Ferrocytochrome *c* and deoxyhemoglobin in the reaction with the iron cysteamine nitrosyl complex $\{Fe_2[S(CH_2)_2NH_3]_2(NO)_4\}SO_4 \cdot 2.5H_2O$

N. A. Sanina, L. A. Syrtsova, B. L. Psikha, N. I. Shkondina, T. N. Rudneva, 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. Fax: +7 (496) 522 3507. E-mail: syrtsova@icp.ac.ru

By an example of the iron cysteamine nitrosyl complex $\{Fe_2[S(CH_2)_2NH_3]_2(NO)_4\}SO_4 \cdot 2.5H_2O$ (CAC), it was shown for the first time that the hydrolysis of this NO donor in the presence of ferrocytochrome *c* (cyt c^{2+}) affords the iron nitrosyl complex NO-cyt c^{2+}, which serves as the NO depot. The rate constant of NO release from CAC was determined from the kinetics of the formation of NO-cyt c^{2+}. At pH 3.0 the rate constant is $(2.7 \pm 0.1) \cdot 10^{-3}$ s^{-1}. Ferrocytochrome *c* produces a less stabilizing effect on CAC than deoxyhemoglobin (Hb). Thus in the presence of cyt c^{2+}, the reaction is completed in 1 h, whereas NO is released from a solution of CAC ($2 \cdot 10^{-4}$ mol L^{-1}) in the presence of Hb during 40 h. The previously unknown stabilization of iron nitrosyl complexes by hemoglobin was found.

Key words: cytochrome *c*, iron nitrosyl complexes, NO donors, nitrosyl complexes of cytochromes.

Numerous studies in the recent past have shown that nitrogen monoxide (NO) is an important bioregulatory agent in various physiological processes.[1,2] This generated interest in the synthesis and investigation of new compounds that can easily deliver NO to biological targets at physiological pH (see Ref. 3) and, consequently, can serve as the basis for new generation drugs. Among these compounds there are the iron thiolate nitrosyl complexes $[Fe_2(SR)_2(NO)_4]$ (for example, R is benzimidazole, benzothiazole, or pyrimidine), which, like S-nitrosothiols[4] and dialkylaminodiazenium diolates (NONOates),[5,6] are hydrolyzed to form NO in protic media.[7] These complexes belong to a new class of universal NO donors for the pharmacological use.[8—10] Nitrogen monoxide plays a large role in the biology of cancer. This molecule can also act as an effector of antitumor protection and as the inducing agent of the neoplasia in the chronic inflammation or infection. Nitrogen monoxide can have the positive or negative feedback effect depending on its concentration in endothelial cells, the type of target cells, and the concentration of metal ions in the cells.[11] However, the concentration plays the major role. Thus nanomolar amounts of NO initiate apoptosis; in micromolar amounts, NO directly or indirectly causes the development of a malignant tumor through different mechanisms.[12] Hence, there are two approaches to the design of antitumor agents: NO synthase inhibitors and NO donors.

Previously, we have found that in the absence of additional activation, iron sulfur nitrosyl complexes decompose to release NO in protic media containing deoxyhemoglobin (Hb),[7,13] the reaction rate constants depending on the molecular structures of the complexes. It was found[7,13] that iron nitrosyl complexes nitrolyze Hb through the interaction with the Hb heme at the free coordination site 6. The HbNO complex serves as an NO depot. This complex not only provides the form of storage of NO (the lifetime of free NO in cells is a few seconds[14]) but also is responsible for the prolonged action of iron nitrosyl complexes as NO donors. Besides Hb, cytochrome *c* (cyt *c*) can also form rather stable nitrosyl complexes in spite of the fact that the heme in cytochrome *c* is six-coordinate. The reactions of NO with cyt *c* are of great importance for the functioning of the mitochondrial respiratory chain.[15] Thus the formation of nitrosyl complexes with both ferro- and ferricytochrome *c* (cyt c^{2+} and cyt c^{3+}, respectively) can inhibit the electron transfer chain. The reaction of NO with cyt *c* leads to a change in the peroxidase activity of cyt *c*, which can be used for the regulation of apoptosis.[16]

