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CONTROL OF THE GENERATION AND REACTIONS OF FREE RADICALS IN BIOLOGICAL SYSTEMS BY KINETIC AND THERMODYNAMIC FACTORS

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Quantifiable redox properties are useful predictors of substrate reactivity in enzyme-catalysed redox reactions of e.g. nitroreductases or peroxidases. Redox properties may also control the rates of electron-transfer reactions between radical products of reduction and oxidation, and endogenous oxidants and reductants respectively. However, in numerous instances prototropic properties of substrate or radical may have profound kinetic consequences, protonation of radicals frequently slowing down electron-transfer reactions. Further, reactions which are thermodynamically extremely *un*favourable may still proceed if radical products are removed from the pre-equilibrium efficiently. Thus kinetic considerations often outweigh the purely thermodynamic viewpoint.

KEY WORDS: Redox properties, Marcus theory, quinones, nitro compounds, superoxide.

INTRODUCTION

The thermodynamics of electron-transfer reactions involving free-radical intermediates are characterized by the difference in reduction potentials between electron donor and acceptor:

$$A^{\cdot -} + B \rightleftharpoons A + B^{\cdot -} \quad (1)$$

$$\Delta E_1 = E(B/B^{\cdot -}) - E(A/A^{\cdot -}) \quad (2)$$

$$\Delta E_1/V \sim 0.059 \log K_1 \quad (3)$$

Pulse radiolysis has proven to be of immense value in characterizing the position of electron-transfer equilibria (1) involving transient radical species, both of oxidant/radical[1] and radical/reductant[2] couples. However, only the *position* of equilibrium (1) can be calculated from a knowledge of E; in some instances the *rate* of approach to the potential equilibrium can be negligibly slow even though thermodynamically favourable. On the other hand, very useful correlations between rates and energetics of radical reactions have been demonstrated.

This short paper outlines some of the correlations, and draws attention to examples where thermodynamically facile reactions are kinetically sluggish, and of reactions

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proceeding to completion even though the pre-equilibrium (1) is energetically unfavourable. It is intended to be didactic rather than a comprehensive survey.

METHODS

Computer simulations of reaction kinetics utilized a FORTRAN program based upon the Gear numerical integration algorithm,[3,4] running on a Microvax II computer and Sigma 5000 graphics display. The pulse radiolysis technique has been reviewed.[5]

RESULTS AND DISCUSSION

Marcus theory of electron-transfer reactions[6]

The rate constant k_1 of reaction (1) can be related to the equilibrium constant K_1 (i.e. ΔE_1) by the Marcus relationship (4):

$$k_1 = A \exp(-\Delta G_1^*/\mathbf{R}T) \quad (4)$$

where A is a collision number and ΔG_1^* defined in its simplest form (one reactant uncharged) by:

$$\Delta G_1^* = (\lambda/4)(1 + \Delta G_1/\lambda)^2 \quad (5)$$

where λ is a reorganisation parameter. Since ΔG_1 is defined by ΔE_1:

$$\Delta G_1/\mathrm{kJ\,mol^{-1}} \sim -96.5\,(\Delta E_1/\mathrm{V}) \quad (6)$$

for a one-electron transfer reaction at 298 K, if the individual couples defined in equation (2) are known, then k_1 as well as K_1 can be predicted *in principle*. We shall see that particular problems arise when protons are involved in the overall reaction.

Examples of the successful application of the Marcus theory to simple free-radical reactions in aqueous solution

Semiquinones were some of the earliest examples of free radicals to be identified in solution and especially in biological systems. The simplest reactions of a semiquinone in solution are electron-exchange between the radical-anion and ground-state molecules, or electron transfer between one quinone and another differing in $E(\mathrm{Q}/\mathrm{Q}^{\cdot -})$:

$$\mathrm{Q}_a^{\cdot -} + \mathrm{Q}_b \rightleftharpoons \mathrm{Q}_a + \mathrm{Q}_b^{\cdot -} \quad (7)$$

electron transfer between $\mathrm{Q}^{\cdot -}$ and the important acceptor, oxygen:

$$\mathrm{Q}^{\cdot -} + \mathrm{O}_2 \rightleftharpoons \mathrm{Q} + \mathrm{O}_2^{\cdot -} \quad (8)$$

and semiquinone disproportionation k_{-9}:

$$\mathrm{Q} + \mathrm{QH}_2 \rightleftharpoons 2\mathrm{Q}^{\cdot -} (+ 2\mathrm{H}^+) \quad (9)$$

