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Reversible Reduction of Nitroxides to Hydroxylamines: the Roles for Ascorbate and Glutathione

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Abstract

Biological applications of stable nitroxyl radicals, NR, include their use as contrast agents for magnetic resonance imaging, spin labels, superoxide dismutase mimics, and antioxidants. The rapid reduction of NR in biological samples into hydroxylamines, HA, significantly limits their application. In its turn, reoxidation of HA back to the NR has been used for detection of reactive oxygen species, ROS. In this work comparative studies of the reduction of pyrrolidine, imidazoline and imidazolidine NR by ascorbate were performed taking advantage of recently synthesized tetraethyl substituted NR with much higher stability towards reduction both *in vitro* and *in vivo*. Surprisingly, these NR kept 10-50% of initial intensity of electron paramagnetic resonance signal for about 1 h in the presence of hundred fold excess of ascorbate. To explain this data, reoxidation of the corresponding HA by ascorbate radical and dehydroascorbic acid back to the NR was proposed. This hypothesis was supported by direct measurement of the NR appearance from the HA upon ascorbate radical generation by ascorbate oxidase, or in the presence of the dehydroascorbic acid. The reversible reaction between NR and ascorbate was observed for the various types of the NR, and the rate constants for direct and reverse reactions were determined. The equilibrium constants for one-electron reduction of the tetraethyl substituted NR by ascorbate were found to be in the range from 2.65×10^{-6} to 10^{-5} which is significantly lower than corresponding values for the tetramethyl substituted NR (less or about 10^{-4}). This explains an establishment of EPR-detectable quasi-equilibrium level of tetraethyl substituted NR in the presence of excess of ascorbate. The redox reactions of the NR-HA couple in ascorbate containing medium was found to be significantly affected by glutathione, GSH. This effect was attributed to the reduction of ascorbate radical by GSH, and the rate constant of this reaction was found to be equal to $10 \text{ M}^{-1}\text{s}^{-1}$. *In summary*, the data provide new insight into the redox chemistry of NR and HA, and significantly affect interpretation and strategy of their use as redox- and ROS-sensitive probes, or as antioxidants.

Keywords

nitroxyl radicals; nitroxide reduction; EPR; hydroxylamine; ascorbate radical; ascorbic acid; glutathione; antioxidants

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Introduction

A number of studies of the reduction of nitroxyl radicals (NR) have been reported (for reviews see [1,2]) because of the importance of this class of the compounds in biology and medicine where they are used as contrast agents for magnetic resonance imaging, MRI [3,4], spin labels [5], superoxide dismutase (SOD)-mimics [6], and antioxidants [7,8]. Chemical reduction of NR to EPR (electron paramagnetic resonance) -silent hydroxylamines (HA) in many cases is an unfavorable factor that significantly limits their applications in biological systems. On other side, EPR-measured rates of NR reduction have been shown to provide information on tissue redox status [9-11], and reactive oxygen species (ROS) generation *in vivo* [11,12]. In its turn, oxidation of HA to NR also has been used for *in vivo* EPR detection of ROS [13-15]. NR being introduced in the biologically relevant systems are predominantly observed in the radical and hydroxylamine forms [2]. Nevertheless, the product of one-electron oxidation of the nitroxides, oxoammonium cation, plays important role in the SOD-mimic activity of cyclic nitroxides [16]. The highly oxidizing oxoammonium cation undergoes fast one-electron reduction back to the nitroxide or two-electron reduction to hydroxylamine, and is apparently responsible for the prooxidative activity and potential adverse effects of the nitroxides [17].

The reduction of the nitroxides by cells is primarily intracellular [2]. Ascorbate plays significant role in NR reduction in erythrocytes, hepatocytes and kidney cells [2,18,19], which are rich in this compound. The reduction rates of NR by ascorbate normally correlate with its electrochemical reduction potential [20] and depend on nature of the radical ring [4,19-21], charge of the radical [1,19], and steric shielding of nitroxyl fragment [22-24]. The only product of stoichiometric reduction of the NR by ascorbate in the absence of oxygen is the hydroxylamine [1]. The reduction of nitroxide by ascorbate is reversible and may deviate from pseudo-first order in the presence of oxygen [25,26]. The involvement of ascorbate radical in the NR reduction was also proposed based on the kinetic studies in alkaline solutions [27,28].

The reaction of the nitroxides with glutathione (GSH) is of particular interest due to the importance of GSH in regulation of intracellular redox status [10,29]. Appreciable chemical reduction of NR by glutathione does not occur over a few hours [1,30-32]. However glutathione can significantly contribute in the reduction of NR in biological systems indirectly by acting as a secondary source of reducing equivalents [10].

