OXYGEN INHIBITION OF NITROREDUCTASE: ELECTRON TRANSFER FROM NITRO RADICAL-ANIONS TO OXYGEN

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SUMMARY

The inhibition of many nitroreductases by oxygen has been explained by Mason and Holtzman in terms of electron transfer to oxygen from the nitro radical-anions, which have been identified as the first intermediate in some reductase systems. We have used the pulse radiolysis technique to measure the bimolecular rate constants of this electron-transfer reaction for over 20 nitro compounds, including substituted 2- and 5-nitroimidazoles of interest as antiprotozoal drugs and radiosensitizers, nitrofurans in use as antibacterial agents, and substituted nitrobenzenes previously used as model substrates for nitroreductases. The logarithm of the rate constant for the reaction of the nitro radical-anion with oxygen is linearly related to the one-electron reduction potential of the nitro compound.

INTRODUCTION

Recent work has suggested that the radical-anions (one-electron reduction products, RNO_2^-) of nitroaromatic and nitroheterocyclic compounds (RNO_2) are obligate intermediates in the enzymatic reduction of these compounds to hydroxylamines or amines (1-3). Oxygen inhibits nitroreductase activity in e.g. hepatic microsomal incubations and measurements of the rate of O_2 uptake have provided evidence (3) that the inhibition results from the electron-transfer reaction [1]:

$$RNO_2^- + O_2 \longrightarrow RNO_2 + O_2^-, \qquad [1]$$

although alternative mechanisms have been proposed (4).

In addition to the widespread use of nitroheterocyclics in medicine (5), these compounds - especially nitroimidazoles - are of current interest as hypoxic cell radiosensitizers of potential clinical value in radiotherapy (6). Biaglow et.al. (7) have shown that nitro compounds can either stimulate or

inhibit O_2 metabolism in cellular preparations, depending on properties related to the electron affinities of the compounds. These variations may arise partly from differences in the rate of reaction [1] with different nitro compounds, and may be an important consideration in radiosensitization if the catalytic turnover of O_2 to H_2O_2 and H_2O by nitro compounds is sufficient to alter the radiosensitivity of tumours (8).

Further, if the toxicity of nitro compounds is related to the production of a reduction product containing e.g. a nitroso, hydroxylamine or amine group, (9,10), then there may be a different toxicity between oxygenated and hypoxic cells (11-14) which may vary according to the rate of reaction [1], which protects RNO_2 against reductive damage. We now report measurements of the rate constants of reaction [1] for over 20 nitro compounds, which reveal a linear free energy relationship between the logarithm of the rate constant and the one-electron reduction potential of the nitro compound.

METHODS

Nitro radical-anions were generated by pulse radiolysis and detected by kinetic spectrophotometry (15). When solutions containing RNO_2 (1-2 mmol dm^{-3}) and formate (0.2 mol dm^{-3}) are irradiated, RNO_2^- and O_2^- (if the solution is not deaerated) are the only free radicals in the system a few microseconds after the end of the 0.2 μs radiation pulse. Both OH and H react with HCO_2^- to give CO_2^- which, like e^-_{aq}, reacts very rapidly with RNO_2 to give RNO_2^-. The absorption of RNO_2^- was generally monitored at slightly longer wavelengths than the UV absorption edge of RNO_2, e.g. 400-440 nm for nitroimidazoles (16,17) and nitrofurans (18), or near the reported maxima for some nitrobenzene derivatives (19,20). Most solutions were buffered at pH 8-8.5 (e.g. 4 mmol dm^{-3} Na_2HPO_4), so that in the absence of O_2 the decay of RNO_2^- was negligible compared with the decay in air or O_2-saturated solutions.

One-electron reduction potentials at pH 7, $E_7^1(RNO_2/RNO_2^-)$ were measured using the method described by Meisel and Neta (21). The pulse radiolysis technique was used to measure the equilibrium constant of the one-electron transfer reaction between RNO_2^- and a redox indicator of known E_7^1 such as a quinone (21) or a viologen (22).

RESULTS

The concentrations of RNO_2^- generated by pulse radiolysis was typically ca. 5 μmol dm^{-3}. At pH 8-8.5, the radical-anions of most of the compounds

studied have first half-lives of ca. 10-100 ms under these conditions. Exceptionally, the anion of p-nitroacetophenone decayed over milliseconds with good second-order kinetics: analysis of the decay at pH 8.5 according to $-d[RNO_2^-]/dt = 2k[RNO_2^-]^2$ gave $2k = 1.1 \times 10^7$ dm^3 mol^{-1} s^{-1}.

The absorption of RNO_2^- decayed exponentially in the presence of O_2, as expected if reaction [1] occurs since $[RNO_2^-] \ll [O_2]$ and O_2^- does not absorb at the wavelengths used. The observed first-order rate constants, k_{obs}, were shown to be first order in $[O_2]$ and the bimolecular rate constants $k_1 = k_{obs}/[O_2]$ were calculated for each compound.