Patel and Willson[7] used pulse radiolysis to characterize K_9 for Q = duroquinone (leading to the definitive calculation of $E(\mathrm{O}_2/\mathrm{O}_2^{\cdot -})$;[8,9] Meisel[10] and Meisel and Fessenden[11] showed kinetic data for all three reaction types could be satisfactorily defined using the Marcus relationships (4) and (5). Values of λ = 60 to 75 kJ mol^{-1} fitted the

data well, varying somewhat on the individual reaction. When $\Delta E = 0$ ($K = 1$), k was of the order of 5×10^7 to $2 \times 10^8\,dm^3\,mol^{-1}\,s^{-1}$ – the "self-exchange" rate for reactions of type (7) when $Q_a = Q_b$.[11]

Nitroaryl radical-anions, $ArNO_2^{\cdot -}$ are important obligate intermediates in the reduction of nitro compounds in biological systems.[12] The simplest reactions and physico-chemical properties of nitro radicals[13] and the chemical basis for the application of nitroaryl compounds as an adjunct to radiotherapy[14,15] have been recently reviewed. Conceptually the simplest reaction of $ArNO_2^{\cdot -}$ is the analogue of (7):

$$(ArNO_2)_a^{\cdot -} + (ArNO_2)_b \rightleftharpoons (ArNO_2)_a + (ArNO_2)_b^{\cdot -} \qquad (10)$$

Our measurements (to be reported in full elsewhere) of k_{10} for electron-exchange between nitroaryl compounds in water, utilizing principally nitroimidazoles typical of those used in chemotherapy against anaerobic organisms, can be described by an equation which is a simple transform of (4) and (5):

$$\log (k_{10}/dm^3\,mol^{-1}\,s^{-1}) = 11 - 4.9(1-0.86\,\Delta E/V)^2 \qquad (11)$$

It is noteworthy that in contrast to the quinone system, equilibrium (7), when $\Delta E = 0$ ($K_{10} = 1$) then $k_{10} \sim 1.3 \times 10^6\,dm^3\,mol^{-1}\,s^{-1}$, i.e. k_{10} was about two orders of magnitude less than k_7 for similar values of ΔE. Thus whilst the relative changes in k_7 or k_{10} for changes in ΔE are broadly similar the absolute values differ considerably. This is reflected in the different values of the reorganisation parameter, λ characterizing the systems: 60–75 kJ mol^{-1} for $Q/Q^{\cdot -}$ compared to about 110 kJ mol^{-1} for $ArNO_2/ArNO_2^{\cdot -}$.

For comparison with linear free-energy relationships between rate and E or ΔE in biological systems, it is useful to bear in mind the rate of change of k with ΔE. In the case of reaction (10), equation (11) indicates $d(\log k_{10})/d(\Delta E)$ varies between about 6 and 12 V^{-1} for $\Delta E \sim 0.2$ to -0.4 V, i.e. *about* an order of magnitude change in rate constant for 0.1 V change in E. A variety of biological or biochemical properties of nitroaryl compounds exhibit redox dependencies which are within this range:[15,17] the redox coefficient may thus give *quantitative* support for a property involving one-electron transfer as a rate-limiting step. In these particular examples[15–17] the properties of nitroaryl compounds – cytotoxicity, mutagenicity, etc. – may all be controlled primarily by the redox-controlled rate of nitroreduction. In simpler chemical[18] or isolated enzyme[19] systems modelling nitroreduction rather precise rate/redox relationships are demonstrable, e.g. for:

$$ArNO_2 + 2FMNH_2 \longrightarrow ArNHOH + 2\,FMN \qquad (12)$$

However, the apparent simplicity – even predictability – of some biological redox relationship depending on one-electron transfer could lead one to oversimplify these processes. Thus the rate of reduction of nitroimidazoles by extracts of *Trichomonas vaginalis* showed a redox dependence characteristic of one-electron transfer reactions only in the absence of ferredoxin,[17] although further studies[20] indicated the usefulness of measurements of $E(ArNO_2/ArNO_2^{\cdot -})$ in explaining variations in activity in the systems.