Recently we synthesized the series of tetraethyl-substituted NR with enhanced stability towards reduction [23]. In the present paper we performed mechanistic studies of the reduction of these and other NR (see Scheme 1 for the structures) in deaerated solutions of ascorbate. For the first time reoxidation of the HA by ascorbate radical and dehydroascorbic acid back to the nitroxide was observed for the radicals of different types. The redox reactions of the NR-HA couple in ascorbate-containing medium was found to be significantly affected by glutathione. The data provide new insight into redox chemistry of the nitroxides and hydroxylamines, and may significantly affect an interpretation of their use as redox- and ROS-sensitive probes, or as antioxidants.

Material and Methods

Reagents

Bovine Cu, Zn-superoxide dismutase (SOD), was obtained from ICN Biomedical Inc (Costa Mesa, CA). L-Glutathione (GSH), dehydroascorbic acid, and ascorbate oxidase were obtained from Sigma-Aldrich Inc. L-ascorbic acid and diethylenetriaminepentaacetic acid (DTPA) were purchased from Acros Organics. NR **1** (3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidine-1-oxyl) and corresponding HA, **1H** (1-Hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine) were purchased from Alexis. TAM Ox 063 (methyl tris(8-carboxy-2,2,6,6-tetrakis-(2-hydroxy-

ethyl)-benzo[1,2-d:4,5-d']bis(1,3)dithiol-4-yl), triarilmethyl radical derivative) was a gift from Nycomed Innovations (Sweden).

Synthesis

The NR **2** (4-Methyl-2,2,5,5-tetraethyl-2,5-dihydro-1H-imidazol-1-yloxy) and **4** (3,4-Dimethyl-2,2,5,5-tetraethylperhydroimidazol-1-yloxy) were synthesized as described in the reference [23]. The synthesis of the NR **5** (3,4-Dimethyl-2,2,5,5-tetramethylperhydroimidazol-1-yloxy), and **6** (4-Methyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-1-yloxy) was described previously [33]. Synthesis of the NR **3** (2,2-Diethyl-3-methyl-1,4-diaza-spiro[4,5]dec-3-en-1-oxyl) and corresponding HA **3H** (2,2-Diethyl-3-methyl-1,4-diaza-spiro[4,5]dec-3-en-1-ol) was performed according to synthetic route shown in the Scheme 2 and described below.

2,2-Diethyl-3-methyl-1,4-diaza-spiro[4,5]dec-3-en-1-ol(3H)

A suspension of 3-hydroxyamino-3-ethylpentan-2-one hydrochloride **3a** (5 g, 27.5 mmol) and ammonium acetate (7.7 g, 100 mmol) in a mixture of cyclohexanone (10 ml, 93 mmol) and methanol (8 ml) was stirred under argon for 24 h. The resulting solution was poured into water (20 ml), neutralized with NaHCO₃ and extracted with diethyl ether (3×10 ml). The ether extract was thoroughly washed with water (5×10 ml) and dried over Na₂CO₃. The ether was removed under reduced pressure and the residue was titrated with hexane at -5°C, the crystalline precipitate of **3H** was filtered off and washed with hexane, yield 3.8 g, 60 %, colorless crystals, m.p. 129-133 (hexane) (Found: C, 69.54; H, 10.48; N, 12.44. Calc. for C₁₃H₂₄N₂O: C, 69.60; H, 10.78; N, 12.49); $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$ 2924, 2870, 1655, 1472, 1451, 1428, 1384, 1282, 1266, 1175, 1034, 962, 934, 900 and 851; $\delta_{\text{H}}(200 \text{ MHz}; \text{CDCl}_3)$ 0.85 (6 H, br t, J 7, 2 × CH₃, Et), 1.23–1.87 (14 H, br m, 2 × CH₂, Et and (CH₂)₅), 1.90 (3 H, s, CH₃C=N) and 6.00 (1 H, br s, OH); $\delta_{\text{C}}(50 \text{ MHz}; \text{CDCl}_3)$ 8.75 (CH₃, Et), 27.09 (CH₂, Et), 25.00, 22.81, 35.57 and 16.23 (CH₃-C=N), 78.24 (C⁵), 91.43 (C²) and 171.19 (C=N).

2,2-Diethyl-3-methyl-1,4-diaza-spiro[4,5]dec-3-en-1-oxyl (3)

Manganese dioxide (2 g) was added to a stirred solution of **3H** (1 g, 4.5 mmol) in chloroform (20 ml). The suspension was stirred for 0.5 h, manganese oxides were filtered off and filtrate was evaporated under reduced pressure to leave orange crystalline solid, which was purified by column chromatography on silica (Kieselgel 60, Merck) using diethyl ether – hexane 1: 20 mixture as eluent to give nitroxide **3** (0.95 g, 95%), orange crystals, m.p 86-88 (hexane) (Found: C, 69.89, H, 10.32; N, 12.41. Calc. for C₁₃H₂₃N₂O: C, 69.91; H, 10.38; N, 12.54); $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$ 2972, 2961, 2935, 2853, 1637, 1452, 1423, 1386, 1376, 1356, 1325, 1294, 1263, 1210, 1172, 1142, 1109, 962, 933, 912, 845 and 813.

