Original article

Electron transfer in *N*-hydroxyurea complexes with iron(III)

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Abstract

Redox behaviour of the iron(III) complex with the antitumour drug hydroxyurea was studied by cyclic voltammetry. The complex underwent a one-electron reduction, followed by an irreversible chemical reaction (EC mechanism) in which a ligand was released. In addition, it was found that the hydroxyurea gave up an electron to iron(III) in solution. Differential-pulse voltammetry revealed an increase in the concentration of the generated iron(II) species. Electron paramagnetic resonance (EPR) spectroscopy studies of the oxidative degradation of hydroxyurea confirmed formation of the radical species $H_2N–CO–NHO^.$. Electrochemical data for iron(III) complexes of hydroxyurea and its structural analogue 3-ethylhydroxyurea, which also exhibits antitumour activity, show the same mechanism involved in the electron transfer. The observed redox properties indicate that hydroxyurea may interfere with electron transfer processes in biological systems after binding to iron-containing ribonucleotide reductase.
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1. Introduction

Hydroxyurea has been extensively used in medical practice for treating a number of cancers, especially chronic myelogenous leukaemia, and sickle cell diseases [1]. Hydroxyurea inhibits ribonucleotide reductase, which catalyses the reduction of ribonucleotides to the corresponding deoxyribonucleotides required for DNA synthesis [2,3]. In light of its ability to inhibit DNA synthesis and to induce cell cycle arrest, hydroxyurea has recently emerged also in the treatment of HIV infections [4].

Protein R2, a small subunit of ribonucleotide reductase, contains a dinuclear μ-oxo-bridged Fe(III) centre and a tyrosyl free radical essential for activity. This radical participates in the enzymatic activity whereas the dinuclear iron centre is required for its generation and stabilisation [5]. The precise mechanism of hydroxyurea action is still not completely clear. The data available concerning the reduction of the diferric/radical site are contradictory. In active *Escherichia coli* protein R2, hydroxyurea scavenges the tyrosyl radical without affecting the iron centre [6,7]. In contrast, hydrox-

yurea reacts with both the iron centre and the radical on a similar time scale in mouse protein R2, and this is accompanied by the release of iron from the protein in the ferrous form [8].

Hydroxyurea is known to form a complex with iron(III) [9]. Some reports in the literature suggest that only hydroxyureas that are capable of complexing metals show antitumour activity [10]. The molecular basis for the selectivity of hydroxyurea is attributed to the sequestering of iron required for the activity of ribonucleotide reductase. An investigation of the electron transfer processes in hydroxyurea complexes with Fe(III) may provide a further insight into the mode of hydroxyurea's action since iron(III) in the active site of the enzyme participates in the redox reactions. The electrochemical behaviour of the hydroxyurea iron(III) complex has not been studied to date, except that the reduction potential of this complex has been reported. However, it was found that the in situ generated complex was reduced at the same potential as a free iron(III) ion in the solvent used, indicating that the redox potential determined was observed for the reduction of free iron species [11].

The primary goal of the present study is to investigate the electrochemical properties of the hydroxyurea complex with iron(III) and to determine the mechanism involved in the elec-
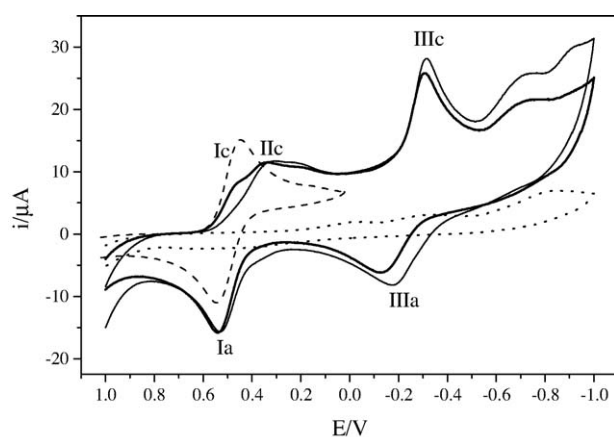
Fig. 1. Cyclic voltammograms of hydroxyurea (- - - -), its complex with iron(III) for $c_L/c_{Fe} = 1:1$ (—) and $c_L/c_{Fe} = 5:1$ (—) and free iron(III) species (·········). Conditions: $c_{Fe} = 5 \times 10^{-3}$ M, dimethylformamide and 0.1 M LiClO$_4$; Pt working electrode at 100 mV/s scan rate, potential expressed with respect to Ag/AgCl.

