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Reductive role of glutathione in the redox cycling of oxidizable drugs

A number of common drugs undergo facile one-electron oxidation to produce relatively stable free radicals: oxidation of phenothiazines and aminopyrine (4-(dimethylamino)-1,2-dihydro-1,5-dimethyl-2-phenyl-3*H*-pyrazol-3-one) by enzymes such as horseradish peroxidase (HRP) is well established [1]. Eling *et al.* [2] have recently demonstrated the formation of the radical cation of aminopyrine (AP) catalysed by prostaglandin (PG) H synthase and showed that the radical-cation $AP^{+\cdot}$ reacted rapidly with glutathione (GSH).

The formation of the radical-cation, $AP^{+\cdot}$ by oxidases and its subsequent reduction by GSH forms a redox cycle conceptually similar to the reductive "futile metabolism" of nitroaryl compounds or quinones in oxic environments [1], and involves the thiyl radical, $GS^{\cdot}$ [2]:

$$AP \xrightarrow{\text{HRP or PG H synthase}} AP^{+\cdot} \quad (1)$$

$$AP^{+\cdot} + GSH \rightleftarrows AP + GS^{\cdot} + (H^{+}) \quad (2)$$

Equilibrium (2) appears to be well over to the right since $AP^{+\cdot}$ disappears rapidly in the presence of GSH [2]. However, we now show by direct measurements of the kinetics of both forward and back reactions (2) that *thermodynamically* the equilibrium is well over to the left, and is *kinetically* driven to the right only because $GS^{\cdot}$ is lost from the system via other reactions.

Materials and methods

Aminopyrine, glutathione, horseradish peroxidase (HRP) Type VI, (all Sigma), KBr (BDH, Aristar) and other inorganic chemicals (BDH, AnalaR) were used as received. Nitrous oxide was BOC Special Gases grade (<10 ppm O_2). Methods for pulse- and γ-radiolysis were similar to those described [3]; a stopped-flow spectrophotometer based upon an Applied Photophysics mixer-drive unit was employed, with data capture methods similar to those used in pulse radiolysis [3]. Pye-Unicam SP8-200 and PU-8600 spectrophotometers were used.

Results and discussion

Preliminary experiments generated $AP^{+\cdot}$ at pH 7 (50 mM phosphate) by addition of HRP (2–10 U/ml) to aqueous solutions of H_2O_2 (0.5 mM) and AP (0.5 mM) in a spectrophotometer cell. The absorption of $AP^{+\cdot}$ (λ_{max} 565–570 nm) was observed [2, 4, 5] and it was confirmed that the absorption disappeared rapidly upon addition of GSH [2]. To investigate the kinetics of reaction (2) and characterize the natural decay pathway (3) [2]:

$$2\,AP^{+\cdot} \rightarrow \text{products} \quad (3)$$

it was desirable to generate $AP^{+\cdot}$ in the absence of other potentially reactive species such as H_2O_2 and HRP intermediates, and all subsequent experiments utilized $AP^{+\cdot}$ generated radiolytically.

Halogen or pseudo-halogen radical-anions, $X_2^{-\cdot}$ were found to oxidize AP to $AP^{+\cdot}$:

$$X_2^{-\cdot} + AP \rightarrow 2X^{-} + AP^{+\cdot} \quad (4)$$

with e.g. the value of the rate constant, $k_4 \simeq 5 \times 10^8\,M^{-1}\,sec^{-1}$ ($X^{-} = SCN^{-}$). Measurements a few microseconds after pulse radiolysis (0.2 μsec, 1.6 Gy pulse) of N_2O-saturated solutions containing KSCN (0.1 M), AP (0.25–0.5 mM) and phosphate (4 mM, pH 7) gave absorptions of $AP^{+\cdot}$ with a maximum at 570 nm. (In such solutions radiolytically-produced e_{aq}^{-} and $OH^{\cdot}$ are converted to $X_2^{-\cdot}$ within 1 μsec after the pulse, and reaction (4) is complete in <30 μsec [6]). The ratio of extinction coefficients of $AP^{+\cdot}$ at 570 nm to that of $(SCN)_2^{-\cdot}$ at 472 nm was found to be 0.232:1, and using $\varepsilon = 7580\,M^{-1}\,cm^{-1}$ at 472 nm for $(SCN)_2^{-\cdot}$ [7] we calculate $\varepsilon = 1760\,M^{-1}\,cm^{-1}$ for $AP^{+\cdot}$ at 570 nm. Similar results were obtained using $Br_2^{-\cdot}$ to oxidize AP. This value is rather lower than a value ($2230\,M^{-1}\,cm^{-1}$) determined from integration of electron spin resonance signals [5].