The aim of the present work was to investigate the reaction of iron nitrosyl complexes with cyt c^{2+} and Hb. It should be noted that the nitrosylation of cyt c^{2+} with NO donors has not been studied yet. In the present study, we used the water-soluble cationic iron nitrosyl complex with the cysteamine ligand, $\{Fe_2[S(CH_2)_2NH_3]_2(NO)_4\}SO_4 \cdot 2.5H_2O$ (CAC), which induces apoptosis of human erythroblastic leukemia K562 cells.[16] The structure and physicochemical properties of CAC have been described previously.[17]

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Experimental

Horse heart cyt *c* (Serva, Germany) as a mixture of 90% of cyt c^{3+} and 10% of cyt of c^{2+}, bovine hemoglobin (MP Biomedicals) as a mixture of oxygenated hemoglobin (HbO_2) and methemoglobin (metHb), Sephadex G-25 (Pharmacia, Sweden), sodium dithionite (Merck, Germany), $Na_2HPO_4 \cdot 6H_2O$ and $NaH_2PO_4 \cdot H_2O$ (MP Biomedicals, Germany), $Na_2[Fe(CN)_5NO] \cdot 2H_2O$ and $NaNO_2$ (Aldrich, USA), L-ascorbic acid and citric acid (Serva, Germany) were used. Solutions were prepared with the use of distilled water (Bi/Duplex distillation apparatus, Germany).

The synthesis of CAC was carried out according to a known procedure.[17] The elemental analysis of the polycrystalline powder was performed in the Analytical Center of the Institute of Problems of Chemical Physics of the Russian Academy of Sciences. Found (%): C, 8.53; H, 2.77; N, 15.70; S, 17.71. $C_4H_{19}Fe_2N_6O_{10.5}S_3$. Calculated (%): C, 9.10; H, 3.60; N, 15.93; S, 18.27.

The technique of operation under nitrogen atmosphere has been described previously.[7]

Preparation of an Hb solution. A homogeneous solution of bovine Hb was prepared from the commercial product according to a known procedure.[13]

Preparation of a cyt c^{2+} solution at pH 3.0. Dry sodium ascorbate (10 mg) was added to a solution of commercial cyt *c* (9 mg) in a 0.05 *M* citrate phosphate buffer, pH 3.0 (3 mL), and the absorption spectrum of the solution was recorded. The solution of cyt c^{2+} was kept in the frozen state in liquid nitrogen. Before use, the cyt c^{2+} solution was thawed in 5 mL vessels under a stream of nitrogen.

Reaction of NO with cyt c^{2+}. A solution of cyt c^{2+}, pH 3.0, (2.5 mL) was placed into a 4-mL anaerobic cell with an optical path length of 1 cm. Then NO was introduced into the cell from a gas container at 1 atm for 3 min with shaking. The completeness of the conversion of c^{2+} into NO-cyt c^{2+} was monitored by spectrophotometry; the absence of changes in the absorption spectra was indicative of the establishment of the equilibrium.

Hydrolysis of CAC at pH 7.0. A 0.05 *M* phosphate buffer, pH 7.0 (2 mL) was placed into a 4-mL anaerobic cell with an optical path length of 1 cm. Then an anaerobic 0.05 *M* phosphate buffer, pH 7.0, was added to CAC in a vessel filled with nitrogen in such a way that the concentration of the complex was $6 \cdot 10^{-4}$ mol L^{-1}. The reaction mixture was stirred for 15 min until the complex completely dissolved, and then the solution of the complex (1 mL) was added to the cell. The final concentration of CAC was $2 \cdot 10^{-4}$ mol L^{-1}. The reference cell contained the anaerobic buffer (3 mL). After the addition of CAC, the absorption spectra were recorded at 15 min intervals for a total of 3 h.

