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Interactions of Superoxide Anion with Enzyme Radicals: Kinetics of Reaction with Lysozyme Tryptophan Radicals and Corresponding Effects on Tyrosine Electron Transfer

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The kinetics of $O_2^{\bullet-}$ reaction with semi-oxidized tryptophan radicals in lysozyme, $Trp^{\bullet}$(Lyz) have been investigated at various pHs and conformational states by pulse radiolysis. The $Trp^{\bullet}$(Lyz) radicals were formed by $Br_2^{\bullet-}$ oxidation of the 3–4 exposed Trp residues in the protein. At pH lower than 6.2, the apparent bimolecular rate is about $2 \times 10^8\,M^{-1}\,s^{-1}$; but drops to $8 \times 10^7\,M^{-1}\,s^{-1}$ or less above pH 6.3 and in CTAC micelles. Similarly, the apparent bimolecular rate constant for the intermolecular $Trp^{\bullet}$(Lyz) + $Trp^{\bullet}$(Lyz) recombination reaction is about $(4–7 \times 10^6\,M^{-1}\,s^{-1})$ at/or below pH 6.2 then drops to $1.3–1.6 \times 10^6\,M^{-1}\,s^{-1}$ at higher pH or in micelles. This behavior suggests important conformational and/or microenvironmental rearrangement with pH, leading to less accessible semi-oxidized $Trp^{\bullet}$ residues upon $Br_2^{\bullet-}$ reaction. The kinetics of $Trp^{\bullet}$(Lyz) with ascorbate, a reducing species rather larger than $O_2^{\bullet-}$ have been measured for comparison. The well-established long range intramolecular electron transfer from Tyr residues to Trp radicals-leading to the repair of the semi-oxidized $Trp^{\bullet}$(Lyz) and formation of the tyrosyl phenoxyl radical is inhibited by the $Trp^{\bullet}(Lyz) + O_2^{\bullet-}$ reaction, as is most of the $Trp^{\bullet}(Lyz) + Trp^{\bullet}(Lyz)$ reaction. However, the kinetic behavior of $Trp^{\bullet}$(Lyz) suggests that not all oxidized Trp residues are involved in the intermolecular recombination or reaction with $O_2^{\bullet-}$. As the kinetics are found to be quite pH sensitive, this study demonstrates the effect of the protein conformation on $O_2^{\bullet-}$ reactivity. To our knowledge, this is the first report on the kinetics of a protein-$O_2^{\bullet-}$ reaction not involving the detection of change in the redox state of a prosthetic group to probe the reactivity of the superoxide anion.

Keywords: Oxidative stress, pulse radiolysis, proteins, electron transfer, conformation

Abbreviations: CTAC, Cetyl tetramethyl ammonium chloride; SDS, sodium dodecyl sulphate

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INTRODUCTION

The kinetics and biological consequences of radical damage to proteins are of particular interest because such processes are implicated in several diseases as well as in the aging mechanism.[1] Radical formation in or radical attack on proteins may lead to denaturation, fragmentation, aggregation and loss of activity. The mechanisms underlying these alterations are poorly understood although it has been established that residues such as Trp, Tyr and Cys act as targets for oxidative stress.[2] Tryptophan is of particular interest since it can be directly oxidized by UV light or indirectly oxidized via type I photodynamic reactions. In both processes, the semi-oxidized $Trp^{\bullet}$ radical and the $O_2^{\bullet -}$ radical-anion are the initial intermediates produced in a reaction chain leading to biological damage.[3] The $Trp^{\bullet}$ radical can also be produced by biochemical reactions in several enzymatic processes such as mitochondrial cytochrome C peroxidation and the DNA photolyase. This literature is summarized in Santus *et al.*[4]

