 20 °C. Air was removed from the working solutions (a buffer, DMSO, solutions of reactants) by evacuation and passage of nitrogen with magnetic stirring for 30—40 min. All vessels and quartz cells were sealed with rubber septa (Sigma, USA) allowing the introduction of a gas or required components through a needle. Solutions were transferred from one vessel to another using syringes with soldered needles, or the solutions were transferred under excess nitrogen pressure through two needles connected by Teflon capillaries. The excess pressure was discharged through another needle connected to a Teflon capillary immersed in water. Quartz cells and 4—14-mL vessels containing weighed samples of NMO or solutions of other reagents were purged with nitrogen through needles for 30 min.

Preparation of a human Hb solution. A homogeneous Hb sample was isolated from fresh human donor blood according to a known procedure[12] and was obtained as oxygenated Hb (HbO_2) in water at a concentration of 0.7 mmol L^{-1}. The latter was used for the preparation of Hb. A 0.05 *M* phosphate buffer, pH 7.0, was used in all steps of the further purification of Hb and in all experiments with Hb. To transform HbO_2 into Hb, a 2×15-cm column packed with Sephadex G-25 was prepared and transferred into an anaerobic state. For this purpose, one column volume (50 mL) of the anaerobic buffer and then the buffer (40 mL) containing a sodium dithionite solution (5 mL, 100 mg mL^{-1}) were passed through the column. The column was kept for 30 min and then washed from dithionite by eluting with the anaerobic buffer (50 mL) until the negative reaction for dithionite with methyl viologen was achieved.[13] A solution of freshly prepared HbO_2 (5 mL, 1.7 mmol L^{-1}) in the buffer was purged with nitrogen for 30 min with stirring, and a solution of dithionite (2 mL, 100 mg mL^{-1}) was added. The absorption spectrum of an aliquot of the solution was recorded to confirm that HbO_2 was converted into Hb. The spectrum of HbO_2 has two absorption peaks in the visible region at $\lambda_{max} = 541$ nm ($\varepsilon = 13.8$ $mmol^{-1}$ L cm^{-1}) and $\lambda_{max} = 577$ nm ($\varepsilon = 14.6$ $mmol^{-1}$ L cm^{-1}), whereas the absorption spectrum of Hb has one peak at $\lambda_{max} = 556$ nm ($\varepsilon = 12.5$ $mmol^{-1}$ L cm^{-1}) (ε are given per heme for human Hb[14]). Then excess dithionite and its decomposition products were removed on a column packed with Sephadex G-25. A Hb solution (5 mL) with a concentration of approximately 0.7 mmol L^{-1} was collected at the exit of the column. Hemoglobin was stored in the frozen state in liquid nitrogen as beads. Before use, Hb was thawed in 5-mL vessels under nitrogen. The nativity and homogeneity of Hb were confirmed by the coincidence of the extinction ratios for all absorption maxima with those published in the literature.

Kinetics of the reaction of NMO with Cys. The reaction was carried out in a 20-mL glass vessel equipped with a temperature-controlled jacket. A solution of Cys (20 mmol L^{-1}) was prepared from cysteine hydrochloride by dissolving it in a 0.05 *M* phosphate buffer (pH 7.0) followed by the addition of 1 *M* NaOH to pH 7.0. A freshly prepared Cys solution was used, its concentration being checked by titration with *p*-hydroxymercuribenzoate.[15] A solution of NMO (90 mmol L^{-1}) in DMSO was prepared. The reaction was initiated by the addition of the nitrate with magnetic stirring at 25 °C. The final concentration of DMSO was 3.3%. Aliquots of the reaction mixture (0.2 mL) were withdrawn with a syringe at certain intervals and frozen by injecting the aliquots into test tubes immersed in liquid nitrogen. Before analysis for NO_2^-, the tubes were kept at −18 °C.