The natural lifetime of $AP^{+\cdot}$, reaction (3), was investigated by generating $AP^{+\cdot}$ using γ-radiolysis of N_2O-saturated solutions containing KBr (0.1 M), AP (1 mM) and phosphate (4 mM, pH 5) (similar results in unbuffered solution). The absorption of $AP^{+\cdot}$ at 570 nm was recorded for about 40 min following cessation of radiolysis (approx. 40–50 Gy), and was found to conform to second-order kinetics ($-d[AP^{+\cdot}]/dt = 2k_3[AP^{+\cdot}]^2$) for >2 half-lives with $2k_3 = 52\,M^{-1}sec^{-1}$ assuming $\varepsilon = 1760\,M^{-1}\,cm^{-1}$ at 570 nm. Second-order kinetics are consistent with reaction (3) being a disproportionation reaction to yield an iminium cation (giving formaldehyde on *N*-demethylation) and aminopyrine [2]. Using a biological system, Eling *et al.* [2] reported a value of $2k_3$ (their "k_d") of $426\,M^{-1}\,sec^{-1}$; this assumes $\varepsilon = 2230\,M^{-1}\,cm^{-1}$ [5] and would be $340\,M^{-1}\,sec^{-1}$ assuming $\varepsilon = 1760\,M^{-1}\,cm^{-1}$.

The first half-life of reaction (3) ($=1/2k_3[AP^{+\cdot}]_0$) is about 11 min with $2k_3 = 52\,M^{-1}\,sec^{-1}$ and $[AP^{+\cdot}]_0 = 30\,\mu M$. This relative stability of $AP^{+\cdot}$ facilitated generation of the radical-cation radiolytically as described above, followed by mixing the solution with buffered, N_2O-saturated solutions of GSH in a stopped-flow spectrophotometer. The result of a typical experiment is shown in Fig. 1 (a), showing the rapid bleaching of the absorbance of $AP^{+\cdot}$ at 570 nm after mixing $AP^{+\cdot}$ (final concentration $\simeq 13\,\mu M$) with GSH (final concentration 200 μM). Doubling the GSH concentration approximately doubled the rate of decay of $AP^{+\cdot}$, corresponding to an apparent $k_2 \simeq 2\text{–}3 \times 10^4\,M^{-1}\,sec^{-1}$: a detailed study will be reported elsewhere.

These observations are consistent with earlier reports [2] studying $AP^{+\cdot}$ in a biological preparation at steady-state, and should enable a value of k_2 to be obtained. However, analogous experience with phenothiazines (PZ) show radical-cations, $PZ^{+\cdot}$ to be generated upon oxidation of PZ by thiyl radicals, $RS^{\cdot}$ [8]. We therefore generated $GS^{\cdot}$ by pulse radiolysis (3 Gy) of N_2O-saturated solutions of GSH (2 mM) and phosphate (4 mM, pH 4.9), and monitored the formation of $AP^{+\cdot}$ at 570 nm when low concentrations (0.1–0.3 mM) of AP were also present. The formation of $AP^{+\cdot}$ as shown in Fig. 1(b), was exponential and first-order in [GSH], yielding an estimate of k_{-2} of $3 \times 10^8\,M^{-1}\,sec^{-1}$.

Neglecting the problem of ionization of GSH and the relative reactivity of GSH vs GS^{-} it thus appears that K_2 ($=k_2/k_{-2}$) is of the order of 10^{-4} from measurements at pH $\simeq$ 5, i.e. equilibrium (2) is well over to the left. In spite of this, $AP^{+\cdot}$ is rapidly removed by GSH. This clearly arises because $GS^{\cdot}$ is removed from the system much more rapidly than $AP^{+\cdot}$, *via* reactions such as (5)–(8):

$$GS^{\cdot} + GS^{\cdot} \rightarrow GSSG \quad (5)$$

$$GS^{\cdot} + GS^{-} \rightleftarrows GSSG^{-\cdot} \quad (6)$$

$$GSSG^{-\cdot} + AP^{+\cdot} \; GSSG + AP \quad (7)$$

$$GS^{\cdot} + O_2 \rightarrow GSO_2^{\cdot} \quad (8)$$

Stimulation of GSSG formation and consumption of O_2 in the HRP-catalysed *N*-demethylation of AP has been previously reported [9], consistent with the above reaction sequence. A detailed comparison of the effects of pH, O_2 and reactant concentrations in these radiolytic systems is underway, in conjunction with numerical integration routines to model the complex reaction sequences. It is hoped to identify the steps which are controlling the removal of $GS^{\cdot}$ radicals in the system and hence the efficiency of reaction (2).

