Biochemical Pharmacology, Vol. 40, No. 4, pp. 799–802, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

EPR STUDIES ON THE OXIDATION OF HYDROXYUREA TO PARAMAGNETIC COMPOUNDS BY OXYHEMOGLOBIN

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(*Received* 27 *December* 1989; *accepted* 9 *March* 1990)

Abstract—*N*-Hydroxyurea forms methemoglobin from oxyhemoglobin with concomitant formation of the aminocarbonylaminooxyl radical H_2N—CO—NHO$^\bullet$, as detected with electron paramagnetic resonance spectroscopy (EPR). This radical could be detected for several hours in a low steady-state concentration. Approximately 1 hr after the reaction had been started, the EPR spectra of two additional paramagnetic intermediates could be detected at low temperature (77°K), a low-spin ferric methemoglobin complex with hydroxyurea (MetHb—NHOH—CO—NH_2) and the hemoglobin–nitric oxide adduct (Hb^{2+}–NO). The intensities of their EPR spectra increased steadily over the range of more than 64 hr. The low-spin ferric methemoglobin complex was immediately formed when hydroxyurea was dissolved in a methemoglobin solution whereas the nitric oxide complex was possibly an oxidation product of the MetHb–hydroxyurea adduct. Its oxidative degradation is known to lead to the very toxic compounds nitric oxide and nitrogen dioxide which can therefore contribute to the toxic action of hydroxyurea.

N-Hydroxyurea is an important antitumor drug that inhibits ribonucleotide reductase [1]. Unfortunately serious side effects such as lipid peroxidation of cell membranes, increased osmotic fragility of erythrocytes and the formation of methemoglobin after hydroxyurea treatment have been reported to occur [2]. Since the coapplication of free radical scavengers such as tocopherol and sodium benzoate have a protective effect, the toxicity of hydroxyurea is at least partly due to the formation of free radical intermediates. For instance the inhibitory function on ribonucleotide reductase has been ascribed to result from one-electron transfer from hydroxyurea to the enzyme-bound tyrosine radical, thereby forming the aminocarbonylaminooxyl radical (H_2N—CO—NHO$^\bullet$) [1]. Lassmann and Liermann [3] reported the formation of the same radical (H_2N—CO—NHO$^\bullet$) from hydroxyurea and hydrogen peroxide or Cu^{2+} ions. In previous papers we described the formation of paramagnetic intermediates from oxyhemoglobin and unsubstituted hydroxylamine [4] as well as methyl-substituted hydroxylamines [5]. All the investigated compounds formed the aminooxyl radical ($R_1R_2NO^\bullet$), free methemoglobin and a low-spin ferric methemoglobin complex with excess hydroxylamine (MetHb—NR_1R_2OH). With unsubstituted hydroxylamine a third paramagnetic intermediate was detected, the hemoglobin–nitric oxide adduct. In view of the fact that *N*-hydroxyurea is structurally related to the hydroxylamines and hydroxamic acids [6] a free radical mechanism of the methemoglobin formation from oxyhemoglobin can be expected, including paramagnetic complexes similar to those observed with hydroxylamine.

MATERIALS AND METHODS

N-Hydroxyurea was obtained from the Aldrich Chemical Co. Bovine hemoglobin was prepared in a modified procedure described by Eyer *et al.* [6] for human hemoglobin. Bovine red cells were washed five times with twice the amount of 0.2 M phosphate buffer, pH 7.4. The cells were sonicated in distilled water and 10 g of Celite were added to 250 mL of the hemolysate. The mixture was stirred for 20 min and then centrifuged for 30 min at 15,000 *g*. Purified hemoglobin was prepared by chromatography of the hemolysate on $DEAE_{52}$-cellulose. Ten mL of the hemolysate were applied to a column (26 mm i.d.) containing 50 g of $DEAE_{52}$ cellulose (Serva) preequilibrated with 10 mM Tris–HCl pH 8.3 and eluted with 0.1 M Tris–HCl pH 7. The fractions were tested for catalase and SOD activity [7] and only those with a catalase activity $k < 1$ and no detectable SOD activity were pooled. If necessary, the chromatographic procedure was repeated. The pooled fractions were chromatographed on Sephadex G-25 with 0.2 M phosphate buffer, pH 7.4. Oxyhemoglobin was determined at 540 nm, the methemoglobin content by the increase in absorbance at 540 nm caused by the addition of cyanide [8]. The EPR experiments were carried out in a Bruker ER 200 D-SRC 9/2.7 spectrometer operating at 9.6 GHz with 100 kHz modulation frequency equipped with a rectangular TE_{102} microwave cavity. For the measurements of the g-values at room temperature, 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) was used as an internal standard (g = 2.0055) [9]. For the liquid