tron transfer processes. Comparison of the data obtained with the electrochemical behaviour of complexes with closely related ligands such as 3-ethylhydroxyurea, which also exhibits antitumour activity, and acetohydroxamic acid is given.

2. Results and discussion

2.1. Stoichiometry of the hydroxyurea iron(III) complex

The ligand to metal ratio in the complex was studied by the molar ratio method using the same experimental conditions under which its voltammetric measurements were performed. Spectral changes accompanying the increase of hydroxyurea in solution show that the absorption peak at 580 nm increases with the amount of ligand added. The molar ratio plot at 580 nm shows inflection at [HU]/[Fe(III)] = 1, indicating a stoichiometry of 1:1 for the complex studied.

2.2. Electrochemistry of the hydroxyurea iron(III) complex

Cyclic voltammetry was used to study the mechanism involved in the electron transfer in the hydroxyurea complex with iron(III). Due to its instability in water, the complex was examined in dimethylformamide, in which it was stable on the timescale of the cyclic voltammetry measurements [9]. The voltammograms were recorded in the range +1.5 to –1.5 V, employing scan rates between 10 mV/s and 1 V/s. Ferrocene was used as a standard both to evaluate the number of electrons involved in the electrochemical process and to assign the potential values. On the platinum electrode, the ferrocenium/ferrocene couple exhibited a potential separation of $\Delta E_p = 80$ mV, and an i_p^c/i_p^a current ratio equal to the one with $E_{1/2} = +0.50$ V vs. Ag/AgCl.

The cyclic voltammograms of hydroxyurea, its complex with iron(III) and free iron(III) species are shown in Fig. 1. The cyclic voltammogram of hydroxyurea shows that the free

ligand is electrochemically inactive in the potential range investigated. The voltammogram of the complex exhibits one quasi-reversible (peak Ic/Ia) and one irreversible reduction wave (peak IIc). The peak couple Ic/Ia can be unambiguously assigned to the reduction of Fe(III) to Fe(II) in noncomplex form [12]. The difference between the potential of the cathodic and the anodic peaks ($\Delta E_p = 98$ mV) and the i_p^c/i_p^a current ratio (≈ 0.8) remained constant when the scan rate was varied between 0.01 and 1 V/s. All these data are diagnostic for a simple quasi-reversible one-electron charge transfer controlled by diffusion. The second cathodic process was assigned to the Fe(III)/Fe(II) redox potential of the chelated iron(III) species. The potential of the cathodic peak was shifted cathodically by 30 mV when the scan rate was changed from 10 mV/s to 1 V/s. This observation and the irreversibility of the cathodic peak current indicate that the charge transfer was followed by a fast homogeneous chemical reaction. Consequently, hydroxyurea was released from the complex during the reduction step due to lower stability of the Fe(II) complex formed. The rate constant for the irreversible dissociation decomposition of the reduced complex could not be estimated due to poorly resolved waves Ic and IIc. The redox potential of the chelated iron was shifted to less positive values only by about 0.1 V. Addition of ferrous salt in solution of hydroxyurea did not change the voltammetric response owing to the lability of the Fe(II) complex, so the reduction of iron(II) bound to hydroxyurea could not be observed. The cyclic voltammograms registered for the iron(III):ligand 2:1, 1:1, 1:5 molar ratio solutions show changes in the electrochemical response related to the ratio of Ic/IIc current peak heights. The increase in ligand concentration led to a decrease in the peak current Ic and a mutually related increase in the peak current IIc. These findings confirm that wave IIc is associated with the reduction of iron(III) bound to hydroxyurea.