Electron transfer from $ArNO_2^{\cdot -}$ to O_2 is of exceptional importance in defining the selectivity of nitroreduction to anaerobic systems, the "futile metabolism" step (13) being well established:[12,21,22]

$$ArNO_2^{\cdot -} + O_2 \longrightarrow ArNO_2 + O_2^{\cdot -} \qquad (13)$$

Our preliminary study[22] of the kinetics of reaction (13) has been considerably extended, and measurements with Mr. E.D. Clarke (to be reported in detail elsewhere) show k_{13} can be described fairly well by the Marcus relationship (4) with $A = 10^{11}\,dm^3\,mol^{-1}\,s^{-1}$ only if $\lambda = 140$–$150\,kJ\,mol^{-1}$ – double the values for typical reactions of semiquinones. When $\Delta E = 0$ k_{13} is extrapolated to be about $1 \times 10^5\,dm^3\,mol^{-1}\,s^{-1}$, and consequently k_{13} is typically 2–3 orders of magnitude slower than k_8 ($Q^{\cdot -} + O_2$) for similar ΔE.

Radical/reductant couples. The ease of oxidation of a one-electron donor nominally described by $D^{2-} \rightarrow D^{\cdot -} + e^-$ is quantified by the value $E(D^{\cdot -}/D^{2-})$, the reduction potential of the radical $D^{\cdot -}$; the lower this value, the easier D^{2-} is oxidized. Reduction potentials of phenoxy and anilino or arylamino radicals have been measured,[2] but for kinetic reasons (see below) the values are often derived from measurements using solutions at high pH. Extrapolating to physiological conditions is not always possible (radical and ground state pK_a's are needed) but perfectly satisfactory redox correlations can be established using Hammett σ constants in a given series. Good examples are found in the work of Dunford *et al.*,[23] where rates of one-electron oxidation of anilines and phenols by Horseradish peroxidase Compound I were rather well correlated with σ. In the case of anilines at pH 7.0, 27 C:

$$\log (k/dm^3\,mol^{-1}\,s^{-1}) = 5.8 - 7.0\sigma \qquad (14)$$

and an almost identical slope (rho value) was found for oxidation of phenols.

The rates of oxidation of phenols by an oxidized deuteroferriheme paralleled those of the HRP Compound I series;[24] the authors considered the oxidizing species, formally of Fe(V) order, could be most satisfactorily assigned to a Fe(III)-porphyrin π-cation radical. Certainly there seems no question that the oxidation is a one-electron process. In fact, the magnitude of the redox dependence of k_{14} (6 orders of magnitude change in k for variation in σ of 0.8) does appear to be predictable from the simplest Marcus relationships, in spite of the complication of proton transfer accompanying electron transfer. We can make a tentative transformation of the Hammett relationship of the form of (14) into the redox dependence (15) using the correlation (16) between $E(PhO^{\cdot}/PhO^-)$ (at high pH)[2] and σ derived from literature data:

$$\log k = \text{constant} + b_1(E/V) \qquad (15)$$

$$E(PhO^{\cdot}/PhO^-)/V \sim (0.64 \pm 0.06) + (0.62 \pm 0.11)\sigma \qquad (16)$$

yielding an estimate of $b_1 \sim 11\,V^{-1}$. A value of $d(\log k)/d(\Delta E)$ of the order of $10\,V^{-1}$ is typical of many one-electron transfer reactions.

Ascorbate, H_2A is an important one-electron reductant and Pelizzetti *et al.*[25] showed the overall reaction (17) proceeded through a rate-limiting one-electron step of the form of (18) via the ascorbyl radical $AH^{\cdot}$. The rate equation was well characterized by the Marcus expression (4) with appropriate adjustments:

$$2\,Ox + H_2A \longrightarrow 2\,Red + A + 2H^+ \qquad (17)$$

$$Ox + HA^- \longrightarrow Red + AH^{\cdot} \qquad (18)$$

In these experiments Ox = one-electron oxidants such as $IrCl_6^{2-}$, $Mo(CN)_8^{3-}$, $Fe(bpy)_2(CN)_2^+$ *etc*

Examples of radical- or ground-state-protonation influencing the rates of electron-transfer reactions