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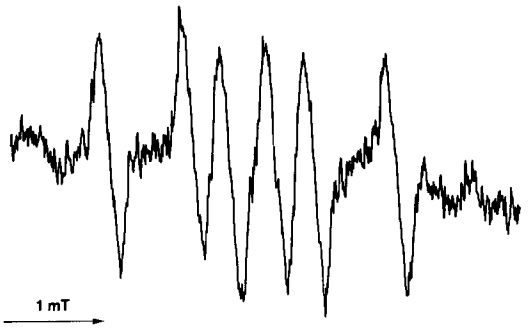

Fig. 1. EPR spectrum of the aminocarbonylaminooxyl radical (H_2N—CO—NHO·). The incubation mixture contained oxyhemoglobin (2.0 mM) and hydroxyurea (0.2 M) in 0.2 M phosphate buffer pH 7.4, containing 1 mM DETAPAC. The spectrometer settings were: scan range, 5.0 mT; modulation amplitude, 0.1 mT; receiver gain, 1×10^6; microwave power, 20 mW; time constant, 0.33 sec; scan rate, 1.78 mT/min; 72 scans.

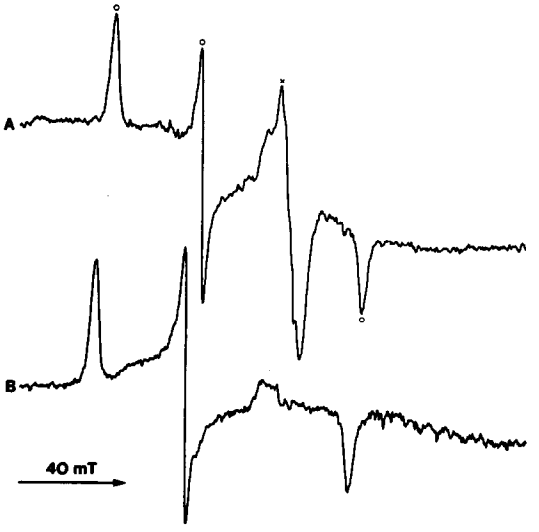

Fig. 2. EPR spectra of the low-spin methemoglobin–hydroxyurea complex (○) and the hemoglobin–nitric oxide adduct (×). (A) EPR spectrum recorded after 40 hr incubation. The incubation mixture contained oxyhemoglobin (2.0 mM) and hydroxyurea (0.2 M) in phosphate buffer pH 7.4. The mixture was frozen after 40 hr and recorded at 77°K. The spectrometer settings were: scan range, 200 mT; modulation amplitude, 0.5 mT; receiver gain, 6.3×10^5; microwave power, 20 mW; time constant, 1.3 sec; scan rate, 17.9 mT/min. (B) EPR spectrum recorded at 77°K immediately after freeze-quenching a 1:1 mixture of a methemoglobin solution (2 mM, pH 7.4, N_2-gassed) and a hydroxyurea solution (1 M in 0.2 M phosphate buffer pH 7.4, N_2-gassed) in liquid nitrogen. The spectrometer settings were: scan range, 200 mT; modulation amplitude, 0.5 mT; receiver gain, 1×10^4; microwave power, 10 mW; time constant, 1.3 sec; scan rate, 17.9 mT/min. The spectrum shows the low-spin methemoglobin–hydroxyurea complex.

nitrogen temperature measurements, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used (g = 2.0036) [10]. For the flow experiments a quartz mixing flat-cell was chosen and for the liquid nitrogen temperature measurements a finger dewar equipped with a quartz test tube. Computer simulations of EPR spectra and multi-scan experiments were carried out with the Bruker data system ESP 1600.