The third cathodic peak (IIIc) is related to the product of the oxidative degradation of hydroxyurea because it was at the same potential as the first cathodic peak of hydroxyurea alone when the current recording was started from +1.5 V (Fig. 2). At this much more positive potential, *N*-hydroxyureas were oxidised in the 2e$^-$ process yielding the aminocarbonyl cation, which was decomposed to the corresponding amine, carbon dioxide and nitrous oxide [13]. The presence of iron(III) ions caused the appearance of the IIIc cathodic peak current, although the initial cathodic scan was applied from +1 V, which is lower than the potential required for anodic oxidation of free hydroxyurea. Considering that the product of hydroxyurea oxidation could not be generated below the voltage applied, the obtained results indicate that the redox reaction occurred between hydroxyurea and iron(III) in solution. This oxidative product is supposed to have been released via an intermolecular redox process in which electron transfer occurred from hydroxyurea to iron(III) ion.

Analysis of the peak couple IIIc/IIIa shows that the ratios of i_p^a/i_p^c were changed from 0.20 to 0.99 for scan rates between 0.2 and 1.2 V/s. Thus, an EC mechanism (electrochemical reaction followed by a chemical reaction) could be
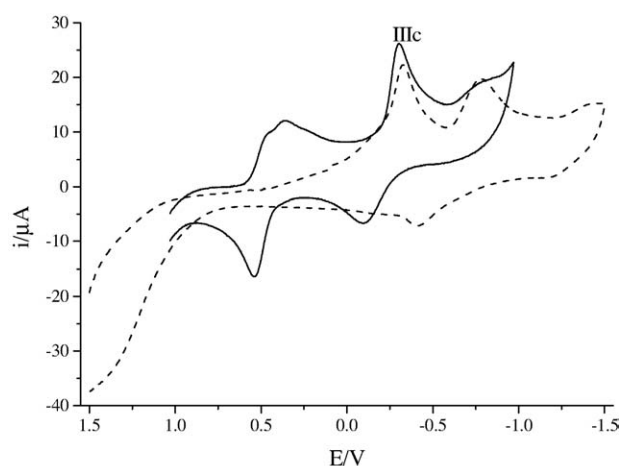
Fig. 2. Cyclic voltammograms of 5×10^{-3} M hydroxyurea solutions (- - - -) and its complex with iron(III) for c_L/c_{Fe} = 1:1 (—) at different starting potential. Same conditions as Fig. 1.

involved. The increase of i_p^a with the scan rates is due to the fact that at higher scan rates there is less time for an irreversible chemical reaction. Addition of a base to a solution of the complex led to disappearance of peak IIIc while peak IIIa remained unchanged, indicating that peak IIIa is not the anodic counterpart of the cathodic peak IIIc (Fig. 3). Oxidation of hydroxyurea has been well studied [14–18]. All reports show that treatment of hydroxyurea with hydrogen peroxide in the presence of various enzymes or heme proteins produces the aminocarbonylaminooxyl radical (H_2N–CO–$NHO^{.}$). Further single electron oxidation produces the C-nitroso species (H_2N–CO–N=O) as an intermediate. Finally, the formation of carbon dioxide, ammonia and nitrogen oxides (N_2O, NO, nitrite and nitrate as stable oxidative decomposition products of NO) was evidenced during these reactions. Therefore, peak IIIc could be attributed to the reduction of nitrogen oxides. It is difficult to identify the process to which peak IIIc/IIIa is due, for this is obviously a very complicated oxidation mechanism with a number of processes occurring in solution.

The complex was immediately formed after mixing the reagents and addition of a base to the solution produced a
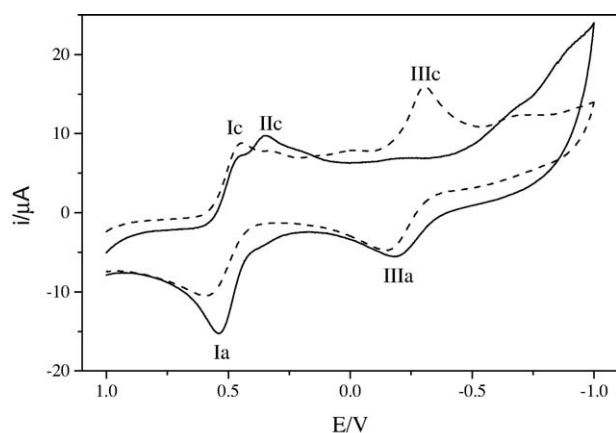

