Free-radical Formation in the Pulse-radiolysis Oxidation of Inactive *Escherichia coli* Met-R2 Ribonucleotide Reductase†

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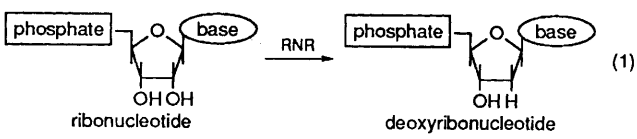
The regeneration of a tyrosyl radical from the inactive *Escherichia coli* met-R2 enzyme using pulse radiolytically generated azide radicals $N_3^{\bullet}$ has been studied. Tryptophan and tyrosine amino acid residues on the protein are reactive towards $N_3^{\bullet}$ with the formation of either a tryptophan radical (peak at 510 nm) or a tyrosyl radical (peak at 410 nm). The combined rate constant (19 °C) obtained for the formation of both these deprotonated species in N_2O-saturated solution, $[N_3^-] = 0.012$ M, pH 7 (40 mM phosphate), and $I = 0.100$ M, is 1.75×10^9 M^{-1} s^{-1}. Biphasic decay of the tryptophan radical is then observed, rate constants 2.9×10^3 and 7.3×10^2 s^{-1}, which are assigned as intramolecular electron-transfer steps. In the faster of these the tryptophan radical receives an electron from a tyrosine to give a tyrosyl radical product. The tyrosyl radicals formed in both the primary and secondary processes decay within 1 s in contrast to the much longer-lived tyrosyl-122 radical present in active R2 enzyme. Similar results were obtained on pulse radiolysis of the mutant Tyr122Phe R2 protein. Thus the stable Tyr-122 radical form of R2 does not appear to be formed in any of these reactions, and the specificity of the regeneration of the radical in the native enzyme involving reaction of the diiron(II) site with O_2 is highlighted.

Ribonucleotide reductase (RNR) [1] catalyses the first unique step of DNA synthesis leading to the reduction of ribonucleotides to the corresponding deoxyribonucleotides [equation (1)].[1–7] The



(1)

enzyme isolated from *Escherichia coli* consists of two proteins, R1 and R2. The larger R1 ($M_r = 2 \times 85\,400$) contains redox-active cysteines as well as substrate and effector binding sites. The R2 protein is also a dimer ($M_r = 2 \times 43\,300$) each subunit of which can bind a binuclear iron centre and stabilise a tyrosyl radical (Tyr$^{\bullet}$) at residue 122.[8–10]

A number of studies on the mechanism of action of the assembled R1R2 enzyme have been reported.[11–13] Evidence for the involvement of the radical in the enzyme cycle has been obtained from the dependence of activity on the presence of the tyrosyl radical in R2,[14] and the use of a ribose analogue substituted at the 2′ position with azide, which behaves as a suicide inhibitor.[15,16] According to the mechanism proposed, a substrate radical is formed at the 3′ position by a process involving hydrogen abstraction. The prime role of the tyrosyl radical is to initiate abstraction of this 3′-hydrogen. The last step in the catalytic cycle is for the 3′-hydrogen to be transferred back to the nucleotide. However, no decrease in the tyrosyl radical signal has been detected yet during turnover.[15,17,18]

The crystal structure of R2 has shown that the tyrosyl radical is buried some 10 Å below the nearest point of a hydrophobic surface, with no access to this site clearly defined.[19] Although this explains in part the stability of the radical, at the same time because of its location it is more difficult to explain how the radical can participate in the required enzymatic changes. One possibility is that there is a conformational change in R2 when it binds to R1, resulting in a somewhat greater exposure of the radical to substrate. However binding of R1 alone is not sufficient to change the EPR spectrum of the Tyr$^{\bullet}$, or significantly affect the susceptibility of the radical to reduction by substrates such as hydroxyurea, suggesting that the structure around the Tyr$^{\bullet}$ is not significantly changed by such binding.[20,21]