Reaction (10) is kinetically facile providing pH > pK_{19}, i.e. the pK for dissociation of the conjugate acid of the radical-anion:

$$(ArNO_2^{\cdot -})H^+ \rightleftharpoons ArNO_2^{\cdot -} + H^+ \tag{19}$$

An example of this is electron transfer from the radical-anion of metronidazole to the more powerful oxidant, 1-methyl-2-nitroimidazole-5-carboxaldehyde, a reaction of the form of (10) with $\Delta E = 0.24$ V ($K_{10} \sim 10^4$). As quite well predicted from equation (11), we measured $k_{10} = 6 \times 10^7 dm^3 mol^{-1} s^{-1}$ at pH $\geqq$ 8 but the measured rate constants fall sigmoidally with decreasing pH to $\sim 3 \times 10^6 dm^3 mol^{-1} s^{-1}$ at pH $\sim$ 4. The data are fitted accurately assuming the conjugate $(ArNO_2^{\cdot -})H^+$ has a value of k_{10} some 20-fold lower than that of $ArNO_2^{\cdot -}$ in this instance, taking a value of p$K_{19} = 6.1$, exactly that found from measurements[26] of the absorption spectrum of the radical from metronidazole.

Similarly, although not quite as dramatic, our colleague, Mr. E.D. Clarke recently found k_{13} to decrease around two-fold between pH 8 and 5 for Ar-NO_2 = metronidazole. A much more complex dependence of rate constant upon pH was observed by Cabelli and Bielski[27] for the oxidation of ascorbate by $HO_2^{\cdot}/O_2^{\cdot -}$ radicals. Other examples of protonation slowing down energetically facile reactions include electron transfer between phenols and phenoxy radicals,[2] and between arylamines and their radical-cations.[28]

Examples of energetically unfavourable reactions proceeding to completion by the removal of products from reaction.

We recently reported[29] kinetic studies which explained the observations of Eling *et al.*[30] concerning the rapid disappearance of the radical-cation of the antipyretic drug, aminopyrine (AP) upon the addition of glutathione:

$$AP^{\cdot +} + GSH \rightleftharpoons AP + GS^{\cdot} + H^+ \tag{20}$$

We measured the back reaction to have a rate constant, $k_{-20} \sim 3 \times 10^8 dm^3 mol^{-1} s^{-1}$ (neglecting H^+ in the rate equation), but the forward reaction had an apparent $k_{20} \sim 2$–$3 \times 10^4 dm^3 mol^{-1} s^{-1}$ at pH 5.6. Hence reaction (20) might be expected to lie well over to the left. We suggested, however, that the reaction proceeded to completion to the right simply because $GS^{\cdot}$ was removed from the equilibrium rapidly, via its equilibrium with GS^- (and addition to O_2 in oxygenated systems).

Of wider interest, and conceptually very simple, is the effect of oxygen in inhibiting quinone reduction via appropriate reductases. Consider the model system:

$$Q \longrightarrow Q^{\cdot -} \tag{21}$$

$$Q^{\cdot -} + O_2 \rightleftharpoons Q + O_2^{\cdot -} \tag{8}$$

$$O_2^{\cdot -} + H^+ \longrightarrow \tfrac{1}{2}H_2O_2 \tag{22}$$

$$Q + QH_2 \rightleftharpoons 2Q^{\cdot -} (+ 2H^+) \tag{9}$$

$$O_2^{\cdot -} + Q^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + Q \tag{23}$$

Suppose $E(Q/Q^{\cdot -}) \gg E(O_2/O_2^{\cdot -})$, e.g. $E(Q/Q^{\cdot -}) = -0.035\,V$ so that $K_8 = 0.01$.[8,9] One might expect that low concentrations of O_2 would have little effect in inhibiting the production of QH_2 via reductases generating $Q^{\cdot -}$, since equilibrium (8) was so much over to the left. However, as Winterbourn has pointed out,[31] rapid removal of $O_2^{\cdot -}$ from the equilibrium may change the picture dramatically, in much the same way as removal of $GS^{\cdot}$ from equilibrium (20). In Figure 1 we show the results of numerical modelling the reaction sequence above, which shows this effect.

