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Mechanism of the 2-ethyl-3-hydroxy-6-methylpyridinium 2-nitroxysuccinate reduction in nitrite-generating systems

Olesya V. Pokidova,*[a] Boris L. Psikha,[a] Alexandra Yu. Kormukhina,[b] Alexander I. Kotel'nikov[a,b] and Boris S. Fedorov[a]

[a] *Institute of Problems of Chemical Physics, Russian Academy of Sciences, 142432 Chernogolovka, Moscow Region, Russian Federation. E-mail: pov@icp.ac.ru*

[b] *Department of Fundamental Physical and Chemical Engineering, M. V. Lomonosov Moscow State University, 119991 Moscow, Russian Federation*

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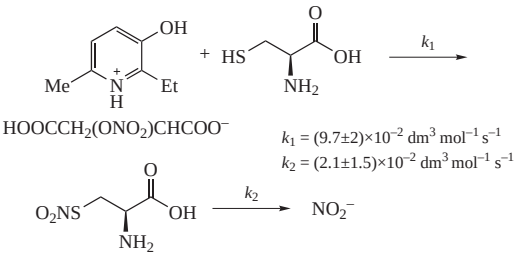
The nitrite-generating activity of 2-ethyl-3-hydroxy-6-methylpyridinium 2-nitroxysuccinate as a promising NO-donor has been investigated in reactions with various reducing agents. Reduction of the NO-donor with cysteine was analyzed using a kinetic modeling method. The calculated rate constants satisfactorily describe the experimental data, thereby confirming the proposed reaction mechanism.



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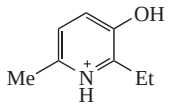
Organic nitrates as exogenous donors of nitric oxide (NO), which represent a signaling molecule involving in regulation of vascular tone, are widely used in clinical practice for the complex treatment of cardiovascular diseases.[1–3] One of the promising examples of this class of compounds is 2-ethyl-3-hydroxy-6-methylpyridinium 2-nitroxysuccinate **1**, a multifunctional substance[4] with combined properties of organic nitrate and effective antioxidant.[5] Compound **1** is a nitroxy derivative of the known antioxidant Mexidol or 2-ethyl-3-hydroxy-6-methylpyridinium succinate, widely used as pharmaceutical in Russia. The nitroxy derivative demonstrates effective NO-donor and nitrite-generating activities *in vitro* compared with Nitroglycerin and Nicorandil.[6] Besides, compound **1** has proved to be an effective inhibitor of phosphodiesterases,[7] which along with guanylate cyclase represent key enzymes in regulation of the cyclic nucleotides level.



$HOOCCH_2(ONO_2)CHCOO^-$

1

For further employment of compound **1** as a drug, it is critical to investigate its biotransformation in the presence of biological substrates *in vitro* and *in vivo*. From this point of view, an important step is to explore the interaction of compound **1** with thiols, in particular with cysteine (Cys), which is a component of the catalytic centers of enzymes involved in metabolism of organic nitrates, such as the aldehyde dehydrogenase enzyme family.[8,9] Non-protein thiols are also able to reduce organic nitrates.[10,11] Moreover, for all thiols including the protein ones, it has been experimentally confirmed that during the interaction with organic nitrates a thionitrate is formed as unstable intermediate.[8,12] Its further reduction leads to release of nitrite ions,[13,14] which are easily converted to NO *in vivo* by deoxyhemoglobin and other heme-containing proteins as well as by xanthine oxidoreductase and various thiol-containing enzymes.[15,16] Thus, thiols play an important role in the initial stage of the transformation of organic nitrates into NO, and depletion of the thiol pool can result in tolerance for the corresponding group of drugs.[17]

Based on the foregoing, the aim of this work was a detailed investigation of the compound **1** reduction into nitrite ion under the action of Cys. Quantitative determination of the nitrite ion content in solution was performed using a convenient and widely used Griess method.† To avoid nitrite oxidation by atmospheric oxygen, the experiments were carried out under anaerobic conditions.‡

First, the reactivity of compound **1** was explored in model systems with various reducing agents. From the kinetic dependence for the accumulation of nitrite ions [Figure 1(*a*)] formed during the interaction of compound **1** with Cys, sodium ascorbate and