Another possibility is that electron transfer between the Tyr$^{\bullet}$ and the hydrophobic surface (which is where interaction with R1 occurs) rather than hydrogen-atom transfer is occurring. A possible route from the surface involving one of the irons (FeI), *via* Trp-48 to the Tyr$^{\bullet}$ of residue 122, has been suggested from the crystal structure of R2.[19] As far as R1 is concerned it has been demonstrated that Cys-439 plays a prominent role in the redox processes involved.[13,22]

Intramolecular electron-transfer reactions of synthetic peptides (X),[23–25] which incorporate a tyrosine (Tyr) and tryptophan (Trp) at either extremity separated by a specific number n of prolines (n = 1–6), have been studied. Here also reaction was initiated by the azide radical generated by pulse radiolysis of solutions containing N_3^-.[26] Since the indole group of Trp is more reactive than the phenol of Tyr towards $N_3^{\bullet}$, the initial dominant reaction is the one-electron oxidation of the tryptophan to the radical Trp$^{\bullet}$, equation (2), where TrpH and

$$N_3^{\bullet} + \text{HTrp-X-TyrH} \longrightarrow {}^{\bullet}\text{Trp-X-TyrH} + N_3^- + H^+ \quad (2)$$

TyrH represent the tryptophan and tyrosine components. Subsequently decay of the deprotonated Trp$^{\bullet}$, which absorbs at 510 nm, and concomitant increase in the absorption at 410 nm due to the formation of the tyrosyl radical Tyr$^{\bullet}$ is observed, equation (3). As in the enzyme the latter has a phenolate group

$${}^{\bullet}\text{Trp-X-TyrH} \longrightarrow \text{HTrp-X-Tyr}^{\bullet} \quad (3)$$

$C_6H_4O^{\bullet}$. Of the $E°$ values reported for the Trp$^{\bullet}$–TrpH couple in the range 0.64–1.08 V, and for the Tyr$^{\bullet}$–TyrH couple in the range 0.78–0.94 V,[23,27–30] the higher values of 1.08 and 0.94 V are believed to apply at pH 7. At this pH the formation of Tyr$^{\bullet}$ is favoured, $\Delta E° \approx 0.14$ V, which value means that intra-

† *Non-SI unit employed:* M = mol dm^{-3}.

molecular electron transfer will proceed to 98% completion. It has been assumed therefore that reaction (3) proceeds to completion.

In the studies described herein on the R2 protein we explore the possibility of regenerating the Tyr-122 radical from met-R2. Although a number of reagents are known to reduce the tyrosyl radical Tyr$^{\bullet}$, and in some cases the Fe^{III}_2 of R2,[14,31,32] oxidation processes in which the tyrosyl radical and/or the Fe^{III}_2 of reduced protein are regenerated are few. One such reaction is the oxidation of the fully reduced R2 protein by O_2, which results in the reformation of both Tyr$^{\bullet}$ and Fe^{III}_2.[8,31] Hydrogen peroxide is also able to oxidise fully reduced R2 to the met form,[33] with some partial regeneration of Tyr$^{\bullet}$.[34,35] However to date only these oxygen-containing oxidants have been found to react with both the Fe^{II}_2 and the tyrosine at position 122. In electrochemical studies there is no evidence for regeneration of reduced R2.[31] From the crystal structure of R2 the Tyr-122 and Fe^{III}_2 are about 10 Å from the closest point of the surface which is hydrophobic. As a result charge impedes the reaction with different reagents, and strong reductants such as $S_2O_4^{2-}$ are not able to reduce either the Tyr$^{\bullet}$ or diiron(III) centres.[31,36,37] It is not surprising therefore that strongly reducing complexes such as $[Co(sep)]^{2+}$ (−300 mV), sep = sepulchrate is the trivial name for the cage ligand 1,3,6,8,10,13,16,19-octaazabicyclo[6.6.6]icosane, and the oxidant $[Fe(CN)_6]^{3-}$ (410 mV) have little or no reactivity.[31,36] In the case of molecules such as O_2 and H_2O_2 their ability to penetrate and co-ordinate at the binuclear iron centre in the regeneration of the protein is an important feature, and very likely a key functional role of the irons.