Determination of NO_2^-. The amount of NO_2^- was determined by the Griess reaction with SA and NEDA.[16] Samples of the reaction mixture were thawn, and SA and NEDA solutions were added. The reaction was completed in 50 min at 20 °C. Then absorption at 548 nm was measured, and the NO_2^- concentration was calculated from the calibration curve that has benn plotted using sodium nitrite.

Kinetics of the reaction of Hb with NMO in the presence of Cys. All experiments were carried out under nitrogen at 25 °C. An Hb solution (0.7 mmol L^{-1}) was introduced into a 4-mL quartz cell with 1-cm path length to the concentration of 6.7—17.4 μmol L^{-1} in a 0.05 *M* phosphate buffer (pH 7.0), and then the absorption spectrum was recorded. Anhydrous DMSO was added under N_2 to a weighed sample of NMO to prepare a solution with a concentration of 90 mmol L^{-1}. An aliquot of a Cys solution (20 mmol L^{-1}) was added to the cell with Hb to the concentration of 10 mmol L^{-1}, and the reaction was initiated by the addition of an NMO solution in DMSO to the final concentration of 3 mmol L^{-1}. The concentration of DMSO in the reaction mixture was 3.3%. The absorption spectra were recorded immediately and then at specified intervals.

Determination of NO. The amount of NO produced in the reaction was estimated by spectrophotometry based on the amount of Hb—NO produced in the reaction. The absorption spectra were recorded on a Specord M-40 spectrophotometer equipped with a computer interface and a temperature-controlled cell compartment. Spectra were recorded at 25 °C. The Hb—NO concentration was estimated from the absorption spectrum of the reaction mixture containing Hb and Hb—NO by decomposition of the experimental absorption spectra into components (spectra of Hb and Hb—NO) by the least-squares method. This is the classical method for the solution of overdetermined systems of equations:

$$\sigma(x) = \sum_i \left\{D(\lambda_i) - \left[(1 - x)D_{\mathrm{Hb}}(\lambda_i) + xD_{\mathrm{Hb\text{-}NO}}(\lambda)_i\right]\right\}^2,$$

where $\sigma(x)$ is the rms deviation, D is the absorption of the tested solution at the wavelength λ_i, D_{Hb} and $D_{\mathrm{Hb-NO}}$ are the absorbances of Hb and Hb—NO solutions, respectively, at the wavelength λ_i, and x is the degree of conversion of Hb into Hb—NO. The calculations were carried out in the wavelength range of 450—650 nm based on 200 experimental points. The rms deviations $\sigma(x)$ for all runs were in the range $2 \cdot 10^{-5}$—$5 \cdot 10^{-6}$, which is indicative of a high quality of the simulation of the absorption spectra of the reaction mixture at each instant of time and a high accuracy of the determination of the Hb and Hb—NO concentrations. In the presence of methemoglobin (metHb) in the reaction system (as evidenced by the absorbance with the maximum at 630 nm), the spectrum was analogously decomposed into three components.

TLC analysis. The reaction mixture was analyzed by TLC using 195×195 mm silica gel plates. The separation was carried out in a chloroform—MeOH—25% ammonia system (6 : 3.5 : 1, v/v). Visualization was performed with UV light (λ = 254 nm).

Results and Discussion

To solve the problem at hand, we initially studied the kinetics of the formation of NO_2^- in the system NMO—Cys (Fig. 1). After 40 h, only NOO was detected by TLC (R_f = 0) in the reaction of NMO with Cys as a product of reduction of one nitrate group NMO. For NMO and MO, R_f were 0.31 and 0.87, respectively. Although only disulfide R′S—SR′ and NO_2^- were found as the reaction products of nitrate RO—NO_2 with thiols R′SH, it has been suggested that the reaction proceeds *via* intermediate cysteine thionitrate R′S—NO_2.[9,10] In the present study, we demonstrated that this reaction does proceed in two steps *via* intermediate cysteine thionitrate.