Solutions

All studies were carried out in 0.1 M Na-phosphate buffer pH 7.6. Solution pH value was adjusted by addition of NaOH if it was necessary.

EPR studies of the NR reduction by ascorbate

The solutions of the NR **1**, **4**, **5** and **6** (1 mM), **2** (0.25 and 1 mM), **3** (50 and 100 μM), and HA **1H** (0, 1, 2, 5 mM) and **3H** (0, 0.1, 0.5 mM) were mixed under anaerobic conditions in glove box (Vacuum Atmosphere Co., Hawthorne, Ca) at oxygen level less than 1 ppm with various concentrations of ascorbate (from 1 to 100 mM). The mixture was immediately transferred to the 50 μl capillary tube, and EPR spectrum was recorded using EMX X-band spectrometer (Bruker). In the kinetics studies the peak intensity of the low field component of the triplet EPR spectrum was monitored over time.

The ascorbate radical generating system

Ascorbate radical was generated from ascorbate (1 mM) in the presence of ascorbate oxidase (from 0.01 to 0.03 U/ml) in air-equilibrated solutions [34]. Under these conditions steady-state concentration of the ascorbate radical was observed for at least 10 min. The EPR spectra were recorded with the following spectrometer settings: scan time 41.94 s, number of scans 16, time constant 40.96 ms, sweep width 5 G, modulation amplitude 0.5 G, microwave power 9.985 mW. The steady-state concentration of the ascorbate radical, $[Asc^{\bullet-}]_{ss}$, was estimated using double integration of its EPR spectrum and the spectrum of known concentration of TAM radical measured in the same conditions. The rate of ascorbate radical generation, V , was calculated from $[Asc^{\bullet-}]_{ss}$, supposing that it is equilibrated by the rate of its bimolecular dismutation, i.e. $V = 2 \cdot k_3 \cdot [Asc^{\bullet-}]_{ss}^2$, where k_3 is bimolecular rate constant of the dismutation reaction [35]. It was found that the rate V is proportional to the enzyme concentration in the experimental concentration range. Note that steady-state concentration of the ascorbate radical was not affected by an addition of SOD (10 or 100 U/ml, data not shown), and, therefore, by possible superoxide anion generation. The kinetics of the HA **1H** oxidation by the ascorbate radical in the presence of ascorbate oxidase and ascorbate was measured as an appearance of the EPR spectrum of the NR **1**. Ascorbate oxidase alone did not oxidize HA **1H**. The influence of the ascorbate radical generation on the kinetics of the reduction of the NR **2** was studied in the presence of 1 mM ascorbate and ascorbate oxidase.

The ascorbate radical reaction with glutathione

The solutions of ascorbate (1 mM), ascorbate oxidase (0.03 U/ml), and glutathione at various concentrations (0, 20, 40, 60 mM) were mixed and then placed into 50 μ l capillary tubes. The steady-state concentration of the ascorbate radical was stable for at least 10 min, and its EPR spectra were recorded under following spectrometer settings: scan time 41.94 s, number of scans 16, time constant 40.96 ms, sweep width 5 G, modulation amplitude 0.5 G, microwave power 9.985 mW. The steady-state concentration of the ascorbate radical in the presence of various concentration of glutathione, $[Asc_{GSH}^{\bullet-}]_{ss}$, was estimated using double integration of its EPR spectrum and of the spectrum of known concentration of TAM radical measured in the same conditions. The rate of the ascorbate radical generation was determined from the steady-state concentration of the ascorbate free radical, $[Asc^{\bullet-}]_{ss}$, measured in the absence of GSH $V = 2 \cdot k_3 \cdot [Asc^{\bullet-}]_{ss}^2$ as it was described above. The rate constant of the reaction of the ascorbate radical with GSH, k_{GSH} , was calculated as follows. Taking into account that the rate of $Asc^{\bullet-}$ formation is equilibrated by its decay in the reaction of dismutation and in the reaction with GSH, one can obtain,

$2 \cdot k_3 \cdot [Asc^{\bullet-}]_{ss}^2 = 2 \cdot k_3 \cdot [Asc_{GSH}^{\bullet-}]_{ss}^2 + k_{GSH} \cdot [GSH] \cdot [Asc_{GSH}^{\bullet-}]_{ss}$. This simple equation was used to calculate the observed rate constant, $k_{obs} = k_{GSH} \cdot [GSH]$, from the steady-state concentrations of ascorbate radical measured in the absence and in the presence of GSH. The rate constant, k_3 , of ascorbate radical dismutation was calculated from Bielski et al. [35] for the values of pH and ionic strength of the solution used in the sample preparation.