Once formed in proteins, however, the $Trp^{\bullet}$ radical can be repaired by long range electron transfer from intact Tyr with the formation of the $TyrO^{\bullet}$ radical. This reaction was originally demonstrated in a large number of proteins by Butler *et al.*[5] in pulse radiolysis studies. The kinetics of Trp repair generally occur on time scales ranging from a microsecond to seconds and are dependent on factors such as protein flexibility and conformation. Previously, it was found in our laboratory that the same reaction occurs after laser flash photolysis of Trp residues in macromolecular assemblies such as the coat protein of the Fd phage.[6]

More recently, we and others have shown by pulse radiolysis kinetics in solutions containing varying O_2 concentrations that the $Trp^{\bullet}$ radical reacts with $O_2^{\bullet -}$ but not with O_2 at diffusion controlled rates.[7,8] Using various Trp derivatives including positively charged tripeptides, we further demonstrated that the rates of the $Trp^{\bullet}/O_2^{\bullet -}$ reaction are strongly dependent on the electrostatic charge of the entity (micelle, DNA) at which the reaction takes place.[4]

Here, we extend the study of peptide radical reactivity to lysozyme as a model protein, using the same techniques. In this investigation we attempt to elucidate the kinetics of reaction between *external* species, such as $O_2^{\bullet -}$ or ascorbate, and the $Trp^{\bullet}$ radical in the more complex environment presented by the protein. The study necessarily examines the extent to which such external species may alter the internal repair of the $Trp^{\bullet}$ radical by electron transfer from Tyr residues. Most interesting is the interplay of these various processes as functions of the protein configuration, which may be seen to alter the accessibility of Trp residue to such external species as well as to change the Trp–Tyr interaction upon which electron transfer depends. Additionally, the kinetics of $Trp^{\bullet}$ recombination may also be seen to be dependent on protein configuration. The results obtained here with $O_2^{\bullet -}$ and ascorbate show that, with such species, the propagation of oxidative stress induced by oxidation of Trp residues may be impeded, thereby diminishing subsequent damage to remote residues, such as Tyr, crucial in the structure and function of the protein.

EXPERIMENTAL

Chemicals

Racemic tryptophan, and lysozyme (3× crystallized) were purchased from Sigma. Cetyl tetramethyl ammonium chloride (CTAC) of the purest grade was supplied by Fluka. Phosphate buffers of various pH and ionic strength were prepared in pure water obtained with a milli/Q system provided by Millipore. Unless otherwise specified the protein concentration used in these experiments was 70 μM. Solutions were saturated with pure N_2O or O_2 prior to and during pulse radiolysis.

Pulse Radiolysis

Pulse radiolysis measurements were carried out with the Notre Dame Radiation Laboratory 8-MeV

linear accelerator, which provides 5-ns pulses of up to 30 Gy. The doses used here were approximately 20 Gy. The principles of the detection system have been previously described[9,10]. A Corning O-51 optical filter, removing all wavelengths shorter than 350 nm, was placed in the analyzing light beam to avoid direct Trp photochemistry.

Radical concentrations calculated from transient absorption data are referenced to $(SCN)_2^{\bullet-}$ dosimetry and recombination kinetics for calibration. The extinction coefficient for $(SCN)_2^{\bullet-}$ is taken to be $7580 \pm 60\,M^{-1}\,cm^{-1}$ at 472 nm, and the G value for $^{\bullet}OH$ in N_2O-saturated solution has been measured as 6.13 ± 0.09.[11] The G value is the number of radicals generated per 100 eV of absorbed energy, and such numbers may be recast as radical concentrations per unit radiation (e.g., a G value of 6.1 corresponds to a concentration of 6 μM/10 Gy). Values used here for $Trp^{\bullet}$ have been determined by comparison of its measured transient absorbance at 520 nm to the peak absorbance of $(SCN)_2^{\bullet-}$ determined under the same experimental conditions of cell geometry and dose. All the pulsed solutions contained 0.1 M Br^- unless otherwise stated. Where necessary, differences in radical G value, arising from the dependence of spur scavenging on Br^- concentration, are taken into account. Additionally, production of superoxide radical by oxygen scavenging of $H^{\bullet}$ is used in calculating the G value for $O_2^{\bullet-}$. Numerical integrations carried out in analyses of rate data were conducted using the *Scientist* software from Micromath Scientific Software.