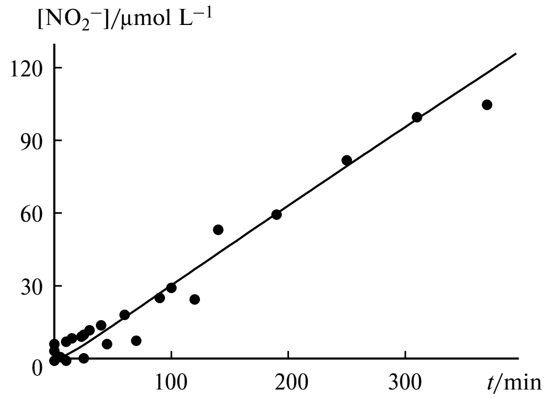


Fig. 1. Kinetics of accumulation of nitrite ions in the NMO—Cys system. Points correspond to experimental data; the line, to calculated data. Conditions: C_{NMO} = 3 mmol L^{-1}, C_{Cys} = 10 mmol L^{-1}; a 0.05 *M* phosphate buffer, pH 7.0, containing 3.3% DMSO as the solvent; T = 25 °C.

The reaction of NMO (RO—NO_2) with Cys (R′SH) was considered as a sequence of two bimolecular steps:[9] NMO reacts with Cys to give thionitrate R′S—NO_2 followed by reduction of the latter to NO_2^-:

$$\mathrm{RO{-}NO_2 + R'SH \xrightarrow{k_1} ROH + R'S{-}NO_2}, \tag{1}$$

$$\mathrm{R'S{-}NO_2 + R'S^- \xrightarrow{k_2} NO_2^- + R'S{-}SR'}. \tag{2}$$

It is known that the Cys molecule dissociates in an aqueous solution

$$\mathrm{R'SH \xrightleftharpoons{K} R'S^- + H^+}, \tag{3}$$

and pK of the SH group of cysteine is 8.2 (see Ref. 17; $K = 6.3 \cdot 10^{-9}$ mol L^{-1}). Experimental runs performed at different pH demonstrated that the accumulation rate of nitrite at pH 8.5 is 34 times higher than that at pH 7.0, whereas the reaction proceeds very slowly at pH 6.0 (Fig. 2). This is evidence that the thiolate ion R′S$^-$ serves as a reductant.

The system of equations corresponding to the reaction scheme (1)—(3), on condition that pH is constant and equal to 7.0 and taking into account the equilibrium K[R′SH] = [R′S$^-$][H$^+$], is as follows:

$$\frac{d[\mathrm{RONO_2}]}{dt} = -k_1[\mathrm{RONO_2}][\mathrm{R'SH}],$$

$$\frac{d[\mathrm{R'SH}]}{dt} =$$

$$= (-k_1[\mathrm{RONO_2}][\mathrm{R'SH}] - k_2[\mathrm{R'SNO_2}][\mathrm{R'S^-}])\frac{[\mathrm{H^+}]}{[\mathrm{H^+}] + K},$$

$$\frac{d[\mathrm{R'S^-}]}{dt} =$$

$$= (-k_1[\mathrm{RONO_2}][\mathrm{R'SH}] - k_2[\mathrm{R'SNO_2}][\mathrm{R'S^-}])\frac{K}{[\mathrm{H^+}] + K},$$

$$\frac{d[\mathrm{R'SNO_2}]}{dt} = k_1[\mathrm{RONO_2}][\mathrm{R'SH}] - k_2[\mathrm{R'SNO_2}][\mathrm{R'S^-}],$$

$$\frac{d[\mathrm{NO_2^-}]}{dt} = k_2[\mathrm{R'SNO_2}][\mathrm{R'S^-}].$$

The initial conditions: $[\mathrm{RONO_2}](0) = [\mathrm{RONO_2}]_0$; $[\mathrm{R'SNO_2}](0) = [\mathrm{NO_2^-}](0) = 0$; the initial concentrations