The use of pulse-radiolytically generated radicals $N_3^{\bullet}$ (+1.33 V) as a means of regenerating Tyr$^{\bullet}$ from the inactive diiron(III) met-R2 protein is explored in this study. Use of such a neutral radical ensures that there is no charge factor, and helps to assess the ability of oxidants other than O_2 in regenerating Tyr$^{\bullet}$. Also a one-electron acceptor should be an ideal oxidant in order to remove a single electron from Tyr-122 and regenerate active R2.

Since the formation and decay of Trp$^{\bullet}$ and Tyr$^{\bullet}$ can be monitored independently at 510 and 410 nm in pulse-radiolysis experiments, it is possible to explore intramolecular electron transfer between these residues. Studies on the mutant R2 protein in which Tyr-122 is substituted by phenylalanine are also reported.

Experimental

Proteins.—Wild type and Tyr122Phe mutant R2 forms were obtained from *Escherichia coli* overproducer strains using literature procedures.[38–40] The mutant is similar to the wild type protein R2 in size, iron content, iron-related visible absorption spectrum and affinity for the R1 subunit. The sole difference observed is that the protein lacks the tyrosyl radical and enzymatic activity. Concentrated solutions of R2 protein were usually stored at −80 °C. Met-R2 was prepared by incubating a concentrated solution of active R2 with hydroxyurea (100 mM) at 25 °C for half an hour. The solution was then dialysed against two portions of 50 mM Tris [tris(hydroxymethyl)methylamine] buffer at pH 7.5 containing 20% glycerol. The volume of each portion was at least 200 times that of the protein solution.

Pulse Radiolysis.—Experiments were carried out using a beam of 2.5 MeV (*ca.* 4×10^{-16} J) electrons produced by a Van de Graaff accelerator at the Cookridge Radiation Research Centre, University of Leeds. Pulse lengths were typically in the range 0.2–1.0 μs. The pulse size was recorded as the secondary emission chamber voltage (V_{sec}). Analysing light from a tungsten filament lamp with filters cutting off light of $\lambda < 385$ and < 450 nm for the studies at 410 and 510 nm respectively was passed through a transparent heat filter before reaching the sample cell. It was then transmitted to a Hamamatsu R446 photomultiplier in the control area *via* front-surface mirrors and a Bausch and Lomb monochromator. The light level was sampled and recorded before an electron pulse was triggered. The voltage changes corresponding to the light absorption changes were monitored using a Gould 4072 digital oscilloscope, with subsequent data transfer onto a magnetic storage disc.

The pathlength of the sample cell was 1.0 cm. The yield of oxidising radical [R] produced for a given pulse was calculated from equation (4), where S_{sec} is the sensitivity (with units Gy

$$[R] = V_{sec} S_{sec} G_R \quad (4)$$

V^{-1}) and G_R is the radiation chemical yield of radicals per joule of energy absorbed. The value of S_{sec} was determined by thiocyanate dosimetry.[41]

Kinetic studies were carried out in 40 mM phosphate buffer at pH 7, $I = 0.100$ M. Buffer solutions were made up from potassium dihydrogen phosphate and dipotassium hydrogen phosphate (both BDH Analar) using Milli-Q ion-exchanged purified water (resistivity > 18 Mohm cm^{-1}). Concentrated samples of R2 protein were dialysed against two portions of deaerated buffer (volume of each portion at least 200 times that of the protein solution), and stored under nitrogen before use. Prior to each experiment, buffer solutions (*ca.* 10 cm^3) were thoroughly saturated with N_2O and argon gas in a tightly sealed vessel for at least 10 min before calculated amounts of concentrated deaerated protein solution (under argon) were added to give the required concentration. When gas is bubbled directly through solutions of the enzyme denaturation is observed. Solutions of the enzyme were standardised by measuring the absorbance at 280 and 310 nm, and using the difference in absorption coefficients ($\varepsilon_{280} - \varepsilon_{310}$) = 1.2×10^5 M^{-1} cm^{-1}. Gas-tight syringes were used for transferring protein solution to the sample cell, and a positive pressure of argon was maintained when loading and draining the cell. Experiments were performed at 19 ± 1 °C on deaerated N_2O-saturated solutions containing 8.7–44.2 μM R2 protein and 0.012 M sodium azide. Azide radicals were generated as secondary products, equations (5) and (6). The G_R value for $N_3^{\bullet}$ is 6.4×10^{-7} mol