GSH influence on the NR reduction by ascorbate

The solutions of the NR **4** (1 mM), ascorbate (0 and 100 mM), and glutathione (0, 5, and 50 mM) were mixed under air-equilibrium conditions. The solutions were immediately transferred to the 50 μ l capillary and kinetics of the nitroxide EPR spectrum intensity was monitored over time.

The kinetics of **1** and **1H** reaction with dehydroascorbic acid

The EPR spectrum peak intensity of the NR **1** was measured in the solutions containing various concentrations of the HA **1H** (5 and 10 mM) or NR **1** (1 mM) and dehydroascorbic acid (1, 2, 5, or 10 mM). The solutions of dehydroascorbic acid were used immediately after preparation.

Calculations

All calculations were performed using Mathcard 2001 and Origin 7 packages.

Results

Reversibility of the NR reduction by ascorbate

Figure 1 shows the kinetics of the reduction of the pyrrolidine NR **1** and tetraethyl-substituted imidazoline NR **2** in deaerated aqueous solution. The observed kinetics clearly deviate from the exponential decay. Particularly NR **2** demonstrates striking ability to resist against the reduction even in 100 times excess of ascorbate, keeping EPR signal intensity at quasi-plateau level for an hour or more. The hydroxylamine is known to be the only product of the NR reduction by ascorbate. Therefore, to explain the incomplete NR reduction by ascorbate anion, one may assume oxidation of the HA back to the parent nitroxide. To test this hypothesis we added 10 mM of the HA **1H** in the reaction mixture of 1 mM nitroxide **1** and 50 mM ascorbate at the time-point when quasi-equilibrium level of the NR **1** was established. This resulted in spectacular reversion of the decay kinetics of the EPR signal of **1** to the kinetics of its growth shown in Figure 2.

Figure 3 shows the kinetics of the reduction of the NR **1** and **3** in the presence of 50 mM of ascorbate and different concentrations of corresponding HA, **1H** and **3H** (see Scheme 1). The quasi-plateau level of the NR was successively increased upon increasing of the HA concentration, further supporting the hypothesized reoxidation of the HA back to the corresponding NR. Two possible oxidizing agents for HA can be proposed. First one is the product of one-electron oxidation of ascorbate, ascorbate free radical. The second one is the product of two-electron oxidation of ascorbate, dehydroascorbic acid.

HA oxidation by ascorbate radical

Ascorbate radical, $\text{Asc}^{\bullet-}$, was generated in ascorbate/ascorbate oxidase system [34], and its steady-state concentration was observed for at least 10 minutes under experimental conditions (see Materials and Methods). Figure 4 shows the kinetics of the NR **3** reduction by ascorbate in the presence and absence of ascorbate oxidase. The observed slowing down of ascorbate-induced nitroxide reduction in the presence of ascorbate oxidase supports the role of ascorbate radical as oxidizing agent for hydroxylamine.

Figure 5 demonstrates direct oxidation of hydroxylamine **1H** in an ascorbate/ascorbate oxidase system. The bimolecular rate constant of the reaction of the ascorbate radical with **1H** was calculated as described in Materials and Methods and was found to be equal to $(1.1 \pm 0.05) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. Note that the rate of **1H** (1 mM) oxidation in the presence of ascorbate oxidase, 0.02 U/ml, and 1 mM ascorbate was not affected by the addition of 10 or 100 U/ml of the SOD (data not shown), therefore supporting negligible contribution of the possible superoxide generation in this process.

HA oxidation by dehydroascorbic acid

Figure 6 shows the kinetics of HA **1H** oxidation in the presence of various concentrations of the dehydroascorbic acid. The data demonstrate an ability of the dehydroascorbic acid to oxidize HA back to the NR at high concentrations of the reagents. However this reaction was

negligible at low experimental concentrations of **1H** and dehydroascorbic acid (1 mM and 0.5 mM, respectively), and can not contribute significantly in the kinetics of the reduction of the NR **1** shown in Figure 1. The shape of the kinetics of the HA oxidation shown in the Figure 6 is complicated due to the decomposition of the dehydroascorbic acid to the product, which is capable of the NR reduction. Figure 7 demonstrates an increase in the reduction rate of the NR **1** in the presence of dehydroascorbic acid supporting assignment of the reducing capability to the decomposition product, apparently diketogulonic acid, rather than to the dehydroascorbic acid itself.