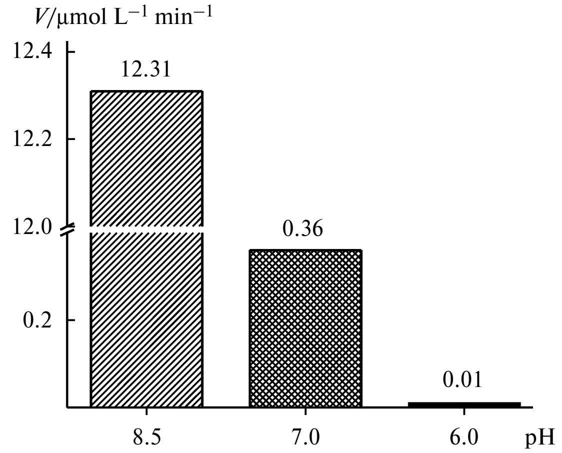


Fig. 2. Accumulation rates of nitrite ions in the NMO—Cys system at different pH. The conditions are given in the caption to Fig. 1.

$[R'SH](0)$ and $[R'S^-](0)$ can be determined from the equilibrium and material balance equations: $[R'SH](0) + [R'S^-](0) = [R'SH]_0$.

The unknown reaction rate constants k_1 and k_2 were estimated from the experimental plot $[NO_2^-](t)$ (see Fig. 1) by minimizing the functional:

$$F(k_1,k_2) = \sum_{j=1}^{N}([NO_2^-]_j^{\mathrm{exp}} - [NO_2^-]_j^{\mathrm{calc}})^2,$$

where N is the number of points in the run, and $[NO_2^-]_j^{\mathrm{exp}}$ and $[NO_2^-]_j^{\mathrm{calc}}$ are the experimental and calculated concentrations $[NO_2^-]$, respectively, at the moment t_j. The rate constants k_1 and k_2 (0.17 and $4.5 \cdot 10^{-3}$ mol^{-1} L s^{-1}, respectively) were determined by processing the experimental data (see Fig. 1).*

Then we studied the kinetics of NO formation in the ternary system Hb—NMO—Cys. Preliminarily, we found that, in the absence of Cys, Hb does not reduce NMO. Since Hb reduces NO_2^- to NO, we examined whether the amount of NO produced in the ternary system corresponds to the amount of NO_2^- produced in the reaction of NMO with Cys, *i.e.*, whether the formation of NO in the system Hb—NMO—Cys can be described by reactions (1)—(6).

$$NO_2^- + Hb + H^+ \xrightarrow{k_3} NO + metHb + OH^-, \qquad (4)$$

$$Hb + NO \xrightarrow{k_4} HbNO, \qquad (5)$$

$$metHb + R'SH \xrightarrow{k_5} Hb + R'S^{\bullet} + H^+. \qquad (6)$$

The kinetics of the reactions giving rise to NO in the ternary system Hb—NMO—Cys was studied. Deoxyhemoglobin, which was a component of the reaction system in the present study, was used for recording the kinetics of NO formation based on accumulation of Hb—NO. This was for several reasons. First, NO is bound to the hemes of Hb at a rate close to the diffusion rate,[14] the binding constant being $3 \cdot 10^{10}$ mol^{-1} L. Hence, Hb serves as a trap for NO. Second, Hb—NO has a characteristic absorption spectrum. The absorption peak of Hb is split into two peaks upon the formation of Hb—NO: λ_{max} = 545 nm (ε = 12.6 mmol^{-1} L cm^{-1}) and λ_{max} = 575 nm (ε = 13.0 mmol^{-1} L cm^{-1}) per heme.[14] We followed the kinetics of NO formation by recording accumulation of Hb—NO and decomposing the absorption spectra into components.

The changes in the absorption spectra of the reaction system containing Hb, NMO, and Cys are presented in Fig. 3. The kinetics of accumulation of Hb—NO in the ternary system Hb—NMO—Cys is presented in Fig. 4. As in the case of the binary system, we studied the reverse problem and estimated the second-order rate constants from the experimental data for the ternary systems. We considered the sequence of reactions (1)—(6). The second-order reaction rate constants k_1 and k_2 were determined for the binary system NMO—Cys as described above. The constants k_3 and k_5 were found from independent experiments (Fig. 5) by studying the kinetics of the reaction of Hb with sodium nitrite (see Fig. 5, *a*) and reducing metHb with Cys (see Fig. 5, *b*).