Fig. 3. Cyclic voltammograms of hydroxyurea complex with iron(III) for c_L/c_{Fe} = 1:1.5 before (- - - -) and after (—) the addition of base (benzyltrimethyl-ammonium hydroxide (final concentration 5.7×10^{-3} M). Same conditions as Fig. 1.
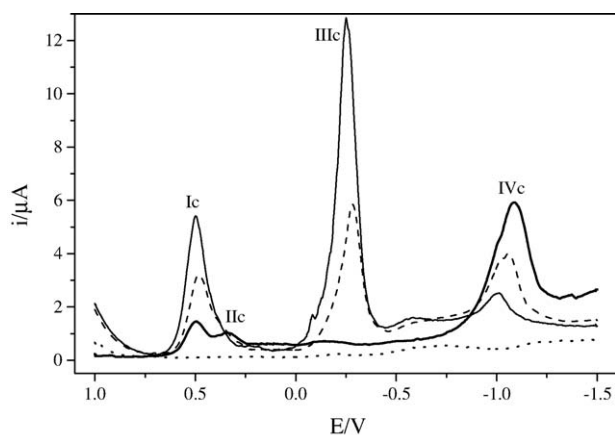

Fig. 4. Differential-pulse voltammograms of hydroxyurea (·········), its complex with iron(III) for c_L/c_{Fe} = 1:1 obtained immediately after mixing the reagents (—), after 24 (- - - -) and 72 hours (—). Same conditions as Fig. 1.

change in voltammograms regarding the ratio of Ic/IIc current peak heights (Fig. 3). With an increase of the solution pH, peak IIc became more pronounced and its height was found to increase, suggesting the release of a proton in the complexation reaction.

Furthermore, the dark blue solution of the complex showed a change in colour over a period of several hours and was decolourised within a few days. The progress of the reduction of iron(III) with hydroxyurea was monitored by differential-pulse voltammetry. As can be seen from Fig. 4, after 72 hours of mixing the iron(III) solution with hydroxyurea, the iron(III) concentration dropped almost to zero and at the same time the peak current IVc of differential-pulse voltammograms at $E_p = -1.05$ V reached the value of the initial iron(III) concentration. This peak was unambiguously assigned to the reduction of iron(II) [12] and its evidence in the solution is a quantitative proof of the proposed mechanism of electron transfer. However, the concentration of the generated oxidative product of hydroxyurea was decreased already after 24 hours in spite of increasing iron(II). A possible explanation is that the gaseous species generated in the oxidation diffuses from the solution or that the initial product of the oxidation undergoes a chemical reaction to yield a substance that cannot be reduced. The differential-pulse voltammograms show that the complex was found in a low concentration after 72 hours upon addition of iron(III) to a solution of hydroxyurea.

