Redox Reactivity of the Tyrosine Radical and Fe^{III}_2 of the B2 Subunit of *E. coli* Ribonucleotide Reductase

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Rate constants have been obtained for the biphasic electron-transfer reduction of Tyr· and Fe^{III}_2 of the B2 subunit of ribonucleotide reductase (RNR) with long-lived radical reductants, *e.g.* methyl viologen, $MV^{+\cdot}$, generated *in situ* by reaction with dithionite.

The enzyme ribonucleotide reductase (RNR) is an essential component of all living cells, being responsible for the conversion of ribonucleotides to deoxyribonucleotides, prior to DNA formation.[1—4] It consists of two subunits, here referred to as B1 and B2, in 1:1 amounts. Protein B1 is dimeric, and in addition to redox active dithiol groups has binding sites for ribonucleotides, and regulatory sites for nucleotide diphosphates. Protein B2 (M_r 78000) has two identical polypeptides (375 amino acids), in which residue 122 is present as a radical Tyr·. The latter exists in the deprotonated phenolate form, and is stabilised in some way not fully understood by the Fe^{III}_2.[5—7] The two Fe^{III}s are μ-oxo bridged, and antiferromagnetically coupled with J −108 cm^{-1}.[8] The UV–VIS spectrum of Fe^{III}_2 has peaks at 325 and 375 nm similar to those observed for hemerythrin,[9] whereas Tyr· gives a sharp band at 410 nm, with some additional contribution to

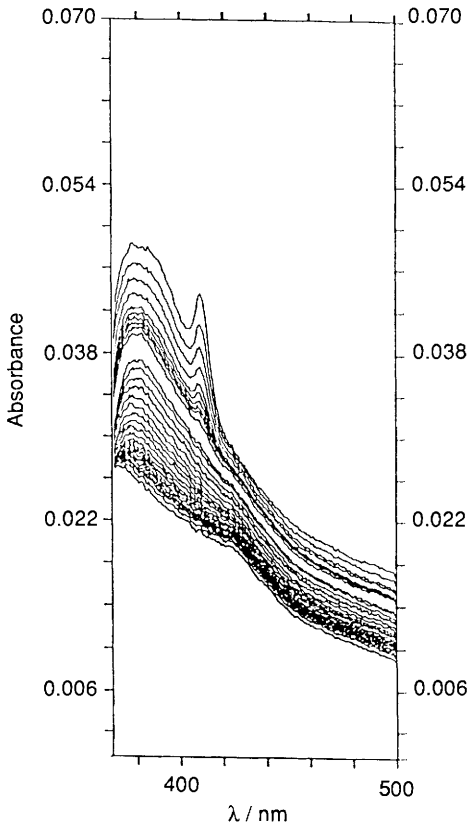


Figure 1. Scan spectra showing absorbance decreases for the reduction of first Tyr$^{\bullet}$ and then the Fe^{III}_2 of *E. coli* B2 ribonucleotide reductase (8 μM) with phenosafarin (1.5 μM) maintained in the reduced form by excess of dithionite (1 mM). Scans recorded every 1.5 min (first 10), and then every 10 min, with a longer time interval before the final spectrum was recorded. Air-free conditions, 25 °C, pH 7.5, I = 0.10 M (NaCl).

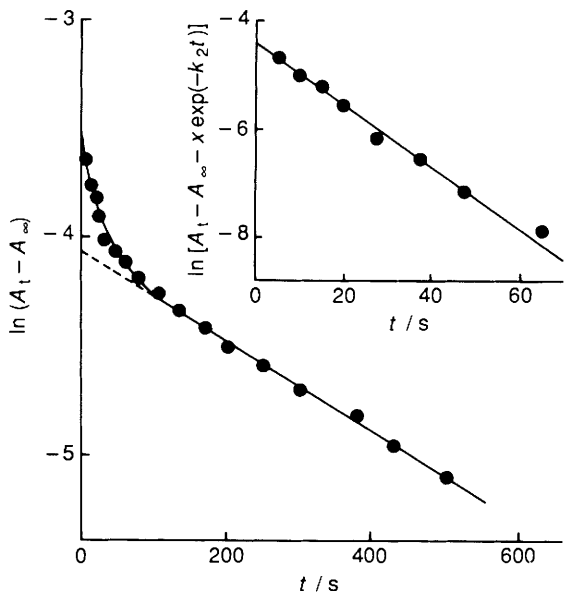
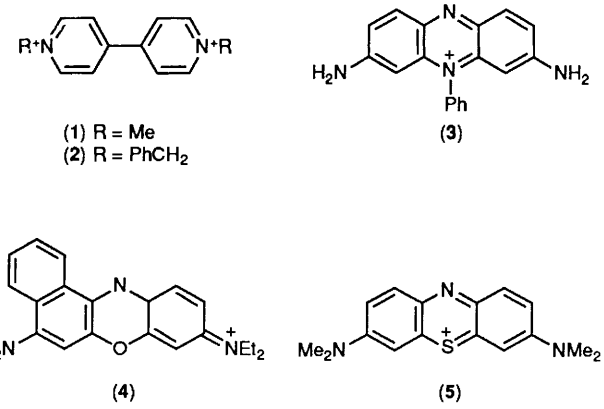


Figure 2. Showing biphasic behaviour for the reaction of the Tyr$^{\bullet}$ and Fe^{III}_2 of the B2 subunit of RNR with the benzyl viologen radical. The latter was kept at constant concentration (2.5 μM) by an excess of dithionite (0.1 mM). Air-free conditions, 25 °C, pH 7.5, I = 0.10 M (NaCl).