We did not make special attempts to describe the kinetics of the reaction of Hb with NO_2^- by one constant (this cannot be done) because it is known that Hb exists in two conformations (T and R), which differ in the redox potential and reactivity.[5] The reaction of Hb having the T conformation with NO_2^- proceeds under allosteric con-

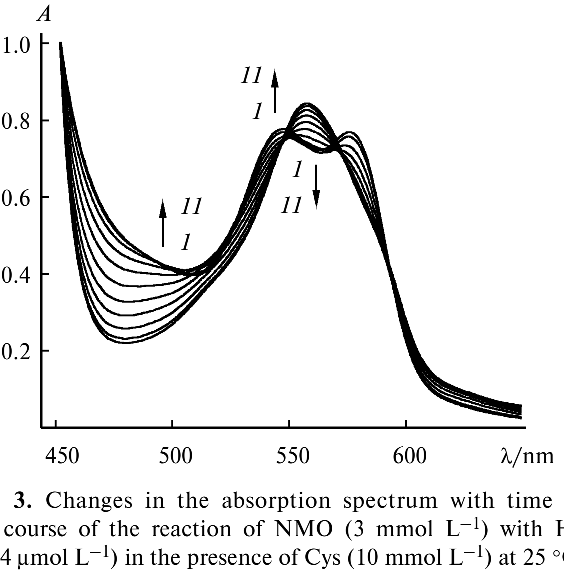


Fig. 3. Changes in the absorption spectrum with time in the course of the reaction of NMO (3 mmol L^{-1}) with Hb (17.4 μmol L^{-1}) in the presence of Cys (10 mmol L^{-1}) at 25 °C; a 0.05 *M* phosphate buffer, pH 7.0, containing 3.3% DMSO was used as the solvent. The first spectrum was recorded at the initial instant of time; the second spectrum, after 15 min; the other spectra, at 60-min intervals.

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* In other cases, the error was ±10%.

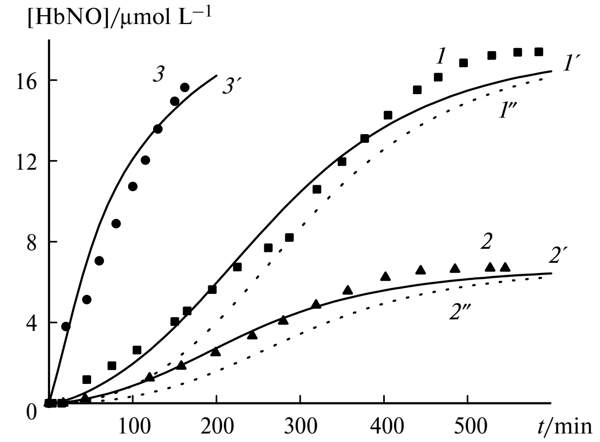


Fig. 4. Comparison of the experimental (*1—3*) points and the calculated (*1*′, *1*″, *2*′, *2*″, *3*′) accumulation curves of Hb—NO in the Hb—NMO—Cys system. The calculations were performed without considering (*1*″, *2*″) and taking into account (*1*′—*3*′) reaction (8). The conditions: C_{NMO} = 3 mmol L^{-1}; C_{Cys} = 10 mmol L^{-1}; C_{Hb} = 17.4 (*1*, *3*), 6.7 μmol L^{-1} (*2*). In the case of (*3*), preincubation of the NMO—Cys reaction system (0.05 *M* phosphate buffer, pH 7.0, containing 3.3% DMSO) for 10 h was followed by the addition of Hb to the concentration of 17.4 μmol L^{-1}.

trol, because the reaction of NO with the heme leads to the transformation of the whole Hb molecule into the more reactive conformation R.[5] Because of this, the reaction rate usually remains unchanged until 50% of the hemes in Hb are consumed in the reaction, and then the reaction rate increases. This was described for the reactions of Hb with both NO_2^- (see Ref. 5) and alkyl nitrites.[18]