RESULTS

Oxygen and Ascorbate Effects on Transient Absorbances

Figure 1A presents the decay of the $Trp^{\bullet}$ radical observed at 520 nm in N_2O- and in O_2-saturated aqueous solutions containing 70 μM lysozyme at pH 6.2. The $Trp^{\bullet}$ radical in Trp alone or Trp-containing peptides, has been shown to react

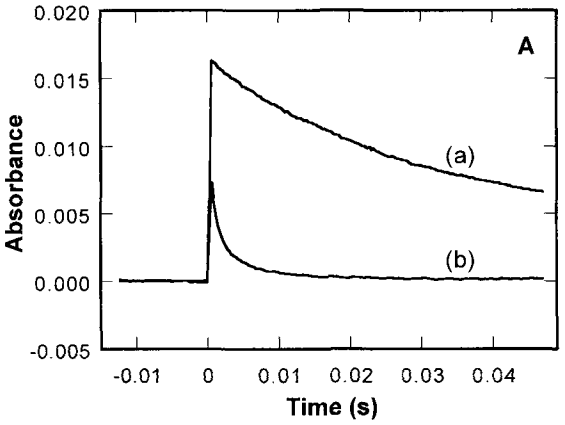

FIGURE 1A Decay of $Trp^{\bullet}$ radical at 520 nm after radiolysis of a 70 μM lysozyme solution in pH 6.2 phosphate buffer (10 mM) containing 0.1 M KBr. Radiolytic dose was ~20 Gy. (a) N_2O-saturated solution and (b) O_2-saturated solution.

only with $O_2^{\bullet-}$ and not with O_2 on this time scale,[7,8] and Figure 1A clearly demonstrates that $O_2^{\bullet-}$ reacts with semi-oxidized Trp residues in lysozyme. In N_2O-saturated solutions containing 0.1 M Br^-, it can be assumed that $Br_2^{\bullet-}$ is the only radical formed that can oxidize the Trp residues.[12] Trp itself reacts with all the $Br_2^{\bullet-}$ radical anions produced giving a yield of $G = 6.1$ (data not shown). Using these data, the G value for the formation of $Trp^{\bullet}$ radicals in N_2O-saturated solutions of lysozyme over the pH range used can be estimated to be 3.8 ± 0.2 by comparing transient absorption with that obtained in 0.5 mM Trp under the same experimental conditions,[13] assuming that the molar extinction coefficient to be the same in the protein as in free amino acid.[4]

On the other hand, at pH 6.3, in O_2-saturated solutions, the $Br_2^{\bullet-}$ radiolytic yield is reduced to $G(^{\bullet}OH) = 2.8$ while $O_2^{\bullet-}$, formed by both reaction of O_2 with the hydrated electron ($G = 2.7$) and with $H^{\bullet}$ ($G = 0.6$), has a total $G(O_2^{\bullet-}) = 3.3$. The smaller transient absorbance observed for the $Trp^{\bullet}$(Lyz) radical at 520 nm in Figure 1A as compared to that obtained in N_2O-saturated solutions is consistent with the reduced $G(Br_2^{\bullet-})$.