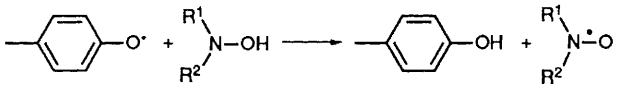
the absorbance at nearby wavelengths. The characteristic green colour of B2 arises from this and the weaker absorbance at ~700 nm. The *X*-ray crystal structure of B2 is about to be published.[10] In a recent paper Sahlin *et al.* have generated radical reductants using dithionite (which is itself unreactive with B2), and shown that the stoicheiometric reactions Tyr$^{\bullet}$ → Tyr and Fe^{III}_2 → Fe^{II}_2 occur.[11] The procedure here reported enables rate constants to be determined for the reactions of Tyr$^{\bullet}$ and Fe^{III}_2 with such one-electron reductants.

The B2 subunit was isolated in good yield from an *E. coli* overproducer.[12] The activity of the isolated enzyme was confirmed in an assay procedure using B1. Concentrations were determined at 410 nm, ε 6600 M^{-1} cm^{-1}. The following procedure has been developed for studying the kinetics under air-free conditions. Degassed buffered (50 mM Tris) solutions of $S_2O_4^{2-}$ and reagent (source of the radical) were prepared in a Miller Howe glove box (O_2<5 ppm). Two optical quartz cells were loaded with the same mix of $S_2O_4^{2-}$ and reagent using a Gilson Pipetman (2—20 μl) and Hamilton syringe (1 ml). After thermostatting (25 °C), the UV–VIS spectrophotometer base line was recorded. A small volume of B2 (8 μM) was then micro-syringed into one of the cells. Conditions selected were with $S_2O_4^{2-}$ (1 mM) in a large excess of reagent (2—250 μM), I = 0.10 M (NaCl). Since reduction by $S_2O_4^{2-}$ is thermodynamically favourable (and fast),[13] the reagent is maintained fully reduced throughout.

Absorbance changes were monitored by conventional spectrophotometry at 380 nm at which wavelength both Tyr$^{\bullet}$ and Fe^{III}_2 absorb, Figure 1. Biphasic kinetics were observed, Figure 2. The kinetics are independent of the concentration of dithionite, and the Tyr$^{\bullet}$ is more reactive than Fe^{III}_2. No evidence was obtained for intermediate formation of Fe^{II}-Fe^{III}, which is presumably rapidly reduced by a second mole of reductant. First-order rate constants $k_{1,obs}$ and $k_{2,obs}$ give linear dependences on reductant, enabling second-order rate constants k_1 and k_2 to be determined, Table 1. No dependence on pH was observed in the range 6.0—9.0.

On admitting O_2 and with careful shaking the absorbance of Tyr$^{\bullet}$ and Fe^{III}_2 is ~90% restored within 10 min. We note that the above electron-transfer rate constants correlate fairly well with driving force,[15] and that in the case of the Methylene Blue radical (11 mV) only the Tyr$^{\bullet}$ is reduced. In the latter (as with hydroxyurea), O_2 is unable to regenerate Tyr$^{\bullet}$.

No reaction of $[Co(sep)]^{2+}$ (−300 mV) and $S_2O_4^{2-}$ (460 mV) is observed with the Tyr$^{\bullet}$ or Fe^{III}_2 of B2, and charge appears to be inhibitory.[11,16] In the enzyme redox cycle



Scheme 1

Table 1. Rate constants (25 °C) for the reduction of $Tyr^{\bullet}$ (k_1) and Fe^{III}_2 (k_2) components of the B2 subunit of *E.coli* ribonucleotide reductase. The reagents listed were one-electron reduced by excess of $S_2O_4^{2-}$ *in situ* to the corresponding radical forms, pH 7.5, I = 0.10 M (NaCl).

Reagent	$E°$/mV	k_1/M^{-1} s^{-1}	k_2/M^{-1} s^{-1}
Methyl Viologen (**1**)	−446	4.5×10^4	2.3×10^3
Benzyl Viologen (**2**)	−359	2.1×10^4	1.1×10^3
Phenosafranine (**3**)	−252	3.0×10^3	373
Nile Blue (**4**)	−110	80	6.3
Methylene Blue (**5**)	11	11.2	—

Stubbe and colleagues[3] have indicated a mechanism involving an H-atom transfer from (and then back to) the 3′ carbon of ribonucleotides. With hydroxyurea (0.44 M^{-1} s^{-1}) and hydroxamic acid derivatives (*e.g.* Didox, 0.40 M^{-1} s^{-1}), only the $Tyr^{\bullet}$ of B2 is reduced.[17] The reduction potential for hydroxyurea has been determined by cyclic voltammetry and is 724 mV (*vs.* normal hydrogen electrode). For such reactions H-atom transfer is a possibility (Scheme 1).[2]

The aromaticity of the radicals in the present studies may be important because of their hydrophobic and/or planar properties. The $Tyr^{\bullet}$ and Fe^{III}_2 sites are buried,[10] and the manner of electron transfer is of interest.

This work is being extended to explore further the features of electron-transfer and H-atom transfer reactivity exhibited by RNR.

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