Hydrolysis of CAC at pH 3.0. The hydrolysis of CAC in a 0.05 *M* citrate phosphate buffer, pH 3.0, was studied as described above.

Kinetics of the reaction of cyt c^{2+} with CAC. A solution of cyt c^{2+}, pH 3.0 (2 mL) was placed into a 4-mL aerobic cell with an optical path length of 1 cm to the concentration of $6.45 \cdot 10^{-5}$ mol L^{-1}. Then aliquots (2 mL) of a solution of CAC ($6 \cdot 10^{-4}$ mol L^{-1}) in a 0.05 *M* citrate phosphate buffer, pH 3.0, (the time of the preparation of the solution under a stream of nitrogen was 5 min) were added to the cell containing the cyt c^{2+} solution and to the reference cell containing the anaerobic buffer. The final concentration of CAC was $2 \cdot 10^{-4}$ mol L^{-1}. The first absorption spectrum was recorded immediately after the addition of CAC, and the the subsequent spectra were recorded at certain intervals.

Kinetics of the reaction of Hb with CAC. A 0.05 *M* phosphate buffer, pH 7.0 (0.9 mL) and an Hb solution (0.1 mL, $6.6 \cdot 10^{-4}$ mol L^{-1}) in the same buffer were placed into a 4-mL aerobic cell with an optical path length of 1 cm. After the dilution, the concentration of Hb was $2.2 \cdot 10^{-5}$ mol L^{-1}. A 0.05 *M* anaerobic phosphate buffer, pH 7.0, was added to a vessel containing CAC and filled with nitrogen to obtain the solution of the complex at a concentration of $6 \cdot 10^{-4}$ mol L^{-1}. The solution was stirred for 5 min. Then 3 mL of the reaction solution were withdrawn under a stream of nitrogen and transferred to an anaerobic vessel containing a 0.05 *M* phosphate buffer, pH 7.0 (3 mL) to obtain the solution of the complex at a concentration of $3 \cdot 10^{-4}$ mol L^{-1}. Aliquots (2 mL) of the solution of the complex were placed into a cell containing Hb and into the reference cell containing the anaerobic buffer (1 mL). The final concentration of CAC was $2 \cdot 10^{-4}$ mol L^{-1}. The absorption spectra were recorded after the addition of CAC at 5 h intervals for a total of 40 h.

Absorption spectra were recorded on a Specord M-40 spectrophotometer equipped with the interface enabling its link to a computer and a temperature-controlled cell holder. The amount of the NO-cyt c^{2+} complex was determined by spectrophotometry using decomposition of the absorption spectrum of the reaction system containing cyt c^{2+} and NO-cyt c^{2+} into the spectra of cyt c^{2+} and NO-cyt c^{2+} with the MATHCAD program as described earlier.[7]

Kinetic modeling. The proposed reaction scheme describing the formation of NO-cyt c^{2+} was considered. The rate constant of NO release from CAC was determined by the least-squares method based on the numerical solution of the corresponding differential equation system. The concentrations of the NO-cyt c^{2+} adduct, which were determined after the decomposition of the absorption spectra of the reaction systems into the spectra of cyt c^{2+} and NO-cyt c^{2+}, were used as the experimental data.

Results and Discussion

The kinetics of the reaction of NO with cyt c^{2+} is briefly described below.

Like deoxyhemoglobin, cytochrome *c* forms nitrosyl complexes both in the oxidized and reduced states (cyt c^{3+} and cyt c^{2+}). The reaction of NO with cyt c^{2+} affords the NO-cyt c^{2+} complex:

$$\text{cyt } c^{2+} + \text{NO} \rightleftharpoons \text{NO-cyt } c^{2+}.$$

The rate of the formation of the NO-cyt c^{2+} adduct depends on the pH of the reaction medium,[18] it decreases with increasing pH. The second-order rate constant of the formation of NO-cyt c^{2+} at pH 3 is 8.3 L mol^{-1} s^{-1}. When an excess of NO was removed from the solution by evacuation, NO-cyt c^{2+} slowly decomposed to cyt c^{2+} and NO with a first-order rate constant of $2.9 \cdot 10^{-5}$ s^{-1}.[18] The equilibrium constant K_{eq} is $3 \cdot 10^5$ L mol^{-1}.