For brevity we have included in Table 1 data only for those compounds of current interest in medicine or radiobiology, or used as model substrates for reductases. Full details of all the measurements will be reported later. In Figure 1 we have plotted $\log_{10} k_1$ for all the compounds whose E_7^1 have been reported previously (21,22) or measured in the present work, against the difference in the one-electron reduction potentials at pH 7 of RNO_2 and O_2, i.e. against ΔG° for reaction [1] (1 mol dm^{-3} reactants). The value of $E_7^1(O_2/O_2^-) = -155$ mV (1 mol dm^{-3} O_2) was used (23-25).

The ratio of RNO_2^- to O_2^- present a few microseconds after the pulse may be varied by altering the concentrations of RNO_2 and O_2; the total concentration of radicals is proportional to radiation dose. These variables were investigated for RNO_2 = B, F and G (see Table), but no changes were detected in k_1 or the exponential decay of RNO_2^-. Hence for these compounds the reaction $RNO_2^- + O_2^- \longrightarrow$ products must have a rate constant $< 10^7$ dm^3 mol^{-1} s^{-1}. This reaction, with a rate constant ca. 1×10^7 dm^3 mol^{-1} s^{-1}, may account for an observed increase in the rate of decay of nitrofuran radical-anions with increase in radiation dose in the presence of O_2.

Evidence that O_2^- is a product of reaction [1] was obtained by pulse radiolysis of a solution containing 200 μmol dm^{-3} metronidazole, 125 μmol dm^{-3} O_2 and 0.2 mol dm^{-3} formate (pH 8.5). The initial spectrum was that expected from equal concentrations of RNO_2^- and O_2^-, and the absorption decayed

Table 1: Reaction of nitro radical-anions with oxygen [a]

Figure code	RNO_2^-	$10^{-6}k_1$ /$dm^3 mol^{-1} s^{-1}$	E_7^1 /mV
A	nitrobenzene	7.7	-486[b]
B	1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole[c]	7.2	-486[d]
C	ethyl[2-(2-methyl-5-nitroimidazol-1-yl)ethyl] sulfone [e]	7.8	-464[d]
D	4-[2-(5-nitroimidazol-1-yl)ethyl]-morpholine[f]	6.1	-457[d]
E	p-nitrobenzoate	2.9	-415
F	1-(2-hydroxy-3-methoxypropyl)-2-nitroimidazole[g]	3.8	-389[b,d]
G	p-nitroacetophenone	1.4	-355[b]
H	p-nitro-(3-dimethylamino)propiophenone [h]	2.0	-315
-	5-nitro-2-furaldehyde semicarbazone [i]	-	-257
I	anti-5-nitro-2-furaldoxime [j] (pH 6.6 or 7.4)	0.15	-253 [b]
-	N-(5-nitro-2-furfurylidene)-1-aminohydantoin [k]	0.25	-
J	5-nitro-2-furfuraldehyde-N'-methyl-N-piperazino-acethydrazone [l]	0.25	-214

[a] In water at 295 K, pH 8.5; [b] Ref.(21); [c] Metronidazole or Flagyl; [d] Ref.(22); [e] Tinidazole; [f] Naxogin or Nitrimidazine; [g] Roche Ro-07-0582; [h] Ro-03-6156 ('NDPP'); [i] Nitrofurazone or Furacin; [j] Nifuroxime; [k] Nitrofurantoin or Furandantin; [l] Nifurpipone or Ro-10-7722.

exponentially ($t_{1/2}$ 0.8 ms) with kinetics invariant between 240-300 and 360-460 nm. The spectrum measured after 4.5 ms (240-300 nm) was within 20 percent of tha expected if RNO_2^- had reacted to produce O_2^- quantitatively. The relatively minor disagreement may arise from artefacts caused by the increase in the scattered light in the detection system when using highly absorbing solutions. Experiments using γ-radiolysis to reduce RNO_2 by CO_2^- showed that RNO_2 was

