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Kinetics of reduction of tyrosine phenoxyl radicals by glutathione

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ABSTRACT

Modification of tyrosine (TyrOH) is used as a marker of oxidative and nitrosative stress. 3,3′-Dityrosine formation, in particular, reflects oxidative damage and results from the combination of two tyrosyl phenoxyl radicals (TyrO·). This reaction is in competition with reductive processes in the cell which 'repair' tyrosyl radicals: possible reductants include thiols and ascorbate. In this study, a rate constant of $2 \times 10^6\ M^{-1}\ s^{-1}$ was estimated for the reaction between tyrosyl radicals and glutathione (GSH) at pH 7.15, generating the radicals by pulse radiolysis and monitoring the tyrosyl radical by kinetic spectrophotometry. Earlier measurements have suggested that this 'repair' reaction could be an equilibrium, and to investigate this possibility the reduction (electrode) potential of the (TyrO·,H^+/TyrOH) couple was reinvestigated by observing the fast redox equilibrium with the indicator 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate). Extrapolation of the reduction potential of TyrO· measured at pH 9–11 indicated the mid-point reduction potential of the tyrosyl radical at pH 7, E_{m7}(TyrO·,H^+/TyrOH) = 0.93 ± 0.02 V. This is close to the reported reduction potential of the glutathione thiyl radical, E_{m7} = 0.94 ± 0.03 V, confirming the 'repair' equilibrium constant is of the order of unity and suggesting that efficient reduction of TyrO· by GSH might require removal of thiyl radicals to move the equilibrium in the direction of repair. Loss of thiyl radicals, facilitating repair of TyrO·, can arise either via conjugation of thiyl with thiol/thiolate or oxygen, or unimolecular transformation, the latter important at low concentrations of thiols and oxygen.

Introduction

Oxidation of tyrosine (TyrOH)[1] to phenoxyl radicals (TyrO·) can lead to coupling of the radicals to form principally 3,3′-dityrosine, a naturally-occurring cross-linked amino acid exhibiting intense fluorescence, facilitating its use as a marker for oxidative stress [1]. Reduced glutathione (GSH) can react with enzymatically-generated tyrosyl radicals, preventing the formation of 3,3′-dityrosine and forming thiyl radicals (GS·) [2]. The effect of GSH on 3,3′-dityrosine formation can be represented by competition between reactions (1, -1) and (2):

$$\mathrm{TyrO^{\cdot}} + \mathrm{GSH} \rightleftharpoons \mathrm{TyrOH} + \mathrm{GS^{\cdot}} \qquad (1,-1)$$

$$2\mathrm{TyrO^{\cdot}} \rightarrow 3,3'\text{-dityrosine}, \qquad (2)$$

(Reaction (–1) is the reverse reaction in equilibrium (1)). Efficient prevention by cysteine of the formation of fluorescent products in radiolysis systems generating phenoxyl radicals from reaction of glycyltyrosine with $N_3^{\cdot}$ radicals was ascribed to the reaction analogous to (1) [3]. However, under physiological conditions where both ascorbate and GSH are present, kinetic factors could preclude GSH being involved in the 'repair' of tyrosyl radicals [4]. Recently, it has been shown that both ascorbate and GSH can scavenge phenoxyl radicals intracellularly, acting synergistically, but ascorbate is favoured over GSH when both are present in physiological concentrations [5].

Ascorbate reduces TyrO· radicals with a rate constant $k_3 = 4.4 \times 10^8\ M^{-1}\ s^{-1}$ [6]:

$$\mathrm{TyrO^{\cdot}} + \mathrm{AscH^{-}} \rightarrow \mathrm{TyrOH} + \mathrm{Asc^{\cdot -}} \qquad (3)$$

but the kinetics of reaction (1) have not been characterized. Indeed, since the reduction potentials of both GS· and TyrO· are thought to be similar at pH ~ 7, reaction (1) may be best viewed as an equilibrium with equilibrium constant K_1 not too far from unity [7]. A rate constant of $(2.4 \pm 1.4) \times 10^3\ M^{-1}\ s^{-1}$ was reported for the reaction of GSH with the tyrosyl radical in *N*-acetyltyrosine at pH 7.0 – 7.4, although supporting data were not provided [8], and a subsequent study indicated a rate constant of $(6 \pm 3) \times 10^5\ M^{-1}\ s^{-1}$ for the corresponding reaction involving cysteine at pH 7.4 [9].