RESULTS

Measurements at room temperature

Figure 1 shows the EPR spectrum obtained from a solution of 0.2 M *N*-hydroxyurea and 2 mM oxyhemoglobin in phosphate buffer (0.2 M, containing 1 mM DETAPAC). The signal intensity after a single scan was below the detection level of the spectrometer but a good signal to noise ratio was obtained after the accumulation of 72 single scans. This is indicative of a very low but long lasting steady-state concentration of the radical species formed in our system. The spectral parameters were as follows: $a_H = 1.17$ mT and $a_N = 0.805$ mT, the g-value was g = 2.0063, practically identical to the values reported by Lassmann for the aminocarbonylaminooxyl radical H_2N—CO—NHO· (1.16 mT, 0.80 mT and 2.0060, respectively). The intensity of its EPR spectrum remained more or less the same for several days (our measurements were extended over 64 hr). A similar time course was observed for the formation rate of methemoglobin, the other direct product detected in our system (see below).

Measurements at liquid nitrogen temperature

When a solution of 0.2 M hydroxyurea and 2 mM oxyhemoglobin in phosphate buffer (0.2 M, 1 mM DETAPAC) was allowed to react for 40 hr and then frozen at 77°K, the EPR spectrum shown in Fig. 2A was obtained. Two different species are present. The first one (marked ○) exhibits the characteristics of a low-spin ferric complex that we assigned to a methemoglobin–hydroxyurea adduct in analogy to our previous results with hydroxylamines [4, 5]. The g-values of the low-spin complex are $g_1 = 2.52$, $g_2 = 2.24$, and $g_3 = 1.86$. The g-values of the second species (marked ×) are $g_{max} = 2.04$ and $g_{min} = 1.98$, practically identical to the values reported by Kon [11] for the hemoglobin–nitric oxide adduct (2.039 and 1.986, respectively). In addition, the absorption of free methemoglobin was detected at g = 6 (not shown). This indicates that the formation of the MetHb–hydroxyurea complex was not complete despite the large excess of hydroxyurea.

In order to prove that the low-spin ferric complex shown above was directly formed from methemoglobin and hydroxyurea without the necessity of oxygen being present we did the control experiment shown in Fig. 2B. We mixed an anaerobic methemoglobin solution (2 mM in 0.2 M phosphate buffer pH 7.4) with a hydroxyurea solution (1 M, pH 7.4) and freeze quenched it within less than 1 sec. The spectrum shows the low-spin ferric methemoglobin-hydroxyurea complex at the same g-values as in Fig. 2A. This proves that the latter complex is immediately formed after the mixing and that no oxygen is involved in this reaction. Accordingly, no nitric oxide adduct could be detected in the reaction mixture.