Facilitating NR reduction by glutathione

GSH does not reduce the NR directly [1,30,31]. Figure 8 demonstrates the stability of EPR signal of 1 mM solution of the tetraethylsubstituted imidazolidine NR **4** in the presence of 50 mM GSH for 40 minutes. However, in the presence of ascorbate, addition of GSH facilitated the ascorbate-induced NR reduction (Figure 8). Note that GSH significantly decreased the “quasi-plateau” level of the NR signal intensity while has low impact on the first rapid phase of the kinetics. This might be explained by the scavenging of the ascorbate radical by GSH resulted in the inhibition of the $\text{Asc}^{\bullet-}$ - induced oxidation of the HA. Figure 9a demonstrates successive decrease of the steady-state concentration of the ascorbate radical observed in the ascorbate/ascorbate oxidase system upon GSH addition in concentration dependent manner, supporting $\text{Asc}^{\bullet-}$ scavenging by GSH. The generation rate and steady-state concentrations of ascorbate radical in the presence of various concentration of GSH were determined, and, therefore, the observed rate constant of the reaction of $\text{Asc}^{\bullet-}$ with GSH was calculated as described in the Materials and Methods. The dependence of k_{obs} on glutathione concentration is in a good agreement with a linear approximation (see Fig. 9b). The bimolecular rate constant of the ascorbate radical reduction by glutathione, k_{GSH} , was found to be equal to $10 \text{ M}^{-1}\text{s}^{-1}$.

Quantitative analysis of the nitroxide reduction by ascorbate

For the simulation of the kinetics of the nitroxide reduction and hydroxylamine oxidation the following reactions were taken into account:

where NR and HA denote nitroxyl radical and its hydroxylamine, respectively; AscH^- , $\text{Asc}^{\bullet-}$, DHA, DGA, and OxDGA denote ascorbate anion, ascorbate radical, dehydroascorbic acid, diketogulonic acid, and product of oxidation of diketogulonic acid by NR, respectively. Excellent fittings of the calculated kinetics using eqs. (1) - (5) to the experimental data were obtained (see Figures 2,3, 6,7) yielding the rate constants shown in the Table 1.

Discussion

Wide-spread applications of the NR as spin probes and labels has established the need for development of structures with enhanced stability towards reduction. Traditionally, the reduction rates of the NR by ascorbate, one of the most important reducing agents in biochemistry, became an essential characteristic of NR utility in various biologically relevant EPR applications. The bimolecular rate constants of ascorbate-induced reduction are significantly higher for six-membered ring NR of piperidine types (e.g., $k_1=3.5 \text{ M}^{-1}\text{s}^{-1}$ for TEMPO [4] and $k_1=7 \text{ M}^{-1}\text{s}^{-1}$ for TEMPOL [36]) than for the five-membered ring NR of pyrrolidine ($k_1=0.07\text{--}0.3 \text{ M}^{-1}\text{s}^{-1}$, see Table 1 and [4]) and imidazolidine (e.g. $k_1=0.85 \text{ M}^{-1}\text{s}^{-1}$ for **5**, see Table 1) types. A presence of the double bond at the position 3 in the five-membered ring NR of pyrroline ($k_1=0.64\text{--}1.6 \text{ M}^{-1}\text{s}^{-1}$ [4]) and imidazoline ($k_1=5.6 \text{ M}^{-1}\text{s}^{-1}$ for **6**, see Table 1) types increases their reduction rates by ascorbate. A negative charge is a factor stabilizing NR against reduction by negatively charge ascorbate anion, AscH^- [1]. Therefore until recently carboxyproxyl NR **1** was reported as one of the most resistant NR against reduction by ascorbate ($k_1=0.1 \text{ M}^{-1}\text{s}^{-1}$, Table 1). A steric protection of the radical NO fragment is another important factor increasing stability of the NR in ascorbate-containing solutions [22–24]. Recently synthesized tetraethyl-substituted imidazolidine NR [23] are probably the most stable NR in respect to reduction in both ascorbate solutions (e.g. $k_1=0.02 \text{ M}^{-1}\text{s}^{-1}$ for the NR **4**, Table 1) and biological fluids. However a comparison of data on NR reduction from various literature sources often is difficult because of the variation of experimental conditions and also due to incomplete understanding of the mechanism of the reaction despite of several decades of the studies.

The main reaction in the mechanism of the NR reduction by ascorbic acid is a forward reaction (1) between ascorbate anion and NR. Ascorbic acid and ascorbate dianion do not contribute significantly in the nitroxides reduction at physiologically relevant pH [1,27]. It is widely accepted that the reduction proceeds according to 2:1 stoichiometry with the formation of the corresponding HA and dehydroascorbic acid [1]. In this work, for the first time, we observed the reaction of HA oxidation by ascorbate radical and by dehydroascorbic acid back to the NR form.