Reaction (1) is also important in understanding tyrosine nitration, since TyrO· is a key intermediate in the complex pathways to 3-nitrotyrosine formation [10–12], and modulation of 3-nitroty-

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[1] *Abbreviations used:* TyrOH, tyrosine; GSH, glutathione.

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rosine levels by GSH could involve reaction (1): in detailed modelling calculations [11], $k_1 = k_{-1} = 3.5 \times 10^5\ M^{-1}\ s^{-1}$ was assumed. The value for k_1 was based on a previous estimate for the rate constant for reaction of the 1-naphthoxyl phenoxyl radical with GSH [13], and it is clearly desirable to have a more direct determination.

In this study we have investigated the kinetics, and position of equilibrium of, reaction (1). Pulse radiolysis was used to generate oxidizing radicals reactive towards TyrOH, generating TyrO$^{\cdot}$, and the latter radical monitored in real time by kinetic spectrophotometry. The redox properties of TyrO$^{\cdot}$ were assessed by measuring the position of an electron-transfer equilibrium with an oxidizable redox indicator of known reduction potential. To help understand the efficiency of GSH as a reductant in systems generating TyrO$^{\cdot}$, analogous experiments used steady-state radiolysis to generate TyrO$^{\cdot}$ at a constant, known rate, and the formation of 3,3′-dityrosine, loss of GSH and formation of oxidized glutathione (GSSG) were measured by HPLC.

Experimental

Chemicals

Chemicals were obtained from Sigma–Aldrich (Poole, UK), except that sodium azide and sodium phosphate buffers were obtained from Fisher, and gases from BOC.

Generation of TyrO$^{\cdot}$ and monitoring its reactions

Radicals were generated by radiolysis of aqueous solutions of sodium azide and tyrosine saturated with nitrous oxide, where the initial radicals (e^-_{aq}, HO$^{\cdot}$, H$^{\cdot}$) are rapidly converted to the oxidizing $N_3^{\cdot}$ radical (yield ~0.7 μM/Gy), which in turn generates TyrO$^{\cdot}$ via reaction (4) in a few microseconds if [TyrOH] ~0.5–1 mM since $k_4 = 1.0 \times 10^8\ M^{-1}\ s^{-1}$ at pH ~ 7 [14,15]:

$$N_3^{\cdot} + \mathrm{TyrOH/TyrO^-} \rightarrow N_3^- + \mathrm{TyrO^{\cdot}}\ (+\ H^+). \quad (4)$$

By selecting concentrations such that, typically, $[N_3^-]/[\mathrm{TyrOH}] \geqslant 100$, direct reaction of HO$^{\cdot}$ with TyrOH was inhibited. Phosphate buffer (20–25 mM) maintained the pH at ~7.2–7.4.

For pulse radiolysis studies, solutions were irradiated using 6 MeV electron pulses (~0.2–0.6 μs) as described previously [7], typically recording absorbance/time profiles as the average of ~8 pulses; TyrO$^{\cdot}$ was monitored at 405 nm. For continuous radiolysis studies, solutions were irradiated with cobalt-60 gamma-rays at 1.2 Gy/min (generating TyrO$^{\cdot}$ at ~0.8 μM/min) for up to 15 min. Samples were removed every 3 min and products measured as described below.

Reaction of TyrO$^{\cdot}$ with GSH

To study the kinetics of the reaction of TyrO$^{\cdot}$ with GSH, azide/tyrosine solutions were prepared as described above but additionally containing GSH (0.1–0.5 mM). Since the unwanted reaction (5) has $k_5 = 9.5 \times 10^6\ M^{-1}\ s^{-1}$ at pH 7, i.e. $k_4/k_5 \sim 10$ at this pH [14]:

$$N_3^{\cdot} + \mathrm{GSH/GS^-} \rightarrow N_3^- + \mathrm{GS^{\cdot}}\ (+\ H^+), \quad (5)$$

and $[\mathrm{TyrOH}]/[\mathrm{GSH}] \geqslant 2$, at most ~5% of the azide radicals formed GS$^{\cdot}$ directly. The decay of the absorbance of TyrO$^{\cdot}$ at 405 nm was accelerated by the presence of GSH and analysed as described below. Studies of the effects of GSH upon 3,3′-dityrosine formation utilizing continuous radiolysis involved ⩽30 μM GSH and so reaction (5) was negligible.