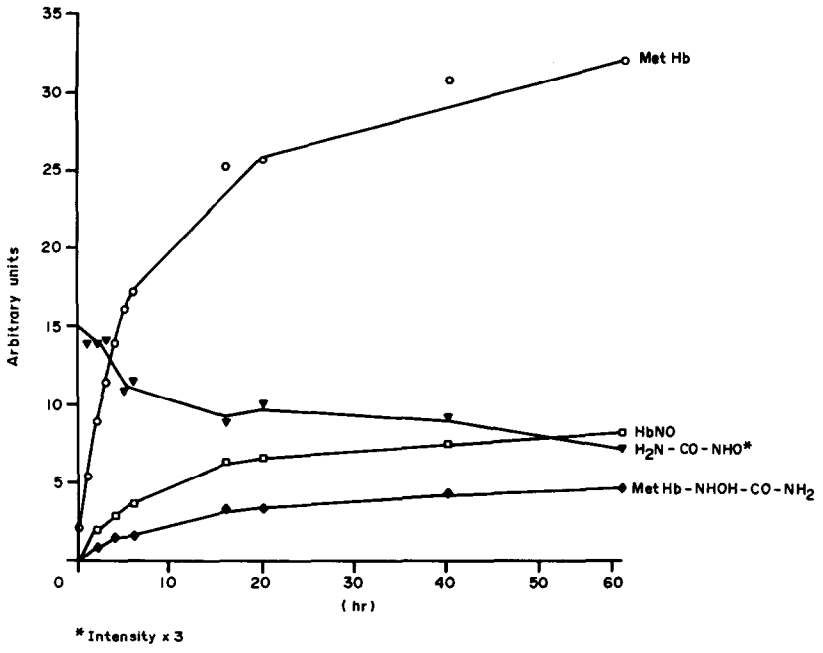

Fig. 3. Time course of the reaction between oxyhemoglobin (2 mM, pH 7.4) and hydroxyurea (0.2 M) in 0.2 M phosphate buffer pH 7.4, containing 1 mM DETAPAC. The samples were taken at different intervals and their EPR spectra were recorded at room temperature (aminocarbonylaminooxyl radical, ▼) or at 77°K (low-spin methemoglobin–hydroxyurea adduct, ◆; hemoglobin–nitric oxide complex, □; and free methemoglobin, ○). The peak-heights of the most intense peaks were measured in arbitrary units.

In Fig. 3 the time course of the appearance of the four paramagnetic species observed is depicted. The aminocarbonylaminooxyl radical (H_2N—CO—NHO·) was present immediately after the reaction had been started. Its concentration reached a maximum after 15 min and then declined gradually until it reached the detection level after 64 hr (marked ▼). The formation rate of methemoglobin had its maximum immediately after mixing and then decreased slowly without reaching a steady state during the observation period of 64 hr (marked ○). At that point the methemoglobin concentration had reached about 35% of the total hemoglobin concentration. The methemoglobin–hydroxyurea complex was first detected after 30 min and its intensity was still increasing after 64 hr when the reaction was stopped (marked ◆). Likewise, the hemoglobin–nitric oxide complex was not observed for the first 30 min and then developed gradually over the 64 hr observation period (marked □).

DISCUSSION

Our investigations show the formation of methemoglobin and three paramagnetic reaction intermediates from oxyhemoglobin and hydroxyurea. First, the aminocarbonylaminooxyl radical (H_2N—CO—NHO·) which can be detected immediately after the reaction has been started:

$$HbO_2 + NH_2\text{—}CO\text{—}NHOH \rightarrow NH_2\text{—}CO\text{—}NHO^{\cdot} + MetHb + HO_2^-.$$

Compared to the previously investigated hydroxylamines [4, 5] the reaction proceeds very slowly and only low steady-state concentrations of the free radical were observed. This is in agreement with the observed methemoglobin formation rate which is high at the beginning and then slowly declines without dropping to zero during the 64 hr observation period. The so-formed methemoglobin reacted with excess hydroxyurea thereby forming a low-spin ferric adduct (MetHb–hydroxyurea) but the equilibrium of this formation was on the side of free methemoglobin as could be seen in the EPR experiments at 77°K:

$$MetHb + NHOH\text{—}CO\text{—}NH_2 \rightleftharpoons MetHb\text{—}NHOH\text{—}CO\text{—}NH_2.$$

A fourth reaction product was found at a later time in the reaction sequence. In our experiments it appeared for the first time after about 30 min and its concentration was still increasing after 64 hr when we stopped the reaction. In comparison with the results obtained with hydroxylamine we suggest that the nitric oxide adduct stems from the oxidative degradation of the low-spin ferric methemoglobin complex:

$$MetHb\text{—}NHOH\text{—}CO\text{—}NH_2 \xrightarrow{[O_2]} Hb^{2+}\text{—}NO + CO_2 + NH_3.$$

This hemoglobin–nitric oxide complex is known to be slowly oxidized and the liberation of the very harmful gases nitric oxide and nitrogen dioxide can cause considerable damage to biological membranes [12]. In summary, hydroxyurea reacts slowly but continuously with oxyhemoglobin and reactive free radicals are produced over an extended period of time. Due to the sensitivity of EPR spectroscopy we had to use much higher concentrations of hydroxyurea than generally used for clinical treatment. Our data may however be a rationale for a better understanding of clinical side effects such as extended methemoglobin formation and structural and functional alterations of erythrocytes.

Acknowledgement—This research was supported by the Österreichische Fonds zur Förderung der wissenschaftlichen Forschung; Project No. P07150-ME.

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