$$N_2O + H_2O + e_{aq}^{-} \longrightarrow OH^{\bullet} + OH^{-} + N_2 \quad (5)$$

$$OH^{\bullet} + N_3^{-} \longrightarrow OH^{-} + N_3^{\bullet} \quad (6)$$

J^{-1}. The range of radiation dose employed was 2.5–3.5 Gy. The amount of $N_3^{\bullet}$ generated in each experiment was normally less than 10% of the total concentration of R2.

Transient absorption changes were monitored at 410 and 510 nm respectively, where the tyrosyl and tryptophan radicals absorb. Spectra of transient species formed as a result of pulsing were recorded over the range 330–550 nm.

Treatment of Data.—The FACSIMILE kinetic package[42] was used to evaluate rate constants. Computer-simulated absorbance *vs.* time traces were generated using different values of rate constants and absorption coefficients. Best values were obtained from computer simulation of experimental traces.

Results and Discussion

A typical transient spectrum recorded 225 μs after pulsing a solution containing 20 μM of met-R2, using a bandwidth of 2 nm is shown in Fig. 1. The peaks at around 410 and 510 nm are assigned to the formation of Tyr$^{\bullet}$ and Trp$^{\bullet}$ respectively as primary products. From previous studies[43] on the flash photolysis of phenolic compounds including tyrosine it has been observed that phenolate radicals have a maximum at 410 nm, and a second broader band at ≈390 nm. These same features are observed for the Tyr$^{\bullet}$ generated in this work. In the Trp$^{\bullet}$ case the peak at 510 nm is accompanied by shoulders at 490 and 530

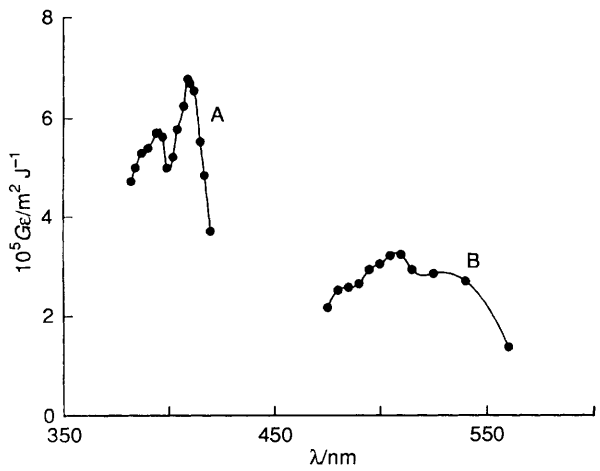


Fig. 1 Transient spectrum indicating formation of tyrosyl radical (A) and tryptophan radical (B) obtained 225 μs after pulsing a solution of R2 (20 μM) in N_2O-saturated solution in the presence of azide (0.012 M), pH 7 (40 mM phosphate buffer), I = 0.100 M, bandwidth 2 nm

nm, in keeping with the broad (unresolved) peak observed previously.[44] The $TrpH^{\bullet+}$ radical with a peak at ≈580 nm[44] does not appear to be formed in the present studies. There is some overlapping in absorbance of the two products. Absorption coefficients of ε_{410} = 3200 M^{-1} cm^{-1} for $Tyr^{\bullet}$ and ε_{510} = 1890 M^{-1} cm^{-1} for $Trp^{\bullet}$ have been reported.[26] However the kinetic studies described below were carried out with the monochromator at increased bandwidth (15 nm) for which we have determined ε_{410} = 2550 M^{-1} cm^{-1} for $Tyr^{\bullet}$ and ε_{510} = 1970 and ε_{410} = 310 M^{-1} cm^{-1} for $Trp^{\bullet}$, and these values were used in the analysis of the data.