Measurements of stable products

Loss of GSH and formation of GSSG were measured by LC–MS calibrated against authentic standards using a Waters 2695 system with a Waters Micromass ZQ mass spectrometer, using single- ion monitoring at m/z $(M + H)^+$ 308 and 613, respectively and a cone voltage of 20 V. Chromatography utilized an ACE column (3 μm, 125 × 3.2 mm) maintained at 35 °C with analytes separated with 10 mM formic acid and 0–20% acetonitrile over 6 min, returning to starting conditions after 0.1 min, with a flow rate of 0.5 mL/min. The formation of 3,3′-dityrosine was monitored by fluorescence (Perkin–Elmer LS50B luminescence spectrometer) with excitation at 320 nm and monitoring emission at 401 nm. Identification of possible TyrOH–GSH adducts used a Waters micromass EMD spectrophotometer, using single-ion monitoring at m/z $(M + H)^+$ 487 and 223 (see Supplementary Data for experimental procedure).

Measurements of the redox properties of TyrO$^{\cdot}$

The thermodynamically-reversible electrode potential for single-electron reduction of the tyrosyl radical was measured as the pH-dependent mid-point reduction potential of the couple, $E_m(\mathrm{TyrO^{\cdot}},H^+/\mathrm{TyrOH})$. This was estimated by establishing a fast electron-transfer equilibrium with a redox indicator of known reduction potential, measuring the equilibrium constant K_6 either from the equilibrium absorbance of the reporter chromophore (A_{eq}) or the kinetics of approach to equilibrium ($K_6 = k_6/k_{-6}$), before a significant fraction of TyrO$^{\cdot}$ radicals could decay by other routes. The method has been described in detail [16] and used extensively, e.g. in recent measurements of the reduction potential of the thiyl radical, $E_m(\mathrm{GS^{\cdot}},H^+/\mathrm{GSH})$ [7]. The redox indicator used was 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate ($ABTS^{2-}$):

$$\mathrm{TyrO^{\cdot}} + \mathrm{ABTS^{2-}}\ (+H^+) \rightleftharpoons \mathrm{TyrOH} + \mathrm{ABTS^{\cdot-}}, \quad (6)$$

the stable radical ABTS$^{\cdot-}$ being monitored at 645 nm and the standard reduction potential of the couple E° ($\mathrm{ABTS^{\cdot-}/ABTS^{2-}}$) taken as 0.68 V vs. NHE [17].

The radicals in the redox equilibrium (6) were generated by pulse radiolysis of N_2O-saturated solutions containing azide, TyrOH, and $ABTS^{2-}$ as described above. The ABTS$^{\cdot-}$ radical was formed concomitantly with TyrO$^{\cdot}$, depending on the relative concentrations of $ABTS^{2-}$ and TyrOH as well as pH, since $k_7 \sim 7 \times 10^9\ M^{-1}\ s^{-1}$ (Folkes, unpublished data) and oxidation of TyrOH by $N_3^{\cdot}$ (4) is markedly pH-dependent, with rate constant k_4 increasing from $\sim 1 \times 10^8\ M^{-1}\ s^{-1}$ at pH ~ 7 to $3.6 \times 10^9\ M^{-1}\ s^{-1}$ at pH 11.8 [14], reflecting $pK_8 \sim 10.4$:

$$N_3^{\cdot} + \mathrm{ABTS^{2-}} \rightarrow N_3^- + \mathrm{ABTS^{\cdot-}} \quad (7)$$

$$\mathrm{TyrOH} \rightleftharpoons \mathrm{TyrO^-} + H^+\ (\text{phenolic OH dissociation}). \quad (8)$$

K_6 was measured as a function of pH between pH ~ 9.1–11.0 (phosphate buffers, 10 mM), using $[\mathrm{ABTS^{2-}}] = 50$ μM and [TyrOH] = 0–1.2 mM.

Data analysis

Data analysis utilized OriginPro software (v.7, OriginLab Corporation, Northampton, MA, USA). Simulation of chemical reaction schemes by numerical integration of the differential equations utilized FACSIMILE software (v.4, MCPA Software Ltd., Farrington, Oxon, UK), which uses the Gear algorithm appropriate to the stiff differential equations found in free-radical chemistry.