In the individual state, ferrocytochrome *c* (cyt c^{2+}) does not react with oxygen; however, all operations with CAC and cyt c^{2+} were carried out under nitrogen, because NO rapidly reacts with atmospheric oxygen[19] (the rate constant is $2 \cdot 10^6$ L mol^{-2} s^{-1}).

To estimate the stability of CAC at pH 7.0 and 3.0, we recorded the changes in the absorption spectra of solutions of CAC in the range of 450—650 nm (Fig. 1). At pH 7.0, CAC is quite stable (see Fig. 1, curve *1*). At pH 3.0, the absorption increases with time, which was attributed to the visually observed formation of a thin suspension in the solution (see Fig. 1, curve *2*).

The absorption spectrum of cyt c^{2+} (*cf.* lit. data[18]) with λ_{max} at 521 and 551 nm ($\varepsilon = 1.68 \cdot 10^4$ L mol^{-1} cm^{-1}) and the absorption spectrum of the NO-cyt c^{2+} complex with λ_{max} at 541 nm ($\varepsilon = 1.1 \cdot 10^4$ L mol^{-1} cm^{-1}) at pH 3 are shown in Fig. 2. The absorption spectrum of the NO-cyt c^{2+} adduct remains unchanged for 1 day, which is attributed to the stability of the adduct in the presence of an excess of NO. According to the data published in the literature,[20] the absorption spectrum of cyt c^{2+} at pH 7.0 shows bands with λ_{max} at 518 and 549 nm ($\varepsilon = 3 \cdot 10^4$ L mol^{-1} cm^{-1}).

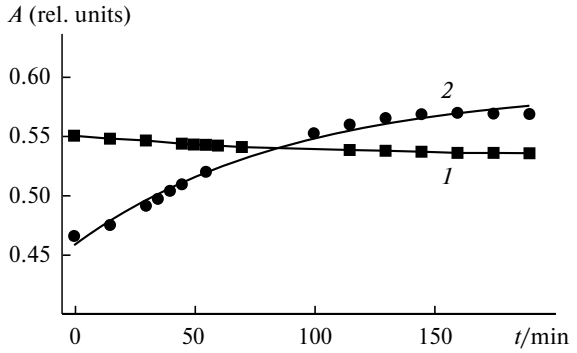

Fig. 1. Kinetics of the hydrolysis of CAC ($2 \cdot 10^{-4}$ mol L^{-1}) at pH 7 (*1*) and pH 3 (*2*) at 23 °C.

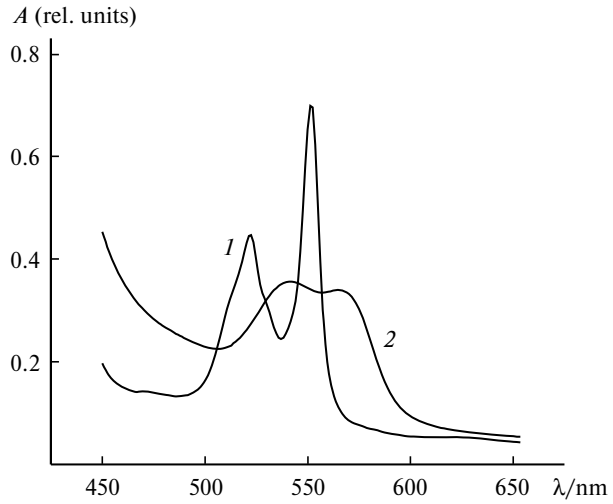

Fig. 2. Absorption spectra of a solution of cyt c^{2+} ($4.15 \cdot 10^{-5}$ mol L^{-1}) (*1*) and a solution of the NO-cyt c^{2+} adduct ($3.23 \cdot 10^{-5}$ mol L^{-1}) (*2*), which was prepared by the equilibration of the starting solution of cyt c^{2+} with gaseous NO (1 atm of NO) in 0.05 *M* citrate phosphate buffer (pH 3.0) at 23 °C.