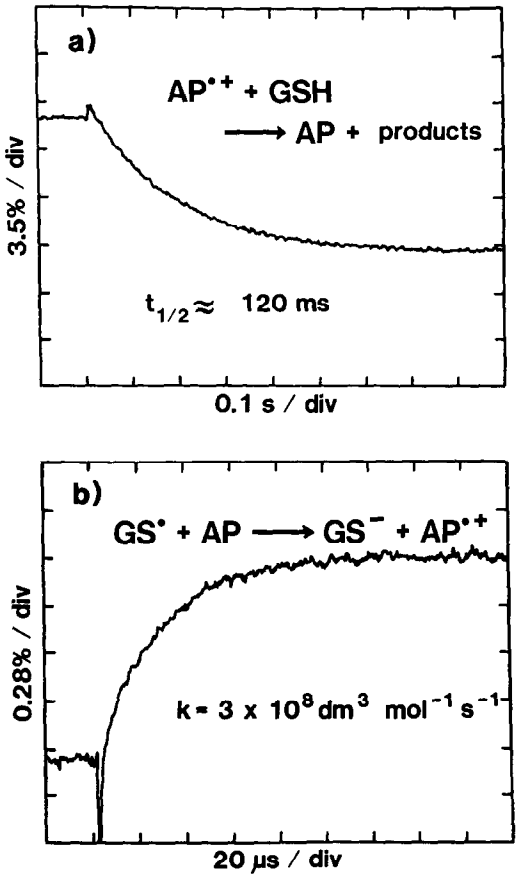

Fig. 1. Oscillograms illustrating transient absorbance changes (ordinate) vs time (abscissae) of aminopyrine cation radical at 570 nm, pathlength = 2 cm: (a) reaction of 13 μM AP^+ with 200 μM GSH in the presence of 0.5 mM AP, 4 mM sodium phosphate, pH 5.6, N_2O saturated, by stopped-flow spectrophotometry, (b) pulse radiolysis of solution containing 2 mM GSH, 100 μM AP and 4 mM sodium phosphate, pH 4.9, N_2O saturated, dose = 3 Gy (≈2 μM radicals).

These studies demonstrate the crucial, reductive role of thiols in redox-cycling oxidizable drugs, and illustrate how reactions which are apparently thermodynamically most unfavourable can be kinetically controlled. An important parallel may be made with the redox cycling of quinones, in which semiquinones (Q^-) are oxidized by O_2:

$$Q^- + O_2 \rightleftarrows Q + O_2^- \quad (9)$$

Since in many biological systems, O_2^- is removed with a half-life of the order of a few tens of microseconds via catalytic disproportionation by superoxide dismutase [10], equilibrium (9) can be driven to the right even if the reduction potential $E(Q/Q^-) > E(O_2/O_2^-)$, i.e. $K_9 \ll 1$. This is possible since the disproportionation of Q^- is much slower than the catalysed decay of O_2^- at likely steady-state concentrations of Q^-. In conclusion, kinetic rather than thermodynamic factors control these redox reactions involving radicals in biological systems.

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Generation of photoemissive species during quinone redox cycling*

The redox cycling of quinones constitutes an oxidative challenge to cells. The field of quinone-induced oxidative injury to cells and tissues has been reviewed (e.g. refs 1 and 2). The generation of reactive oxygen species is thought to be involved in the chemotherapeutic action of quinone anticancer drugs (e.g. ref. 3). We have, in recent years, been interested in the process of the generation of reactive species, taking advantage of their property of photoemissive decay by employing techniques of single-photon counting [4].

The intact hemoglobin-free perfused rat liver was shown to respond to an infusion of a model quinone, menadione (2-methyl-1,4-naphthoquinone), with an increased photoemission [5]. The bulk of the photoemission was in the red spectral region, with about 80% of the intensity being emitted at wavelengths greater than 620 nm; this and other data shown in ref. 5 point to the formation of singlet molecular oxygen during menadione redox cycling in the

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