† The experimental technique consisted in the measurement of optical density for an azo dye formed during the interaction of nitrite ions with sulfanilamide (SA) (Sigma, USA) and *N*-(1-naphthyl)ethylenediamine (NEDA) (MP Biomedicals, Germany). The reaction was initiated by addition of a freshly prepared solution of compound **1** to the solution of reducing agent, namely Cys, sodium ascorbate, glutathione (GSH) or $FeSO_4$ (Sigma, USA), in buffer at pH 7.0. Aliquots of the reaction mixture were taken at certain time intervals, introduced into vessels with a solution of 0.5% SA in 0.25 M HCl (1.5 ml) and incubated for 5 min, then a solution of 0.02% NEDA in 0.5 M HCl (1 ml) was added to the mixture. Absorbance was measured after 10 min at 540 nm using an Agilent Cary 60 spectrophotometer (USA). The concentration of nitrite ion was calculated from the calibration curve plotted for sodium nitrite as a reference.

‡ Kinetic experiments on the interaction of compound **1** with reducing agents were carried out in an argon atmosphere. All vessels and quartz cuvettes used were sealed with Rubber Septa seals (Sigma, USA). The vessels containing phosphate buffer and weighed samples were preliminarily purged with argon for 30 min. All the solutions employed were transferred using syringes with soldered needles.

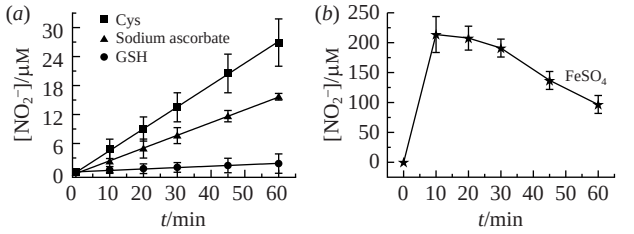


Figure 1 Nitrite ion accumulation *vs.* time in the interaction of compound **1** (3×10^{-3} M) at 23 °C with (*a*) 1×10^{-2} M Cys, sodium ascorbate or GSH in 0.05 M phosphate buffer (pH 7.0) and (*b*) 1×10^{-2} M $FeSO_4$ in Tris–HCl buffer (pH 7.0). The dots represent experimental data.

glutathione (GSH), we can conclude that Cys is characterized by the highest reducing activity of 0.4 μmol dm^{-3} min^{-1} and GSH has the lowest activity of 0.03 μmol dm^{-3} min^{-1}. Sodium ascorbate is 10 times more effective reducing agent (0.3 μmol dm^{-3} min^{-1}) compared with GSH, but weaker one than Cys.

At the same time, kinetic curve for the accumulation of nitrite ions obtained in the reaction of compound **1** with iron(II) sulfate, in comparison to Figure 1(*a*), is far from linear [Figure 1(*b*)]. In this instance, the highest concentration of nitrite ion is achieved in the first 10 min, while the initial reaction rate is 50 times higher than that for reduction with Cys.

Thus, Fe^{2+} as well as sodium ascorbate can be considered as natural reducing agents *in vivo* for compound **1**, though the corresponding mechanisms have not been described yet and require careful consideration.

Further kinetic investigation of the mechanism of compound **1** reduction to nitrite ion was carried out with Cys, since it demonstrated higher reduction ability compared with GSH [see Figure 1(*a*)]. The scheme of the process includes a sequence of two bimolecular reactions:

$$RO{-}NO_2 + R'SH \xrightarrow{k_1} ROH + R'S{-}NO_2 \quad (1)$$

$$R'S{-}NO_2 + R'S \xrightarrow{k_2} NO_2^- + R'S{-}SR', \quad (2)$$

where $RO{-}NO_2$ is compound **1**, R′SH is Cys and $R'S{-}NO_2$ is the cysteine thionitrate intermediate, which is formed at the first step (1) and reduced to nitrite ion at the second step (2).

The Cys molecule is known to dissociate in an aqueous solution with p*K*a = 8.2:[18]

$$R'SH \underset{}{\overset{K}{\rightleftharpoons}} R'S^- + H^+. \quad (3)$$

Moreover, the reducing agent for cysteine thionitrate in reaction (2) is exactly the thiolate ion. Indeed, according to the experimental data obtained at different pH values (Figure 2), with an increase in the solution pH to 7.9, the rate of the nitrite ion accumulation elevates by two times compared with the one at pH 7.0. When pH is lowered to 5.9, the rate decreases by three times.