The reaction of CAC with cyt c^{2+} was studied under anaerobic conditions (under a nitrogen atmosphere) at 23 °C. The time-resolved absorption difference spectra were recorded. Since the solution of CAC is unstable (see above), the reference cell contained only the buffer with pH 3.0 (Fig. 3). Each absorption spectrum was decomposed into the components by the MATHCAD method, after which the concentration of cyt c^{2+} and the degree of conversion into the NO-cyt c^{2+} complex were determined. Based on the calculated concentrations of the NO-cyt c^{2+} adduct, the kinetic curve was plotted (Fig. 4).

To elucidate the mechanism of the reaction of CAC with cyt c^{2+} and to quantitatively describe the reaction, the following reaction sequence was considered:

$$\mathrm{LFe(NO)} \xrightarrow{k_1} \mathrm{LFe + NO} \qquad (1)$$

$$\mathrm{NO + cyt}\ c^{2+} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{NO\text{-}cyt}\ c^{2+}, \qquad (2)$$

where $L = \{Fe[S(CH_2)_2NH_3]_2(NO)_3\}SO_4$.

The corresponding system of equations is as follows:

$$d[LFe(NO)]/dt = -k_1[LFe(NO)],$$

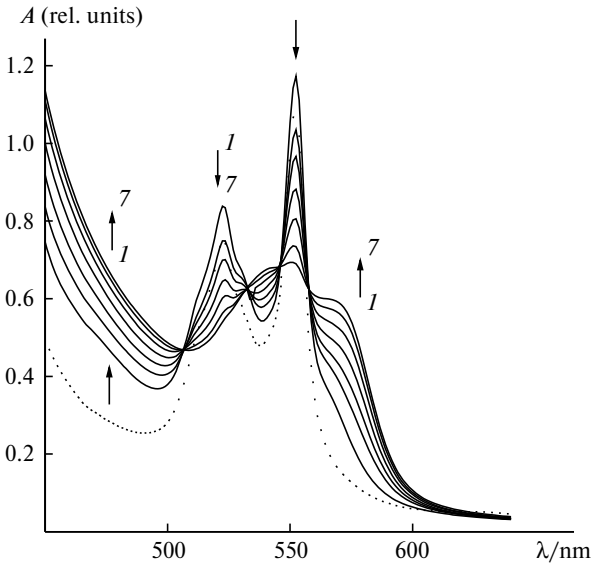

Fig. 3. Time-resolved absorption difference spectra for the reaction of CAC ($2 \cdot 10^{-4}$ mol L^{-1}) with cyt c^{2+} ($6.45 \cdot 10^{-5}$ mol L^{-1}) within 1.33 (*1*), 5.65 (*2*), 10.1 (*3*), 17.33 (*4*), 27.33 (*5*), 42.33 (*6*), and 60.33 min (*7*) after the beginning of the reaction. The absorption spectrum of the starting solution of cyt c^{2+} is shown by the dashed line. The conditions: 0.05 *M* citrate phosphate buffer (pH 3.0), 23 °C.