In this connection, the equation for the rate constant k_3 was written as follows:

$$k_3(t) = k_{3.0} + k_{3.1}\left(1 - \frac{[\text{Hb}]_t}{[\text{Hb}]_0}\right), \qquad (7)$$

where $k_3(t)$ is the rate constant of the reaction of Hb with NO_2^- responsible for the overall reaction pathway, $[\text{Hb}]_0$ is the initial Hb concentration, $[\text{Hb}]_t$ is the running concentration of free Hb, and $k_{3.0}$ and $k_{3.1}$ are the reaction rate constants of the first and second steps, respectively. Equation (7) describes an increase in the constant k_3 with increasing degree of the transformation and includes two constants $k_{3.0}$ and $k_{3.1}$, which were estimated from an independent experiment on the kinetics of the reaction of Hb with $NaNO_2$ (see Fig. 5, *a*). The constants $k_{3.0}$ and $k_{3.1}$ are (5.8±0.7) • 10^{-1} and 1.3±0.2 mol^{-1} L s^{-1}, respectively.

The calculations demonstrated that all experimental data cannot be described by five constants k_1—k_5 (see Fig. 4).

k_1	k_2	$k_{3.0}$	$k_{3.1}$	k_4[14]	k_5
0.17	4.5 • 10^{-3}	(5.8±0.7) • 10^{-1}	1.3±0.2	10^8	0.043

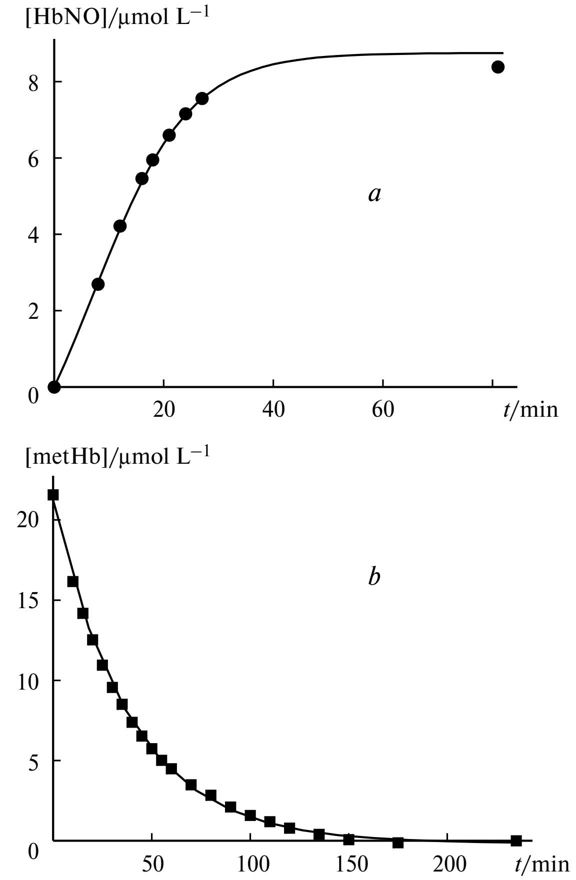


Fig. 5. Determination of the rate constants of reactions (4) and (5). Points correspond to experimental data; curves, to calculated data. (*a*) The kinetics of the reaction of Hb with $NaNO_2$. The Hb concentration was 17.5 μmol L^{-1}; the $NaNO_2$ concentration was 500 μmol L^{-1}. (*b*) The kinetics of reduction of metHb with cysteine. The metHb concentration was 36 μmol L^{-1}; the Cys concentration was 10 mmol L^{-1}. The conditions are given in the caption to Fig. 1.