Predominantly the oxidation of the HA back to the NR proceeds via the reverse reaction (1) with ascorbate free radical (Fig.5). The oxidation of the HA by the dehydroascorbic acid might be significant at high concentrations of the HA (Fig. 6) and is described by the forward reaction (2). In its turn, the reverse reaction (2) of the NR reduction by the ascorbate radical results in the dehydroascorbic acid formation and might contribute in the overall kinetics as well. It was found that this reaction significantly affects the reduction of TEMPO-derivatives of the NR of piperidine type [27] in alkaline medium. However, in most cases the main source of the formation of dehydroascorbic acid is the reaction (3) of the ascorbate radical dismutation studied in details by Bielski et al. [35]. The following dehydroascorbic acid decomposition to diketogulonic acid, described by the reaction (4), was discussed in the literature [37,38]. Being the only irreversible reaction in the proposed scheme of the reactions (1)–(5), the reaction (4) is responsible for the slow decay of the quasi-equilibrium level of the NR (see Figures 1–3). Furthermore, DGA, is apparently responsible for the NR reduction in the presence of dehydroascorbic acid (Figure 7) according to reaction (5). However, this process is slow and insignificantly contributed to all the experimental kinetics except the one shown in Figure 7.

Excellent fittings of the calculated kinetics using eqs. (1)–(5) to the experimental data were obtained (see Figures 2, 3, 6 and 7). The rate constant k_3 for the reaction of ascorbate radical dismutation was calculated using literature data [35] for the experimental values of pH and ionic strength of solution. In its turn, the equilibrium constant K_3 for the reaction (3) was found to be equal to $(4.2 \pm 0.8) \times 10^{-9} \text{ M}^{-1}\text{s}^{-1}$ being within the range $(10^{-9} - 10^{-8}) \text{ M}^{-1}\text{s}^{-1}$ reported

in the literature [39]. The obtained rate constant for DHA hydrolysis, $k_4 = (7.0 \pm 2.0) \times 10^{-4} \text{ s}^{-1}$, measured at pH 7.6, is also in a reasonable agreement with literature value, $(2.5 \pm 2.0) \times 10^{-4} \text{ s}^{-1}$, pH 7.2 [38], particularly taking into account the catalysis of the hydrolysis by hydroxyl anion and weak acids.

The reversibility of the reaction (1) significantly affects the reduction kinetics of the NR. The equilibrium constants, K_1 , for the tetraethyl substituted NR **2-4** are in the range $(0.2-1) \times 10^{-5}$ being at least one order of magnitude less than corresponding values for the tetramethyl substituted NR (see Table 1). As a consequence, establishment of quasi-equilibrium level of the NR in the presence of excess of ascorbate was clearly observed only for the tetraethyl substituted NR (Fig.1). The absolute values of the rate constant of the HA oxidation by ascorbate radical are remarkably high being in the range $(1-5) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for the pyrrolidine HA **1H** and HA of the imidazolidine nitroxides **4** and **5**, and almost two orders of magnitude higher for the HA of the tetraethyl substituted imidazoline NR **2** and **3** exceeding $10^5 \text{ M}^{-1} \text{ s}^{-1}$. Note that the rate constants of oxidation of the HA **1H** by superoxide ($3.2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ [40]) and by ascorbate radical ($1.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, Table 1) are comparable. Steady-state concentrations of the ascorbate radical in the blood [41,42] may significantly exceed those for superoxide radical particularly it is increased up to 3-30 nM during vitamin C supplementation. Therefore, the observed high reactivity of the cyclic HA towards ascorbate radical may significantly contribute in the observed kinetics of both the NR reduction [11] and HA oxidation [15] measured in blood as well as in the tissues with high ascorbate content such as liver and kidney [2,18,19].

Interestingly, the sum of the reverse reactions (1) and (2) represents the dismutation of ascorbate radical catalyzed by the NR. The rate of the NR-facilitated ascorbate radical dismutation might be comparable or even higher than spontaneous dismutation by the reaction (3), e.g., for 1 nM ascorbate radical this rate increases in two orders of magnitude in the presence of 0.1 mM concentrations of the NR **1** and its HA form **1H** (see Table 1). Therefore, NR, may be termed as “ascorbate radical dismutase”-mimics by analogy with their SOD-mimetic activity [16]. This might be of particularly interest due to discussed prooxidant side-effects of the ascorbic acid (vitamin C) probably related to the formation of ascorbate free radical *in vivo* [43]. On other hand, NR itself are considered as potential pharmacological agents with antioxidant activity [16]. The antioxidant SOD-mimetic properties of the NR might be partially compromised by prooxidant side effect of its highly reactive oxidized form, oxoammonium cation [17]. Therefore, from the mechanistical point of view, combined applications of the NR and vitamin C could have synergistic antioxidant effect. The ascorbate have high reactivity to the O-, C-, and S-centered physiologically relevant radicals forming less reactive ascorbate radical, and the NR react with ROS forming, in part, oxoammonium cation. In complimentary way, ascorbate effectively reduces oxoammonium cation to HA, while NR facilitates dismutation of ascorbate free radical.

