Kinetics of Inactivation of the Tyrosine Radical of the B2 Subunit of *E. coli* Ribonucleotide Reductase

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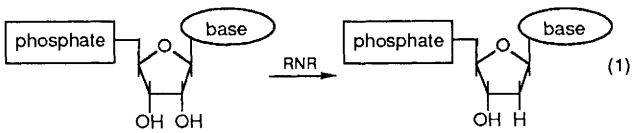
UV-VIS spectrophotometry has been used to study the reactivity of ten reductants with the tyrosine radical of the B2 subunit of ribonucleotide reductase at pH 6—9; charge (positive and negative) on both inorganic and organic reductants, was found to be inhibitory.

The enzyme ribonucleotide reductase (RNR) catalyses the formation of 2′-deoxyribonucleotides from the four different ribonucleotides, a reaction essential to DNA synthesis, equation (1).[1—3] The need for all living organisms to have this capability, and the important key role of RNR cannot be over emphasized. Inhibition of RNR is relevant, therefore, in the prevention of cell growth and is a target in anticancer, antibacterial, and antiviral drug action.[4] The enzyme consists of two subunits one having redox active dithiols as functional (reducing) groups (B1), and the other (unusually) a tyrosine free radical, which is generated and stabilised in some way not yet fully understood by an Fe^{III}_2 unit (B2). The best characterised enzyme is from bacterial *E. coli*, the subunits of which can be used as a model for mammalian M1 and M2 forms.[5] There is already considerable structure information from physical measurements which indicate a μ-oxo bridged Fe^{III}_2 site similar to that in hemerythrin.[6,7] An *X*-ray crystal study of B2 is in progress.[8] To date there are no kinetic studies on the reactivity of RNR or its components.



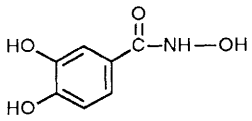
(1)

Ribonucleotide Deoxyribonucleotide

The Tyr· and Fe^{III}_2 containing B2 subunit (*M* 78 000) was isolated from an *E. coli* overproducer.[9] Yields of protein (green in colour) were ~200 mg from 65 g of cells. The protein was stored at −85 °C. The activity was checked by a procedure using B1, and concentrations determined from the UV-VIS spectrum, which has a sharp band due to the Tyr· at 410 nm [ε 6600 M^{-1} cm^{-1} (M^{-1} = mol^{-1} dm^3)].[7] The Tyr· is in a deprotonated phenolate form, and it has been suggested that this is H-bonded in some way to a ligand of the Fe^{III}_2.[10] The B2 subunit consists of two identical polypeptides, each containing Fe^{III}_2. The radical activity is attributed to Tyr 122.[11]

Hydroxyurea

Hydroxamic acids
R = alkyl or aryl



3, 4-Dihydroxybenzohydroxamic acid

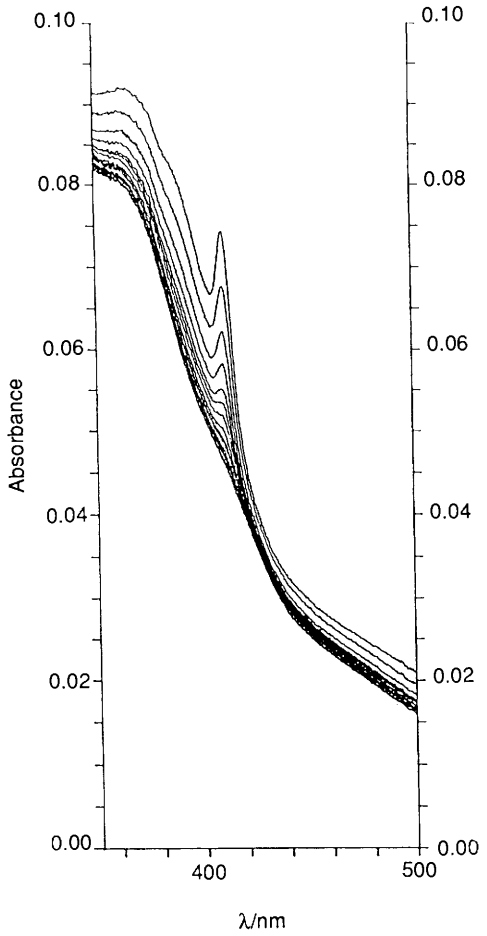
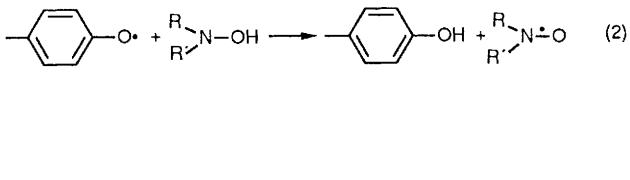


Figure 1. Scan spectra illustrating the decay of the 410 nm peak of Tyr• of the B2 subunit of ribonucleotide reductase (8 μM) on reduction with hydroxyurea (2.0 mM) at 25 °C, pH 7.5, I = 0.10 M (NaCl).