The observed formation rate of Hb—NO in the ternary system is higher than the calculated rate assuming that Hb—NO is formed only in the reaction of Hb with NO_2^- produced in the binary system NMO—Cys. This effect can be attributed to the following three possible facts: 1) Cys increases the reaction rate of Hb with NO_2^-; 2) NO_2^- is reduced to NO in the binary system NMO—Cys; 3) Cys provides another reaction pathway of NMO with Hb. To test the first hypothesis, we studied the reaction of NO_2^- with Hb in the presence of Cys and found that the reaction rate constant remained unchanged. Consequently, this version can be discarded. To test the second possibility, we performed preincubation of NMO with Cys in a phosphate buffer, pH 7.0, containing DMSO at 25 °C, then added Hb (see Fig. 4, curve *3*), and immediately recorded the absorption spectrum of the reaction mixture. The Hb—NO complex was not detected. The concentration of the latter gradually increased (see Fig. 4, curve *3*). The formation rate of Hb—NO was higher than that in an analogous run without preincubation (see Fig. 4, curve *1*). Therefore, the second explanation can also be ruled out. To test the third hypothesis, *viz.*, the involvement of NMO in the reaction with Hb, we included reaction (8), *viz.*, reduction of the intermediate (cysteine thionitrate, R´S—NO$_2$) generated from NMO and Cys in reaction (2), in the sequence of reactions (1)—(6).

$$\mathrm{R'S{-}NO_2 + Hb \xrightarrow{k_6} NO + metHb + R'S^{\bullet}}. \qquad (8)$$

The system of equations corresponding to the overall reaction scheme (k_1—k_6), taking into account the equilibrium

$$K[\mathrm{R'SH}] = [\mathrm{R'S^-}][\mathrm{H^+}], \qquad (9)$$

the constancy of pH ([H$^+$] = const), and quasistationarity of the process with respect to [NO], can be written as follows:

$$\frac{d[\mathrm{RONO_2}]}{dt} = -k_1[\mathrm{RONO_2}][\mathrm{R'SH}],$$

$$\frac{d[\mathrm{R'SH}]}{dt} = k'\frac{[\mathrm{H^+}]}{[\mathrm{H^+}] + K},$$

$$\frac{d[\mathrm{R'S^-}]}{dt} = k'\frac{K}{[\mathrm{H^+}] + K},$$

$$k' = -k_1[\mathrm{RONO_2}][\mathrm{R'SH}] - k_2[\mathrm{R'SNO_2}][\mathrm{R'S^-}] - k_5[\mathrm{metHb}][\mathrm{R'SH}],$$

$$\frac{d[\mathrm{R'SNO_2}]}{dt} = k_1[\mathrm{RONO_2}][\mathrm{R'SH}] - k_2[\mathrm{R'SNO_2}][\mathrm{R'S^-}] - k_6[\mathrm{Hb}][\mathrm{R'SNO_2}],$$

$$\frac{d[\mathrm{NO_2^-}]}{dt} = k_2[\mathrm{R'SNO_2}][\mathrm{R'S^-}] - k_3[\mathrm{Hb}][\mathrm{NO_2^-}],$$

$$\frac{d[\mathrm{Hb}]}{dt} = -2k_3[\mathrm{Hb}][\mathrm{NO_2^-}] + k_5[\mathrm{metHb}][\mathrm{R'SH}] - 2k_6[\mathrm{Hb}][\mathrm{R'SNO_2}],$$

$$\frac{d[\mathrm{metHb}]}{dt} = k_3[\mathrm{Hb}][\mathrm{NO_2^-}] - k_5[\mathrm{metHb}][\mathrm{R'SH}] + k_6[\mathrm{Hb}][\mathrm{R'SNO_2}].$$

The initial conditions: [RONO$_2$](0) = [RONO$_2$]$_0$; [R´SNO$_2$](0) = [NO$_2^-$] = [metHb](0) = 0; [Hb](0) = [Hb]$_0$; the initial concentrations [R´SH](0) and [R´S$^-$](0) are not equal to their starting values [R´SH]$_0$ and to zero and can be calculated from the equilibrium condition (9) and the material balance equation at the initial instant of time: [R´SH](0) + [R´S$^-$](0) = [R´SH]$_0$.