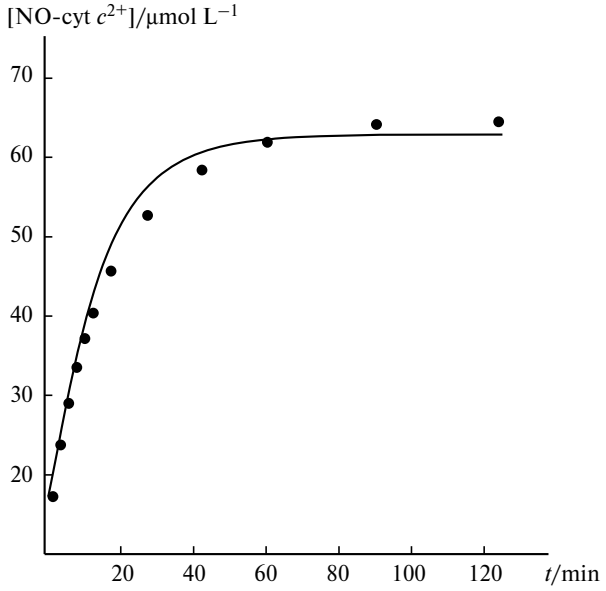

Fig. 4. Kinetic curve of the accumulation of the NO-cyt c^{2+} adduct in the course of the reaction of CAC ($2 \cdot 10^{-4}$ mol L^{-1}) with c cyt c^{2+} ($6.45 \cdot 10^{-5}$ mol L^{-1}). The points correspond to the experimental data.

$$d[NO]/dt = k_1[LFe(NO)] - k_2[NO][\text{cyt } c^{2+}] + k_{-2}[\text{NO-cyt } c^{2+}],$$

$$d[\text{cyt } c^{2+}]/dt = -k_2[NO][\text{cyt } c^{2+}] + k_{-2}[\text{NO-cyt } c^{2+}],$$

$$d[\text{NO-cyt } c^{2+}]/dt = k_2[NO][\text{cyt } c^{2+}] - k_{-2}[\text{NO-cyt } c^{2+}].$$

The rate constants of the reaction (2) were reported:[18] $k_2 = 8.3$ L mol^{-1} s^{-1}, $k_{-2} = 2.9 \cdot 10^{-5}$ s^{-1}. The constant k_1 was determined by the minimization of the functional $\Phi(k_1)$, which is the sum of the squares of the deviations between the calculated and experimental values of [NO-cyt c^{2+}] at specified instants of time t_i:

$$\Phi(k_1) = \sum_{i=1}^{n}\{[\text{NO-cyt } c^{2+}]_{calc}(t_i) - [\text{NO-cyt } c^{2+}]_{exp}(t_i)\}^2.$$

In the calculations, the amount of the NO-cyt c^{2+} complex, which was decomposed during 5 min before the beginning of the measurements, was also taken into account. The numerical solution of the problem gave $k_1 = (2.7 \pm 0.1) \cdot 10^{-3}$ s^{-1}, which satisfactorily describes the experimental data (see Fig. 4).

It was interesting to compare the above-described reaction with the reaction of CAC with Hb. Deoxyhemoglobin traps NO (the binding constant[21] is $3 \cdot 10^{10}$ L mol^{-1}, the rate of binding is close to the rate of diffusion[22]) and has the characteristic absorption spectrum that changes during the binding of NO. Hence, the release of NO from CAC can be followed based on the formation of HbNO.[7,13] In the course of the transformation of Hb into HbNO, as in experiments with cyt c^{2+}, the spectra overlap with each other. Thereby, the concentration of HbNO was estimated by the computer decomposition of the experimental absorption spectra into the components (the spectra of Hb and HbNO) by the least-squares method.[7] The calculations were carried out in the wavelength range of 450—650 nm based on two hundred experimental points. The reaction rate constants were estimated by analyzing the kinetic curves for the reaction of Hb with CAC. Since the absorption of all iron sulfur nitrosyl complexes is observed in the visible region, we recorded the absorption difference spectra of the buffer and the experimental system with Hb, which contained the corresponding complex at the same concentration. Figure 5 shows the time-resolved absorption difference spectra for the reaction of Hb with CAC. The absorbance at the absorption maximum of Hb at 556 nm decreases, whereas the absorbance at 545 and 575 nm increases, which is indicative of the formation of the HbNO complex. The spectra were recorded until they ceased to change. The kinetic curve for the formation of HbNO is shown in Fig. 6. The first-order rate constant (k) for this reaction calculated by the equation $y(t) = a(1 - e^{-kt})$, where a is the final concentration of HbNO, is $7.9 \cdot 10^{-6}$ s^{-1}. This constant is several orders of magnitude smaller than the constants of NO release from other iron nitrosyl complexes,[7,11,23] which were determined based on the reaction with Hb.