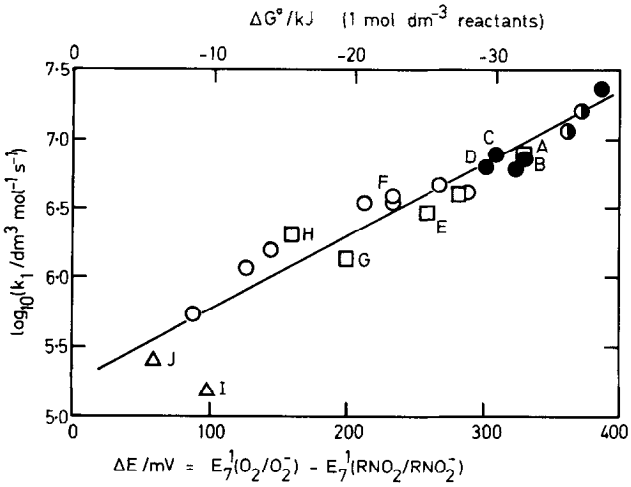

Fig.1 — The rate constants for the reaction of nitro radical-anions with oxygen: variation with the standard free energy change of the reaction. Measurements at 295 K in water at pH 8.5 (○) 2-nitroimidazoles; (●) 5-nitroimidazoles; (◑) 4-nitroimidazoles; (□) nitrobenzenes; (△) 5-nitrofurans

completely protected against reductive damage by concentrations of O_2 too low to react with CO_2^- directly.

The temperature dependence of reaction [1] for RNO_2 = Ro-07-0582 between T = 283 and 362 K fitted the Arrhenius expression $k_1 = A.\exp(-E_a/RT)$, with $\log_{10}A/dm^3\ mol^{-1}\ s^{-1} = 12.07 \pm 0.12$ and $E_a = 31.1 \pm 0.7$ kJ.

DISCUSSION

Our results indicate: (i) the reaction of RNO_2^- with O_2 results in the complete regeneration of RNO_2 and the production of O_2^-, in agreement with earlier observations (1); (ii) the rate constants for reaction [1] are well below diffusion-controlled values, and (iii) these rate constants increase with decreasing values of $E_7^1(RNO_2/RNO_2^-)$. For most of the compounds, the one-electron reduction potential is unchanged between pH 7 and 9 because the pK_a for the dissociation of the protonated radical RNO_2H is < 7 (e.g.2-3 for

nitrobenzenes (19) and ~6 for nitroimidazoles (26). Protonation of RNO_2^- affects the reaction of the nitro radical with O_2 (27), but for nitrobenzenes and nitroimidazoles our measurements of k_1 will apply at pH 7.4.

An earlier value of $k_1 = 1.5 \times 10^9$ dm^3 $mol^{-1}s^{-1}$ for RNO_2 = nifuroxime (18,28,29) is 10^4 higher than our measurements. The reasons for this marked difference are being investigated; preliminary experiments (27) producing RNO_2^- by pulse radiolysis of unbuffered solutions of nifuroxime and t-butanol (18,28,29) suggest the existence of rapid prototropic changes which may not occur in our formate system.

When $\Delta G^o = 0$, our data predict $k_1 \simeq 10^5$ dm^3 $mol^{-1}s^{-1}$, a value similar to that reported (30) for the electron exchange reaction for nitrobenzene anion in a partly aqueous solvent. Electron transfer from the anion of Ro-07-0582 to p-nitroacetophenone ($\Delta G^o \simeq -3.3$ kJ (21) was found to be $< 10^6$ dm^3 $mol^{-1}s^{-1}$ (16). The values of k_1 are 10^2-10^3 slower than the reactions of RNO_2^- with quinones involving similar values of ΔG^o (21,31). The variation of k_1 with ΔG^o may be compared with recent descriptions (31-33) of some electron transfer reactions in terms of the Marcus theory (34). For RNO_2 = Ro-07-0582 (ΔG^o = -22.6 kJ; $k_1 = 3.8 \times 10^6$ dm^3 $mol^{-1}s^{-1}$ at 295 K) the Marcus free energy of activation $\Delta G^* \simeq 28$ kJ if the collision number $Z = 3 \times 10^{11}$ dm^3 mol^{-1} s^{-1} (33). An identical value of ΔG^* is obtained if the Eyring activation energy $\Delta G^{\ddagger}$ (35) = 35.2 $\pm$ 1.0 kJ (calculated from the temperature dependence of k_1) is related to ΔG^* by the expression: $\Delta G^* \simeq \Delta G^{\ddagger} + RT.\ln(Zh/\underline{k}T)$ (34).

In a nitroreductase system, the initial rate of production of RNO_2^- may be faster and the rate of reaction (1) is slower the more positive the value of $E_7^1(RNO_2/RNO_2^-)$. In model systems, the rate of nonenzymic reduction of substituted nitrobenzenes by reduced flavins has been correlated with a parameter which is related to the reduction potential (36). The difference in the rate of reduction of p-nitrobenzoate and its methyl ester by $FMNH_2$ (37) may reflect differences in reduction potential.

Whilst the net changes observed in both *in vitro* and *in vivo* reductase systems will reflect the much more complex situation, the one-electron reductio

potentials of the nitro compounds and the prototropic behaviour of the nitro radicals are clearly of great importance especially in considering reactions with reducing agents of known reduction potential such as $FMNH_2$ (3) or ferredoxin (38).

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