2.3. Electron paramagnetic resonance (EPR) studies of hydroxyurea oxidation with the iron(III) ion

Room temperature EPR analysis of the solution of hydroxyurea and iron(III) revealed a distinct six-line resonance pattern characteristic of the aminocarbonylaminooxyl radical (Fig. 5). The spectral parameters were: $a_N = 8.15$ and $a_H^{NH} = 11.47$ G, the g-value was 2.00819. The spectrum is identical with the reported spectra of the radical generated by the oxidation of hydroxyurea with excess hydrogen peroxide or oxyhaemoglobin [18–20]. The radical was formed immedi-
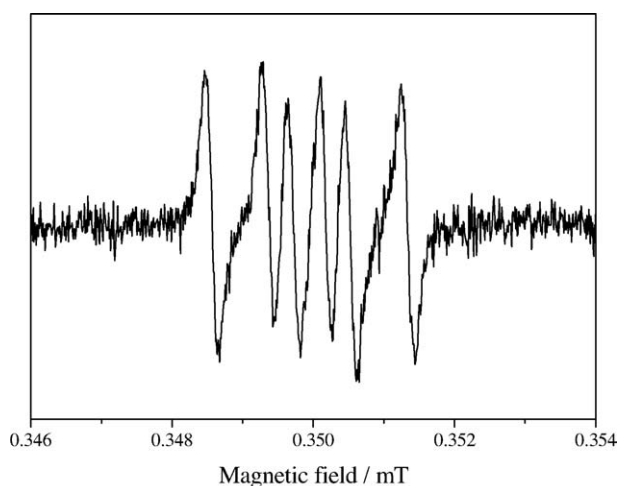
Fig. 5. First derivative EPR spectrum of the aminocarbonylaminooxyl radical (H_2N–CO–$NHO^{\cdot}$). Conditions: $c_{Fe} = 5 \times 10^{-3}$ M, c_L/c_{Fe} = 1:1, DMF, 0.1 M $LiClO_4$, at room temperature with f = 9.836 GHz and 6.4 mW.

ately after the reaction was started as a result of the one-electron transfer from hydroxyurea to iron(III). The radical species Fe(III)–H_2N–CO–$NHO^{\cdot}$ was not found. This observation further proves the proposed electron transfer mechanism of the decomposition of hydroxyurea.

2.4. Comparison with electrochemical properties of other iron(III) complexes

The electrochemical data for iron(III) complexes of hydroxyurea and its structural analogue 3-ethylhydroxyurea show the same mechanism of electron transfer (Fig. 6). Although the overlapping of cathodic peaks Ic and IIc makes it difficult to determine the exact value of the reduction potentials of the complexes, they appear similar. The reduction peaks found for the hydroxyurea and 3-ethylhydroxyurea complexes were at +0.34 and +0.35 V, respectively. These data suggest that the same structure for both complexes can be assumed.
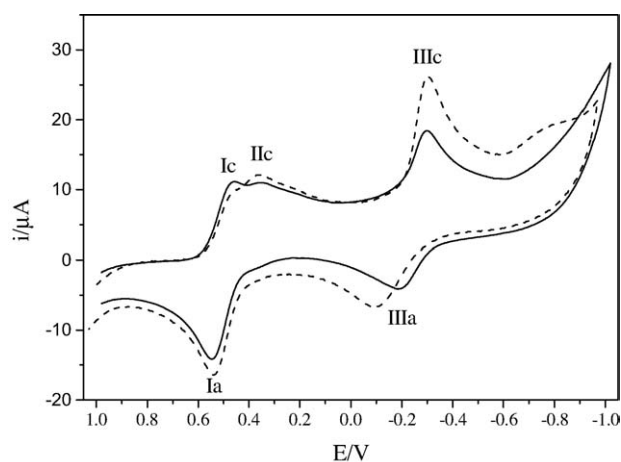

Fig. 6. Cyclic voltammograms of iron(III) complexes with hydroxyurea (- - - -) and 3-ethylhydroxyurea (—). Conditions: $c_{Fe} = 5 \times 10^{-3}$ M, c_L/c_{Fe} = 1:1, others same as Fig. 1.

The behaviour of the iron(III) complex with acetohydroxamic acid differed from that of the hydroxyurea complexes. Although the same ratio c_L/c_M was used, in this system iron was completely bound to the ligand and thus the cathodic peak corresponding to the reduction of solvated iron(III) was not observed. The complex was reduced at a less positive value (E_p = +0.07 V) than the hydroxyurea complexes with iron(III). The lower potential of the acetohydroxamatoiron(III) complex indicates its higher stability constant, which is in agreement with the values determined from equilibrium measurements in aqueous solution [9,21]. Electrochemical studies of iron complexes reveal a strong influence of the type of metal binding groups on the redox potentials, with relatively little influence of the ligand substituent effects [22,23]. Therefore, the comparison of the redox potential values obtained for hydroxyurea complexes with those obtained for the aceto-hydroxamatoiron(III) complex may also support the existence of a different iron to ligand coordination mode. For the latter case, only the (O, O) donor set may be involved in the metal ion coordination. Hence, chelation through the N–O oxygen atom and the amine group can be assumed for hydroxyurea complexes. On the other hand, it is reasonable to anticipate that the electron donating effect of the ethyl group on nitrogen involved in complexation should be reflected in the difference of the redox potential. Due to the increased electron density at the iron centre, reduction at a less positive potential would be expected for the ethyl hydroxyurea complex. However, it is possible to speculate that the redox potential of the complexes may be related, in addition to the electron donating ability of the substituent, to steric effects of the ligands.