The synergistic effect of antioxidant action of the NR and vitamin C is a simple example of the balanced antioxidant network. Glutathione is one of the main player in orchestrated cellular antioxidant defense [10,29]. In this paper we observed GSH-facilitated NR reduction and explained it by the scavenging of the ascorbate radical by GSH, resulted in the inhibition of the $\text{Asc}^{\bullet-}$ -induced oxidation of the HA. The observed effect of GSH on steady state concentration of ascorbate radical allows quantitative description in terms of the bimolecular reaction. However, it worth to note that direct reaction of ascorbate radical with GSH with formation of ascorbate and thiyl radical is thermodynamically unfavorable [44], and the mechanism of ascorbate radical scavenging by GSH might be complex. The apparent bimolecular rate constant of the ascorbate radical reduction by GSH was found to be comparatively low, $10 \text{ M}^{-1} \text{ s}^{-1}$, therefore it hardly could play significant role *in vivo*.

The development of the NR with long life-time in living tissues is a long-time goal for the researches working in the field of biomedical EPR application. Normally, the reduction of the NR *in vivo* proceeds towards complete loss of the EPR spectra of the NR limiting their applications. The synthesis of the tetraethyl substituted NR helped us to demonstrate, for the first time, the effective mechanism of the reoxidation of their HA back to the parent radical in the presence of ascorbate. It worth to encourage, therefore, the development of the NR with increased sensitivity of its HA form towards oxidation. Ideally, the reducing and oxidizing processes will equilibrate for these NR keeping significant fraction of the radical form. In its turn, the EPR measured steady-state concentration of NR would reflect redox status of the microenvironment of the probe.

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Glossary

List of abbreviation

Asc^{•-}	ascorbate radical
AscH⁻	ascorbate anione
DGA	diketogulonic acid
DHA	dehydroascorbic acid
DTPA	diethylenetriaminepentaacetic acid
EPR	electron paramagnetic resonance
GSH	L-glutathione
HA	hydroxylamine NR
MRI	magnetic resonance imaging
NR	nitroxyl radical (nitroxide)
ROS	reactive oxygen species
SOD	superoxide dismutase
TAM (Ox063)	

methyl tris(8-carboxy-2,2,6,6-tetrakis-(2-hydroxy-ethyl)-benzo[1,2-d:4,5-d']bis (1,3)dithiol-4-yl), triarilmethyl radical derivative

TEMPO

2,2,6,6-Tetramethylpiperidine 1-oxyl

TEMPOL

4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl

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Scheme 1.

The chemical structures of the NR **1-6** and corresponding HA, **1H** and **3H**.

Scheme 2.
Synthetic route for the HA **3H** and corresponding NR **3**.

Figure 1.

Left. The kinetics of the reduction of the NR 1 and 2 (denoted on the graph) by ascorbate measured by EPR (see Materials and Methods). The solutions of the NR, 1mM, and ascorbate (2 mM, dotted lines; 20 mM, dashed lines, and 100 mM, solid lines) in 0.1 M Na-phosphate buffer, pH 7.6, 0.1 mM DTPA, were mixed under anaerobic conditions. The mixture was immediately transferred to the 50 μ l capillary tube, and the peak intensity of the low-field component of the EPR spectrum was measured over time. **Right.** Enlarged initial part of the reduction kinetics of the NR for 20 mM ascorbate.

Figure 2.

The kinetics of the nitroxide reduction of 1 mM nitroxide **1** by 50 mM ascorbate in deaerated 0.1 M Na-phosphate buffer, pH 7.6, 0.1 mM DTPA, measured by EPR. The time-point of an addition of 10 mM hydroxylamine **1H**, 15.5 min after initiation of the kinetics, is indicated by arrow. Insert: enlarged part of the kinetics around the time-point of the hydroxylamine addition. Solid line is the best fit of experimental data to equations (1) - (4) with parameters: $k_1 = 0.09 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = 1.3 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, $k_{-2} = 0.8 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, $k_2 = 1.0 \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$, $k_3 = 2.6 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $k_{-3} = 1 \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$, $k_4 = 7.6 \times 10^{-4} \text{ s}^{-1}$.

Figure 3.