At 510 nm (Fig. 2) the last formation of $Trp^{\bullet}$ is followed by a biphasic decay to a plateau. At 410 nm (Fig. 3) the initial growth is followed by a slower growth. Analysis of this slower growth by the FACSIMILE program shows it to match the faster phase of the decay at 510 nm. Equations (7)–(13) explain the general

$$N_3^{\bullet} + Tyr(1) \xrightarrow{k_a} N_3^- + Tyr^{\bullet}(1) \quad (7)$$

$$N_3^{\bullet} + Trp(1) \xrightarrow{k_b} N_3^- + Trp^{\bullet}(1) \quad (8)$$

$$N_3^{\bullet} + Trp(2) \xrightarrow{k_c} N_3^- + Trp^{\bullet}(2) \quad (9)$$

$$N_3^{\bullet} + Trp(3) \xrightarrow{k_d} N_3^- + Trp^{\bullet}(3) \quad (10)$$

$$Trp^{\bullet}(1) \xrightarrow{k_e} Tyr^{\bullet}(2) \quad (11)$$

$$Trp^{\bullet}(2) \xrightarrow{k_f} X \quad (12)$$

$$N_3^{\bullet} + N_3^{\bullet} \xrightarrow{k_g} Product \quad (13)$$

features of the kinetic results. Reactions (7) and (11) account for the fast and slow processes in which $Tyr^{\bullet}$ is formed. Reactions (8)–(10) correspond to the formation of $Trp^{\bullet}$ at three different sites. The first of these $Trp^{\bullet}(1)$ decays by electron transfer from tyrosine, (11), and the second $Trp^{\bullet}(2)$ decays without formation of an observed product. A third form $Trp^{\bullet}(3)$ is long-lived and gives rise to the plateau with absorbance above 0.4 × 10^{-3} [Fig. 2(*b*)].

The FACSIMILE program was used to evaluate rate constants for the above mechanism by fitting pulse-radiolysis traces such as those in Figs. 2 and 3 for three concentrations of R2. The rate constant for reaction (13) was set at the literature value of 4.5 × 10^9 M^{-1} s^{-1}.[44] Although each individual trace does not evaluate every rate constant, by employing traces at