Previously,[13] it has been found that the dinuclear iron tetranitrosyl complex with pyridine-2-thiol is stabilized in the presence of Hb. This effect was explained by the absorption of the complex by the Hb macromolecule, which leads to a weakening of the contacts of the complex with

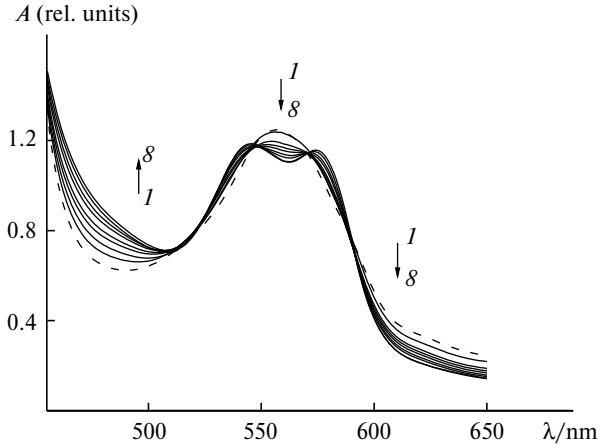

Fig. 5. Time-resolved absorption difference spectra for the reaction of CAC ($2 \cdot 10^{-4}$ mol L^{-1}) with Hb ($2.2 \cdot 10^{-5}$ mol L^{-1}). The first spectrum was recorded within 5 min and the second spectrum was recorded within 5 h after the beginning of the reaction; the subsequent spectra were recorded at 5 h intervals. The absorption spectrum of the starting solution of Hb is shown by the dashed line. The conditions: 0.05 *M* phosphate buffer (pH 7.0), 23 °C.

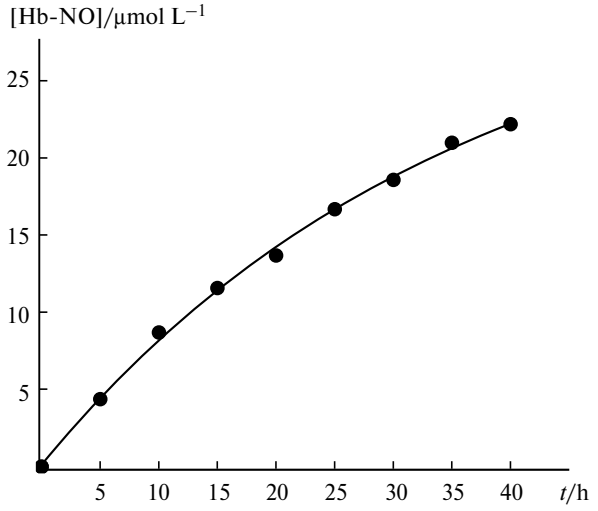

Fig. 6. Kinetics of the formation of Hb-NO in the reaction of ($2 \cdot 10^{-4}$ mol L^{-1}) with Hb ($2.2 \cdot 10^{-5}$ mol L^{-1}). The points correspond to the experimental data, the approximation of the data by the equation $y(t) = a(1 - e^{-kt})$ is represented by the solid line.

the aqueous phase and results in a decrease in the rate of NO release from the complex. The study of the influence of Hb on the NO-donor ability of the μ-N-C-S-type dinuclear tetranitrosyl complexes containing such ligands as methylbenzimidazolethiol $[Fe_2(C_8H_8N_2S)_2(NO)_4]$, benzimidazolethiol $[Fe_2(C_7H_5N_2S)_2(NO)_4] \cdot (C_3H_6O)_2$, and benzothiazolethiol $[Fe_2(C_7H_4SNS)_2(NO)_4]$ showed that the stabilization effect of these Hb complexes is attributed to the basicity of the sulfur-containing ligands.[24] However, ligands containing the free lone pair of the electrons are absent in CAC. In the cysteamine ligands of CAC, two NH_3^+ groups bear positive charges to form the salt structure with the SO_4^{2-} anions.