For illustrative purposes we have generated $Q^{\cdot -}$ at a constant rate of $0.1\,\mu mol\,dm^{-3}\,s^{-1}$, beginning with $[Q]_0 = 100\,\mu mol\,dm^{-3}$ and keeping $[O_2]$ = constant, as might occur *in vivo*. Values of k_8 and k_{-8} can be predicted[10] for any $E(Q/Q^{\cdot -})$ (we used $k_8 = 4 \times 10^6$, $k_{-8} = 4 \times 10^8\,dm^3\,mol^{-1}\,s^{-1}$ in this example, and we set k_{-9} at the typical value of $1 \times 10^8\,dm^3\,mol^{-1}\,s^{-1}$ with the semiquinone formation constant, $K_9 = 5 \times 10^{-7}$ initially. Removing $O_2^{\cdot -}$ in a first-order manner with a half-life of 30 μs because of the presence of superoxide dismutase (SOD)[32] drives equilibrium

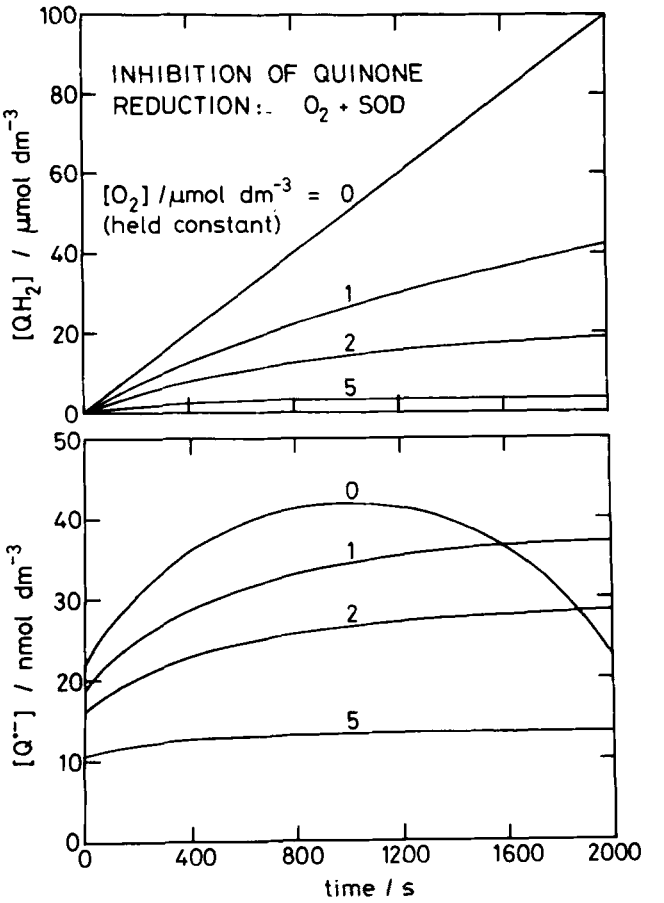

FIGURE 1 Illustration of the effect on reduction of a quinone of a low combination of oxygen (concentration maintained constant), and superoxide dismutase sufficient to remove $O_2^{\cdot -}$ with a half-life of 30 μs. In this example, $E(Q/Q^{\cdot -}) = -0.035\,V$ and the semiquinone formation constant, $K_9 = 5 \times 10^{-7}$; for other conditions, see text.

(8) to the right and we see that a constant $[O_2] = 1\ \mu mol\ dm^{-3}$ is sufficient to inhibit QH_2 production by over 50%. Inhibition is increased to over 90% with this $[O_2]$ if K_9 is increased to 5×10^{-6}.

(Higher semiquinone formation constants, and hence higher concentrations of $Q^{\cdot -}$, may be characteristic of some biologically-important quinones). The model also yields values for the concentration of $Q^{\cdot -}$ and it may be possible to model and gain greater insight into the behaviour of more realistic systems where the appropriate kinetic and thermodynamic parameters are known or can be reasonably estimated. Other factors, such as possible reduction of SOD by semiquinones from low potential quinones[33] require further study. It will also be more representative of cellular systems *in vitro* to set [Q] constant, assuming diffusion of Q is faster than removal. (In this example, since $O_2^{\cdot -}$ is a more powerful oxidant than most $Q^{\cdot -}$, one might expect reaction (23) to be facile. However, it plays little part in the overall scheme since the concentration of $O_2^{\cdot -}$ is kept around 4 orders of magnitude lower than that of $Q^{\cdot -}$.) Numerical simulations of this kind may help indicate which physical properties are optimal for either maximal QH_2 production in bioreductive activation or maximal superoxide formation (redox cycling).

It is hoped these few examples will have illustrated the importance of assessing kinetic, as well as thermodynamic factors in considering electron-transfer reactions of biological relevance.

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