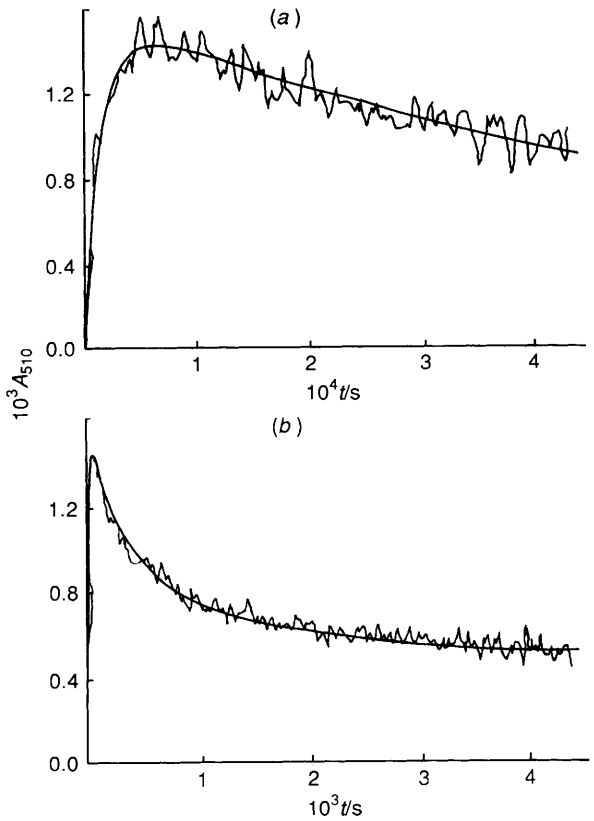


Fig. 2 Absorbance (at 510 nm) *vs.* time traces for the pulse radiolysis of R2 (20 μM) in N_2O-saturated solution in the presence of azide (0.012 M), pH 7 (40 mM phosphate buffer), I = 0.100 M. The traces illustrate the formation and decay of $Trp^{\bullet}$. Sweep speeds are 50 (*a*) and 500 μs per division (*b*). The smooth curves were simulated using the following rate constants: (*a*) k_a = 8.0 × 10^8, k_b = 5.8 × 10^8, k_c = 2.15 × 10^8, k_d = 2.7 × 10^8 M^{-1} s^{-1}; k_e = 2.8 × 10^3 and k_f = 6.8 × 10^2 s^{-1}; (*b*) k_a = 5.6 × 10^8, k_b = 3.5 × 10^8, k_c = 2.4 × 10^8, k_d = 2.7 × 10^8 M^{-1} s^{-1}; k_e = 3.5 × 10^3 and k_f = 7.3 × 10^2 s^{-1}. For both simulations k_g = 4.5 × 10^9 M^{-1} s^{-1}

both wavelengths and at a range of time-scales a set of rate constants is obtained which is both self-consistent and consistent with the proposed mechanism. The data indicate that $Trp^{\bullet}$ and $Tyr^{\bullet}$ are the only radical products, but show that at each of the concentrations of R2 used (13.3, 26.5 and 44.2 μM) significant amounts of the $N_3^{\bullet}$ radicals decay by the self-reaction (13), *i.e.* 27, 14 and 12%, respectively, at the doses used in this study.

The overall rate constant for the reaction of $N_3^{\bullet}$ with R2 is (1.75 ± 0.2) × 10^9 M^{-1} s^{-1} which is partitioned between reactions (7)–(10) as follows: k_a = (7.0 ± 0.7) × 10^8, k_b = (5.2 ± 0.7) × 10^8, k_c = (1.9 ± 0.2) × 10^8 and k_d = (3.2 ± 0.3) × 10^8 M^{-1} s^{-1}, where the errors are standard deviations of the mean for 12 evaluations.

Although the above scheme suggests that intramolecular electron transfer between a tryptophan and tyrosine is feasible, there is no evidence in the present studies for an involvement of either Trp-48 or Tyr-122, where Trp-48 has been suggested as a mediator for electron transfer between the hydrophobic surface and Tyr-122. In fact the $Tyr^{\bullet}$ signal generated is not stable, and on a slow time-base decay of the absorbance at 410 nm is observed (Fig. 4). This result differs from the situation in which a stable tyrosyl radical at position 122 is formed when oxygen-related oxidants (*e.g.* O_2 and H_2O_2) are used. In the latter processes the diiron(II) centre also participates in the reaction.

Results obtained on pulse radiolysis of the Tyr122Phe mutant further support these findings. Since the Tyr-122 residue is replaced by a phenylalanine, any $Tyr^{\bullet}$ signal observed after