Inhibition of the enzyme by inactivation of the Tyr is of both general and medical interest.[2,12,13] Suitable reagents include hydroxyurea (HU), which is in clinical use as an anticancer drug, and hydroxamic acid (RCONHOH) derivatives such as 3,4-dihydroxybenzohydroxamic acid (Didox), which is undergoing clinical trials. Other polyphenolic compounds and thiosemicarbazones are also being investigated.[12]

Different reactions have been monitored by a UV-VIS scan over the range 350—450 nm. Typical absorbance changes for the decay of Tyr• of B2 (8 μM) are shown in Figure 1. At the Tyr• 410 nm peak the absorbance Δε change is 3040 M^{-1} cm^{-1}. First-order rate constants (25 °C) give linear dependences on reagent concentration (5—50 mM). With HU as a one-equivalent reductant, equation (2), second-order rate constants (0.44 M^{-1} s^{-1}) are invariant over the range pH 6.0—9.0, and with Didox (0.40 M^{-1} s^{-1}) over the range pH 6.0—7.5 (Didox is unstable at higher pH). It is concluded that the reactivity of B2 is not dependent on pH. However, with *N*-methylhydroxylamine MeNHOH (0.52 M^{-1} s^{-1}), the rate constant decreases with pH, the protonated form (pK_a 6.15 by independent titration) having little or no reactivity, Figure 2. Rate constants are listed in Table 1. No reduction of Fe^{III}_2 was observed, and the Tyr product is stable in air with no tendency to regenerate Tyr•.

Previously it has been reported that dithionite (−460 mV), which in solution is partially dissociated, $S_2O_4^{2-} \rightleftharpoons 2SO_2^{\bullet-}$,



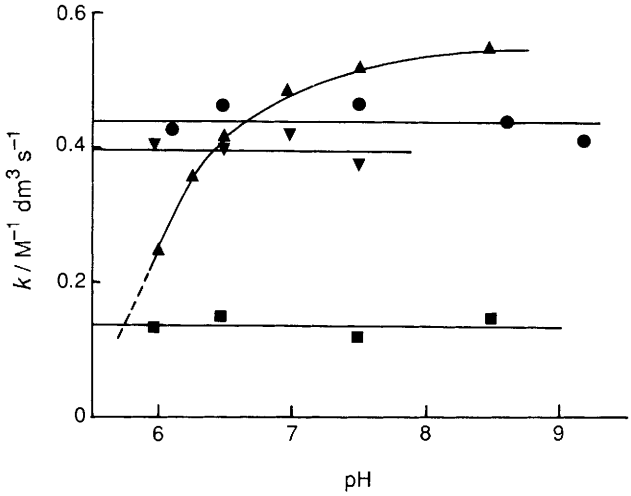


Figure 2. The variation of second-order rate constants k with pH for the reduction of Tyr• of the B2 subunit of ribonucleotide reductase (8 μM) with 5—50 mM reagents hydroxyurea (●), *N*-methylhydroxylamine (▲), Didox (▼), and L-glutamate γ-monohydroxamic acid (■), at 25 °C, I = 0.10 M (NaCl).

Table 1. Rate constants (25 °C) for reduction of the Tyr• of the B2 subunit of *E. coli* RNR.[a]

Reductant	Formula	k /M^{-1} s^{-1}
Hydroxyurea	$NH_2CONHOH$	0.44
N-Methylhydroxylamine	MeNHOH	0.52
Didox	$3,4\text{-}(OH)_2C_6H_3CONHOH$	0.40
Acetohydroxamic acid	MeCONHOH	0.125
L-Glutamate γ-monohydroxamic acid	$^-O_2CCH(NH_3^+)(CH_2)_2CONHOH$	0.135
Aspartate β-monohydroxamic acid	$^-O_2CCH(NH_3^+)CH_2CONHOH$	0.034

[a] pH 7.5, I = 0.10 M (NaCl). No pH dependences were observed in the range pH 6.0—9.0 except for *N*-methylhydroxylamine. Four other reductants were found to be unreactive (see text).

does not react directly with Tyr•.[14] It was suggested that charge is a determining factor, and that the inactivity was due to nearby negative charge on the protein. However we find that the one-equivalent inorganic reductant $[Co(sep)]^{2+}$ (−300 mV) is also unreactive (sep = sepulchrate, which is the trivial name for the cage ligand 1,3,6,8,10,13,16,19-octaazabicyclo[6.6.6]eicosane).[15] This is quite remarkable in view of the favourable thermodynamics, and particularly so since no reduction of Fe^{III} is observed either. H-atom transfer may be relevant in the reaction in equation (2).

Other studies have been carried out with amino-acid derivatives of hydroxamic acid, RCONHOH (R = histidine, L-lysine, glycine, aspartate, and L-glutamate). Such compounds are known to have a high affinity for Fe^{III}, but in the present case do not react with Fe^{III}_2.[16] Charge is again seen to be important. Thus the first three compounds, which have just one C-atom between NH_3^+ and the hydroxamic acid group

–CONHOH, show no activity. With aspartate β-monohydroxamate (0.034 M^{-1} s^{-1}) and glutamate γ-monohydroxamate (0.135 M^{-1} s^{-1}), however, where the corresponding separation is two and three C-atoms respectively, rate constants correlate well with the distance separating $-NH_3^+$ and the hydroxamic acid. These findings are consistent with observations for protonated *N*-methylhydroxylamine, where the positive charge is in close proximity to the hydroxamic acid and there is little or no reactivity.

To summarise, rate constants determined are of similar magnitude, none of the reductants (including HU and Didox) have very high reactivity with Tyr•, and charge (both + and −) is inhibitory. The latter suggests that the Tyr• is protected, and in a hydrophobic region, consistent with crystallographic information.[8] Present evidence is that the Tyr• of mammalion M2 may be more exposed. Thus although B2 and M2 exhibit the same sensitivity to HU, M2 is 75 times more reactive with Didox.[17] Further studies are in progress. Of particular interest is how the Tyr• of B2 can abstract a H-atom from the 3′-carbon of a ribonucleotide associated with B1.

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