The numerical solution of the system of equations allows the calculation of the experimental [HbNO](*t*) from the equation

$$\frac{d[\mathrm{HbNO}]}{dt} = k_3[\mathrm{Hb}][\mathrm{NO_2^-}] + k_6[\mathrm{Hb}][\mathrm{R'SNO_2}].$$

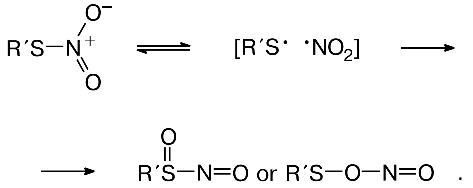
The unknown reaction rate constant k_6 was estimated from the minimization condition for the functional:

$$F_3(k_6) = \sum_{j=1}^{m} p_{ij} \sum_{i=1}^{n_j} ([\mathrm{HbNO}]_i^{\mathrm{exp}} - [\mathrm{HbNO}]_i^{\mathrm{calc}})^2,$$

where m is the number of runs, n_j is the number of points in the *j*th run, p_{ij} are the weighted coefficients, and $[\mathrm{HbNO}]_i^{\mathrm{exp}}$ and $[\mathrm{HbNO}]_i^{\mathrm{calc}}$ are the experimental and calculated concentrations [HbNO], respectively. The average constant $k_6 = (4.5\pm0.3)\cdot10^{-3}$ L mol^{-1} s^{-1} was determined by processing the experimental data (see Fig. 4).

Cysteine thionitrate was reduced to NO$_2^-$ (reaction (2)). However, the calculations showed that the inclusion of step (8) giving rise to NO$_2^-$ in the sequence of reactions (1)—(6) did not allow us to describe the experimental data presented in Fig. 4. At the same time, on the assumption that reaction (8) produces NO, the calculated curves correspond to the experimental points (see Fig. 4). A good agreement was achieved for a large region of the plot, and then the experimental reaction proceeds at a higher rate, which is characteristic of the reactions of Hb under allosteric control.

Let us consider how to represent one-electron reduction of thionitrate to NO. It was suggested[10] that thionitrate undergoes the so-called thionitrate rearrangement and is in equilibrium with sulfenyl or sulfinyl nitrite:



(10)

In the review,[10] the possibility of the production of NO from sulfenyl nitrite through homolytic decomposition was discussed. In some cases (most likely, this is associated with a decrease in pH to values lower than neutral pH or in the case of *tert*-butyl nitrate), a small amount of NO was detected in the reaction of nitrates with thiols. Under our experimental conditions, as mentioned above, NO is not formed in the reaction of NMO with Cys. It is quite probable that Hb exhibits high reactivity to sulfenyl nitrite because this compound is a structural analog of alkyl nitrites. Although Hb does not react with nitrates, *viz.*, alkyl nitrates, it exhibits high reactivity to alkyl nitrites.[18] The reaction rate constants for different alkyl nitrites vary from 14.5 to 1420 mol^{-1} L s^{-1}, which are substantially larger than $k_{3.1}$ for the reaction of Hb with NO_2^-. Interestingly, the products of rearrangement (10) contain trivalent nitrogen, indicating an intramolecular redox reaction. Apparently, due to the latter process, one electron from $R'S^-$ is sufficient for the generation of NO_2^- in reaction (2).

The rate constant for reaction (8) slightly changes with the Hb concentration. The question arises as to why the second-order reaction rate constant k_6 depends on the Hb concentration. In the case under consideration, Hb in the ternary system is coordinated to NO, NO_2^-, Cys and, probably, to NMO and cysteine thionitrate. All these interactions can occur simultaneously, resulting in a very complex system, which apparently influences the reaction rate constant k_6.

The above-described reaction of Hb with cysteine thionitrate is important for metabolism of nitrates. The fact that thiols are necessary for the biotransformation of nitrates remains an open question. This effect can be explained taking into account that Hb is the largest source of the natural reducing agent for NO_2^- and, in addition, provides the possibility of reducing apparently any nitrate in the presence of thiol *via* an intermediate (thionitrate).

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