In summary, we disclosed a previously unknown fact of stabilization of the cationic iron nitrosyl complexes by hemoglobin. It was shown that the NO-cyt c^{2+} complex is formed in the course of the hydrolysis of CAC in the presence of cyt c^{2+}. The rate constant of NO release from CAC was determined from the kinetics of the formation of the NO-cyt c^{2+} adduct.

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References

1. J. A. McCleverty, *Chem. Rev.*, 2004, **104**, 403; P. C. Ford, L. E. Laverman, *Coord. Chem. Rev.*, 2005, **249**, 391.
2. N. M. Crawford, *J. Exp. Bot.*, 2006, **57**, 3.
3. K. M. Davies, D. A. Wink, J. E. Saavedra, L. Keefer, *J. Am. Chem. Soc.*, 2001, **123**, 5473.
4. R. Butler, I. L. Megson, *Chem. Rev.*, 2002, **102**, 1155.
5. A. S. Dutton, J. M. Fukuto, K. N. Houk, *Inorg. Chem.*, 2004, **43**, 1039.
6. A. S. Dutton, Ch. P. Suhrada, K. M. Miranda, D. A. Wink, J. M. Fukuto, K. N. Houk, *Inorg. Chem.*, 2006, **45**, 2448.
7. 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*, 2007, **16**, 181.
8. O. S. Zhukova, N. A. Sanina, L. V. Fetisova, G. K. Gerasimova, *Ros. Bioterapevt. Zh.* [*Russ. Biotherapeutic J.*], 2006, **5**, 14 (in Russian).
9. A. A. Timoshin, A. F. Vanin, Ts. R. Orlova, N. A. Sanina, E. K. Ruuge, S. M. Aldoshin, E. I. Chazov, *Nitric Oxide*, 2007, **16**, 286.
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. L. J. Hofseth, S. P. Hussain, G. N. Wogan, C. C. Harris, *Free Radical Biol. Med.*, 2003, **34**, 955.
12. S. Mocellin, V. Bronte, D. Nitti, *Med. Res. Rev.*, 2007, **27**, 317.
13. 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].
14. A. L. Lehninger, D. L. Nelson, M. M. Cox, *Principles of Biochemistry*, 2nd ed., New York, Worth Publ., 1993, 769 pp.
15. A. N. Osipov, G. G. Borisenko, Yu. A. Vladimirov, *Usp. Biol. Khim.*, 2007, **47**, 259 [*Biol. Chem. Rev.* (*Engl. Transl.*), 2007, **47**, 1491].
16. N. A. Sanina, O. S. Zhukova, Z. S. Smirnova, L. M. Borisova, M. P. Kiseleva, S. M. Aldoshin, *Ros. Bioterapevt. Zh.* [*Russ. Biotherapeutic J.*], 2008, **1**, 52 (in Russian).
17. T. N. Rudneva, N. A. Sanina, K. A. Lyssenko, S. M. Aldoshin, M. Yu. Antipin, N. S. Ovanesyan, *Mendeleev Commun.*, 2009, **19**, 253.
18. M. Hoshino, K. Ozawa, H. Seki, P. C. Ford, *J. Am. Chem. Soc.*, 1993, **115**, 9568.
19. A. M. Miles, D. A. Wink, J. C. Cook, M. B. Grisham, *Methods Enzymol.*, 1996, **268**, 105.
20. W. D. Butt, D. Keilin, *Proc. Roy. Soc. London, Ser. B*, 1962, **156**, 429.
21. R. Cassoly, Q. H. Gibson, *J. Mol. Biol.*, 1975, **91**, 301.
22. E. Antonini, M. Brunori, *Hemoglobin and Myoglobin in the Reactions with Ligands*, in *Frontiers of Biology*, Eds A. Neuberger, E. L. Tatum, North-Holland Publ. Comp., Amsterdam—London, 1971, **21**, 276.
23. 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].
24. 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].

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