We assumed that for the reversible reaction (3) at each moment of time the equilibrium condition was satisfied. Taking into account the detailed equilibrium in reaction (3) and the constant pH = 7.0 (*i.e.*, $[H^+]$ = const), the corresponding system of differential equations was derived as the following:

$$d[RO{-}NO_2]/dt = -k_1[RO{-}NO_2][R'SH]$$

$$d[R'SH]/dt = -(k_1[RO{-}NO_2][R'SH] + k_2[R'S{-}NO_2][R'S^-])\,[H^+]/(K + [H^+])$$

$$d[R'S{-}NO_2]/dt = k_1[RO{-}NO_2][R'SH] - k_2[R'S{-}NO_2][R'S^-]$$

$$d[NO_2^-]/dt = k_2[R'S{-}NO_2][R'S^-]$$

$$[R'S^-] = [R'SH]\,K/[H^+].$$

Considering the equilibrium of reaction (3), the following initial conditions were employed:

$$[R'SH](0) = [R'SH]_0\,[H^+]/(K + [H^+])$$

$$[RO{-}NO_2](0) = [RO{-}NO_2]_0$$

$$[R'S{-}NO_2](0) = [NO_2^-](0) = 0,$$

where $[R'SH]_0$ and $[RO{-}NO_2]_0$ are the initial concentrations of Cys and compound **1**, respectively.

With the known value of equilibrium dissociation constant for Cys ($K = 6.3\times10^{-9}$ mol dm^{-3}),[18] the reaction rate constants k_1 and k_2 were determined from the dependence of NO_2^- concentration *vs.* time in three experiments employing different initial concentrations $[RO{-}NO_2]_0$ (Table 1 and Figure 3). As can be seen from Figure 3, the reaction scheme (1)–(3) with the values determined for rate constants adequately describes the experimental data on the accumulation of nitrite ion.

Thus, based on the data obtained, it can be concluded that for the reduction of compound **1**, Cys represents more active thiol than GSH. The kinetic investigations performed describe satisfactorily the reduction process of compound **1** by Cys, where at the first step cysteine thionitrate is formed with $k = (9.7\pm2)\times10^{-2}$ dm^3 mol^{-1} s^{-1} and further reduced at the second step to nitrite ion with $k = (2.1\pm1.5)\times10^{-2}$ dm^3 mol^{-1} s^{-1}. Comparison of the two constants led us to conclusion, that the reduction of cysteine thionitrate was a limiting step and proceeded almost five times slower than the initial reaction step.

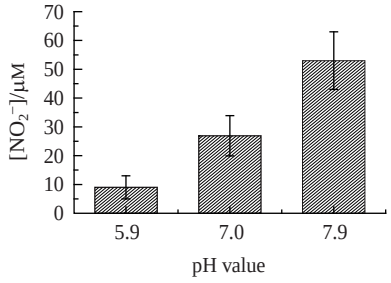


Figure 2 Reduction rate of compound **1** (3×10^{-3} M initially) by 1×10^{-2} M Cys at various pH in 0.05 M phosphate buffer at 23 °C.

Table 1 Rate constants k_1 and k_2 according to the mechanism considered for interaction of compound **1** with Cys.

Run	[compound **1**]$_0$/mol dm^{-3}	k_1/dm^3 mol^{-1} s^{-1}	k_2/dm^3 mol^{-1} s^{-1}
1	3×10^{-4}	8.3×10^{-2}	2.0×10^{-2}
2	9×10^{-4}	1.24×10^{-1}	1.4×10^{-2}
3	3×10^{-3}	8.3×10^{-2}	2.9×10^{-2}

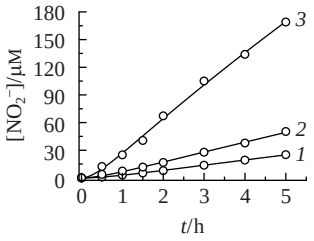


Figure 3 Nitrite ion accumulation *vs.* time for the interaction of compound **1** at (*1*) 3×10^{-4}, (*2*) 9×10^{-4} and (*3*) 3×10^{-3} M with Cys (5×10^{-3} M) in 0.05 M phosphate buffer (pH 7.0) at 23 °C. The dots are experimental data and the solid lines represent theoretical calculation.

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