The kinetics of the reduction of 0.1 mM nitroxide **3** (A) and 1 mM nitroxide **1** (B) by 50 mM ascorbate in the presence of various concentrations of corresponding HA, **3H** and **1H**, measured by EPR spectroscopy (see Materials and Methods). The solutions were prepared in deaerated 0.1 M Na-phosphate buffer, pH 7.6, 0.1 mM DTPA. The concentrations of the hydroxylamines for the kinetics from bottom to top were 0, 0.1, and 0.5 mM for **3H** and 0, 2, 5, and 10 mM for **1H**. Note: 0.5 mM solution of **3H** contained 0.036 mM radical admixture resulted in an increase of initial concentration of the radical. For **1H** the radical admixture was less than 0.1%. Lines are the best fits of the experimental data to the equations (1-4) with parameters: $k_1 = (0.1 \pm 0.01)$

$$M^{-1}s^{-1}, k_{-1} = (1.1 \pm 0.2) \times 10^3 M^{-1}s^{-1}, k_2 = (1 \pm 0.2) \times 10^{-2} M^{-1}s^{-1}, k_{-2} = (0.9 \pm 0.2) \times 10^3 M^{-1}s^{-1}, k_3 = (2.6 \pm 0.3) \times 10^6, k_{-3} = (1.0 \pm 0.2) \times 10^{-2} M^{-1}s^{-1}, k_4 = (7 \pm 2) \times 10^{-4} s^{-1}.$$

Figure 4.

The kinetics of the reduction of 50 μ M of the NR 3 by 1 mM ascorbate in the absence (●) and in the presence (○) of ascorbate oxidase, 0.03 U/ml, in 0.1 M Na-phosphate buffer, pH 7.6, 0.1 mM DTPA, measured by EPR spectroscopy as described in the Materials and Methods. Symbols (■) depict the EPR signal intensity of the NR 3 in the presence of 0.03 U/ml of ascorbate oxidase alone.

Figure 5. Kinetics of the formation of the radical **1** upon oxidation of hydroxylamine **1H**, 1 mM solution, in the presence of 1 mM ascorbic acid and various concentrations of ascorbate oxidase: 0 (■), 0.01 (□), 0.02 (●), and 0.03 U/ml (○), in 0.1 M Na-phosphate buffer, pH 7.6, 0.1 mM DTPA, measured by EPR as described in the Materials and Methods.

Figure 6.

Kinetics of the formation of the NR **1** upon oxidation of 5 mM solution of the HA **1H** in deaerated 0.1 M Na-phosphate buffer, pH 7.6, 0.1 mM DTPA, in the presence of various concentrations of the dehydroascorbic acid, 2 mM (□) and 5 mM (■), measured by EPR as described in the Materials and Methods. Solid lines are the best fits of the experimental data to equations (1-4) assuming the rate constant of monomolecular decomposition of dehydroascorbic acid, $k_4 = (6.8 \pm 0.5) \times 10^{-4} \text{ s}^{-1}$ and the rate constant of hydroxylamine oxidation by the ascorbate radical, $k_{-1} = (1.05 \pm 0.05) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ yielding the bimolecular rate constant of hydroxylamine oxidation by dehydroascorbic acid, $k_2 = (1 \pm 0.25) \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$.

Figure 7.

Kinetics of the reduction of 1mM nitroxide **1** in deaerated 0.1 M Na-phosphate buffer, pH 7.6, 0.1 mM DTPA, in the presence of various concentrations of the dehydroascorbic acid, 5 mM (□) and 10 mM (■), measured by EPR as described in the Materials and Methods. Solid lines are the best fits of the experimental data to equations (4, 5) yielding the rate constant $k_4 = 7 \times 10^{-4} \text{ s}^{-1}$ and $k_5 = (3.0 \pm 0.4) \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$.

Figure 8.

Influence of the glutathione on the kinetics of the reduction of the NR **4** by ascorbate measured by EPR as described in the Materials and Methods. The solutions of ascorbate and GSH in 0.1 M Na-phosphate buffer, pH 7.6, 0.1 mM DTPA, were added to 1 mM solution of the NR **4** in the same buffer. The mixture was transferred to the 50 μ l capillary tube immediately afterwards, and intensity of the low-field peak of the EPR spectrum was monitored over time. The concentrations of the reagents were as following: (a) 50 mM GSH alone; (b) 100 mM ascorbate alone; (c) 100 mM ascorbate and 5 mM GSH; (d) 100 mM ascorbate and 50 mM GSH.

Figure 9a.

The EPR spectra of the ascorbate free radical measured in 0.1 M Na-phosphate buffer, pH 7.6, 0.1 mM DTPA, in the presence of ascorbate oxidase, 0.03 U/ml, ascorbate, 1mM, and various concentrations of GSH: 0, 20, 40, and 60 mM for the spectra from the biggest spectral intensity to the smaller ones. The EPR spectrometer settings were as following: scan time, 41.94 s; number of scans, 16; time constant, 40.96 ms; sweep width, 5 G; modulation amplitude, 0.5 G.

Figure 9b.

The dependence of the observed rate constant, k_{obs} , of the reaction of ascorbate free radical with glutathione on GSH concentration. The bimolecular rate constant, k_{GSH} , was calculated as described in the Materials and Methods and found to be equal to $(10 \pm 5) \text{ M}^{-1}\text{s}^{-1}$.