3. Conclusions

The redox potential of the hydroxyurea complex with iron(III) was shifted to less positive values only by 0.1 V compared to the solvated iron ion, indicating a low stability constant for this complex. During the reduction step, hydroxyurea was released from the complex due to lower stability of the Fe(II) complex formed (EC mechanism). In addition, it was found that the redox reaction occurred between hydroxyurea and iron(III) in solution. Differential-pulse voltammetry revealed an increase in the concentration of the generated iron(II) species. EPR studies of the oxidative degradation of hydroxyurea confirmed the one-electron transfer from hydroxyurea to iron(III) and formation of the radical species H_2N–CO–$NHO^{\cdot}$. The electrochemical data for iron(III) complexes of hydroxyurea and its structural analogue 3-ethylhydroxyurea show the same mechanism involved in the electron transfer processes. The observed redox properties indicate that hydroxyurea may interfere with electron transfer processes in biological systems after binding to iron-containing enzymes and that it is able to directly affect the iron centre of ribonucleotide reductase. If such reaction would occur in vivo, the presence of hydrogen peroxide in the aqueous surroundings may

produce a Fenton-like reaction. The iron released from the protein in the ferrous form can generate through catalysis the hydroxyl free radical. Wherever it is produced, the hydroxyl radical is highly reactive and can cause covalent cross-linking of free radical propagation in a wide variety of biological molecules. Possible biomolecules damage processes may be considered as side effects of described electron transfer mechanism.

4. Experimental

4.1. Materials and reagents

Hydroxyurea and acetohydroxamic acid (min. 98 %) supplied by Sigma were used without further purification. 3-Ethylhydroxyurea was prepared according to the procedure described previously [10]. Iron(III) perchlorate stock solution was prepared from recrystallised solid iron(III) perchlorate hydrate (98 %, Aldrich) and was standardised as described previously [9]. All other chemicals were of analytical grade (Merck and Sigma).

The studied complexes were generated in situ by addition of an appropriate amount of the iron(III) perchlorate stock solution to a solution of ligand in dimethylformamide containing 0.1 M lithium perchlorate as supporting electrolyte. The iron(III) concentration was kept at 5×10^{-3} M, and the ligand was varied from 2.5×10^{-3} to 2.5×10^{-2} M.

4.2. Voltammetric measurements

Voltammetry experiments were performed using an EG&G Princeton Applied Research Model 273 A potentiostat. The three-electrode system was composed of a platinum working electrode ($\varnothing = 2$ mm, EG&G/PAR), a platinum auxiliary electrode and an Ag/AgCl reference electrode, which was separated from the sample solution by a Vycor glass frit.

To provide a reproducible active surface and improve the sensitivity and resolution of voltammetric peaks, the working electrode was polished prior to each electrochemical measurement with 0.5 µm alumina powder on a polishing cloth. Then, it was thoroughly rinsed with methanol and doubly-distilled water, and gently dried with tissue paper. All the solutions examined using electrochemical techniques were degassed with solvent-saturated argon for 5 minutes The samples were blanketed with argon during acquisition. All measurements were performed with positive feedback IR compensation and were carried out at room temperature.

4.3. Spectroscopic measurements

The EPR measurements were carried out using a Bruker ELEXSYS E500 EPR spectrometer. The concentrations were the same as those applied in the voltammetric studies.

Visible absorption spectra were recorded on an Agilent 8453 UV–Visible spectrophotometer diode-array. The changes in absorbance at the wavelength of maximum absorption were measured for different ligand to metal ratios.

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