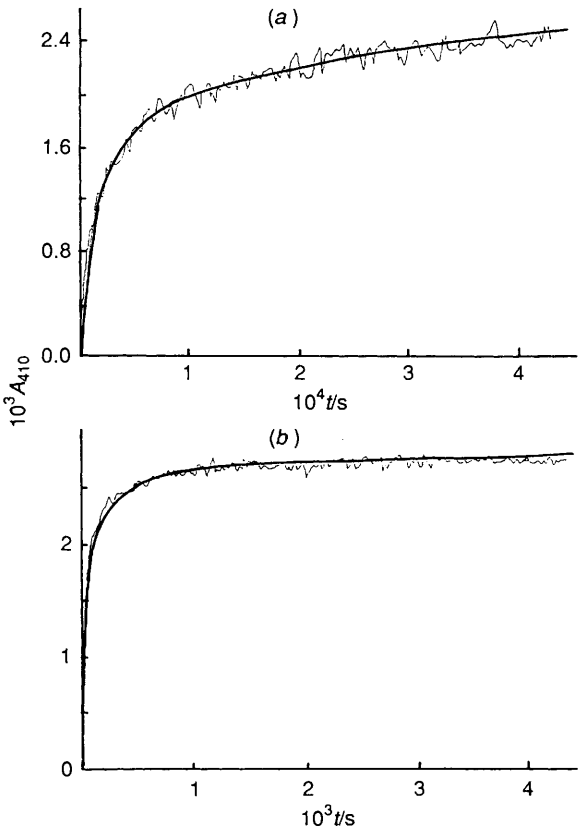


Fig. 3 Absorbance (at 410 nm) *vs.* time traces for the pulse radiolysis of met-R2 (26.5 μM) in N_2O-saturated solution in the presence of azide (0.012 M), pH 7 (40 mM phosphate buffer), $I = 0.100$ M. The traces illustrate the biphasic formation of Tyr$^\bullet$. Sweep speeds as in Fig. 2. The smooth curves were simulated using the following rate constants: (*a*) $k_a = 6.7 \times 10^8$, $k_b = 5.9 \times 10^8$, $k_c = 2.3 \times 10^8$, $k_d = 2.9 \times 10^8$ M^{-1} s^{-1}; $k_e = 2.6 \times 10^3$ and $k_f = 7.0 \times 10^2$ s^{-1}; (*b*) $k_a = 4.8 \times 10^8$, $k_b = 3.1 \times 10^8$, $k_c = 1.0 \times 10^8$, $k_d = 1.7 \times 10^8$ M^{-1} s^{-1}; $k_e = 2.6 \times 10^3$ and $k_f = 1.2 \times 10^3$ s^{-1}. For both simulations $k_g = 4.5 \times 10^9$ M^{-1} s^{-1}

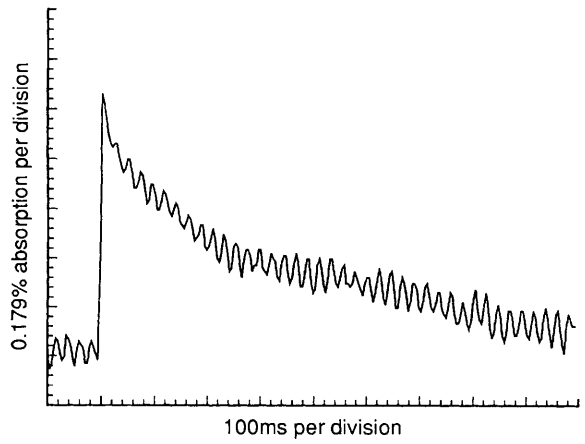


Fig. 4 Pulse-radiolysis trace recorded at 410 nm for R2 (20 μM) in N_2O-saturated solution in the presence of azide (0.012 M), pH 7 (40 mM phosphate buffer), $I = 0.100$ M, illustrating the slow decay of Tyr$^\bullet$

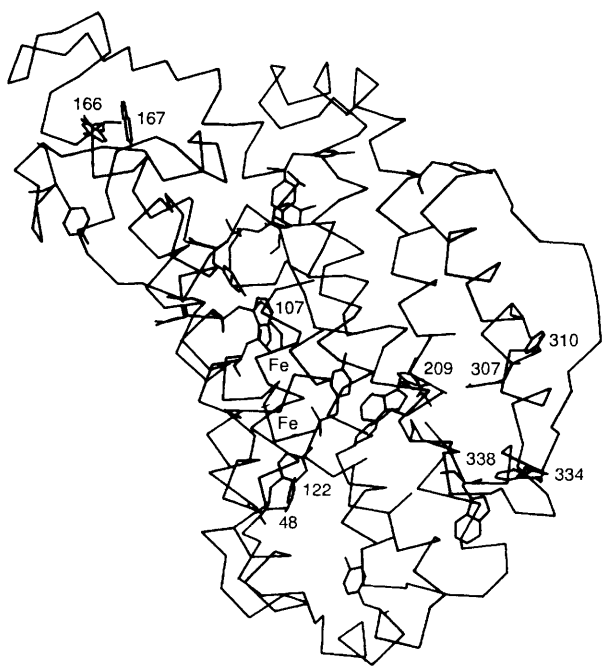


Fig. 5 Molecular graphics view of the R2 subunit of ribonucleotide reductase showing the positions of relevant Trp and Tyr residues and their proximity to the active site

pulse radiolysis cannot be due to Tyr$^\bullet$ formation at position 122. The behaviour observed is in fact very similar to that of the wild-type protein, with intramolecular rate constants k_e and k_f in the case of Tyr122Phe as listed in Table 1. These values are however about half those obtained for the wild-type protein, which raises the question as to whether there might still be a contribution from Tyr-122. If so it is not the stable tyrosyl-122 radical as formed by treating the fully reduced enzyme with O_2. One explanation may be that the extent of hydrogen bonding is different. Alternatively it is possible that the iron population of the Tyr122Phe mutant differs in some way from that of the wild-type enzyme.

Recently two intermediates have been detected in the re-activation of fully reduced R2 with O_2 using stopped-flow spectroscopy and rapid freeze–quench EPR spectroscopy.[45] The first, which exhibits a broad absorption band at 565 nm, is believed to be a peroxodiiron(III) species. The second, with an absorption band at 360 nm and a sharp isotropic EPR singlet ($g = 2.00$), is thought to be an oxygen-derived or a protein-derived free-radical product coupled to the diiron(III) centre. The mechanism proposed involves one (or more) high-valent iron intermediates,[34,35] and is consistent with the reaction of Fe^{II}_2 and O_2 as the initial step. Interestingly, in similar studies on the reactivation of the diiron(II) form of the Tyr122Phe mutant with O_2, not only was the lifetime of the transient 565 nm absorption found to be longer, but a new transient absorption band at 410 nm was observed as the former decayed. These results correlate well with our findings in the pulse-radiolysis studies on Tyr122Phe, namely that at least one other tyrosyl free radical can be generated.

While the studies described yield useful information about the behaviour of R2, no information has been obtained regarding the feasibility of electron transfer in R2 between Tyr-122 and the protein surface. In the reaction with $N_3^\bullet$ more than one Trp and Tyr is oxidised to the radical form, and one of the Trp$^\bullet$ radicals is able to oxidise a nearby tyrosine by intramolecular electron transfer. Using molecular graphics and the crystal structure coordinates of R2 we have attempted to identify the most likely residues involved.[44] Out of the seven R2 tryptophans only three, Trp-167, -334 and -338, are at the surface with their indole nitrogens exposed to solvent (Fig. 5), and therefore favourably positioned to react with $N_3^\bullet$. Although residues Trp-48 and -107 are near to the surface, it can be seen that their indole nitrogens are buried, whereas Trp-111 and -286 are completely buried. Since an $N_3^\bullet$ radical is expected to react directly with the indole side chain of a tryptophan, Trp-167, -334 and -338 are the strongest candidates for such oxidation. Also intramolecular electron transfer between Trp$^\bullet$ and tyrosine is most likely to be observed for those Trp$^\bullet$ which have a tyrosine residue

Table 1 Pulse-radiolysis studies on met-R2 forms of wild-type RNR and its Tyr122Phe mutant in N_2O-saturated solution. Rate constants for intramolecular electron-transfer reactions (11) and (12), pH 7.0 (40 mM phosphate buffer), in the presence of azide (0.012 M), I = 0.100 M, 19 ± 1 °C

	Protein	
Rate constant	Wild type	Tyr122Phe
$10^{-3}k_e/s^{-1}$	2.9 ± 0.3	1.2 ± 0.1
$10^{-2}k_f/s^{-1}$	7.3 ± 0.7	2.3 ± 0.2

nearby. A favourable rate constant $k_e = 2.9 \times 10^3$ s^{-1} has been obtained for this process. Relevant through space distances (d) measured between the nearest points on the aromatic groups are Trp-167 and Tyr-166 (d = 10.09 Å), Trp-334 and Tyr-307 (d = 8.87 Å), and Trp-338 to Tyr-310 or Tyr-209 (d = 13.58–15.61 Å). The hydrophobic surface is to the bottom left-hand edge of the molecule as illustrated in Fig. 5. Based on distance alone the Trp-167/Tyr-166 and Trp-334/Tyr-307 combinations are more likely to participate in intramolecular electron transfer than Trp-338 with Tyr-209 or Tyr-310. For the small driving force (−0.14 V), the direct connection between Trp-167 and Tyr-166 might be most favoured. However both are distant from residue 122, which again makes it difficult to account for the two-fold greater reactivity of wild-type as compared to Tyr122Phe mutant protein (Table 1).

Acknowledgements

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