Figure 1B shows the time evolution of the corresponding transient absorbances at 410 nm

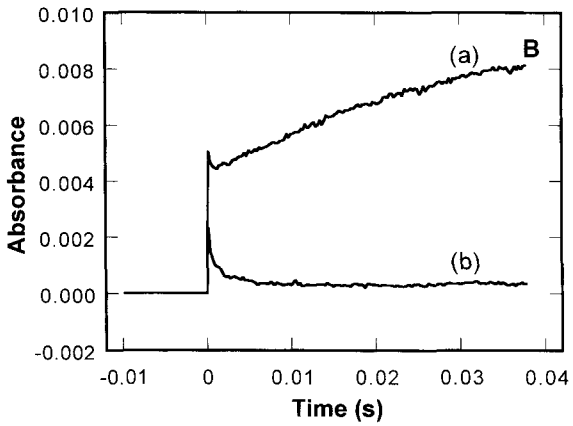

FIGURE 1B Time dependence of the transient absorbances measured at 410 nm under the same conditions as above. (a) N_2O-saturated solution and (b) O_2-saturated solution.

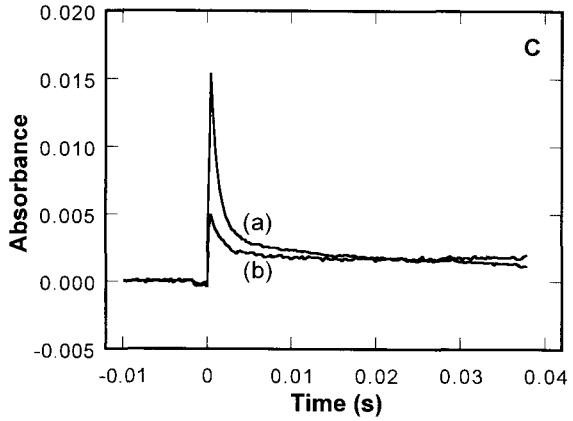

FIGURE 1C (a) decay of the Trp• radical at 520 nm and (b) decay of the TyrO• radical at 410 nm in the presence of 20 μM ascorbate under the same conditions as in (Figure 1A). Ascorbate was prepared as a 1 mM stock solution in water and was added to the solution just before saturation with N_2O.

observed under O_2 and N_2O saturation at pH 6.3. The fast initial growth can be attributed to the formation of both the Trp•(Lyz) and TyrO• radicals since the ratio of the initial absorbances observed at 520 nm, (Figure 1A), and 410 nm (Figure 1B) is 2.9 whereas the ratio of molar extinction coefficients of the Trp•(Lyz) radical at the same wavelengths ($\varepsilon_{520} = 1750$ and $\varepsilon_{410} = 300\,M^{-1}\,cm^{-1}$) is 5.8. Thus, at pH 6.3, half of the initial transient absorbance observed at 410 nm can be attributed to the TyrO• radical presumably formed directly by reaction of Br_2^- with Tyr residues. Assuming that the molar extinction coefficient for the TyrO• radical is $2700\,M^{-1}\,cm^{-1}$ at 410 nm [14], the G value for TyrO• formation can be estimated to be 0.4 and 0.6 at pH 6.2 and pH 9 respectively. The much slower first order transient growth observed at 410 nm in N_2O-saturated solution ($k \sim 50\,s^{-1}$) is consistent with Trp• radical "repair" by intramolecular electron transfer from Tyr residues previously reported under other experimental conditions.[5,14] At pH 5.8–6.2, transfer yields of ~20% can be estimated from the difference in the initial and final transient absorbances at 410 nm after completion of the intramolecular transfer. This yield is about half that reported by Weinstein *et al.*[14] under different experimental conditions. This slow growth may be seen to be quenched in O_2-saturated solutions.

A comparison of the extent of Trp• radical "repair" by $O_2^{\bullet-}$ and ascorbate, two negatively charged species of markedly differing sizes, is interesting since it could provide some information on the reactivity of semi-oxidized Trp in lysozyme at different conformations. The ascorbate anion reacts at a high rate ($1 \times 10^8\,M^{-1}\,s^{-1}$) with the Trp• radical alone.[15] At pH 9 the lysozyme structure is believed to be much more relaxed as compared to that at pH values around 6,[14] which may contribute to the somewhat higher initial yield of TyrO• formation at the higher pH. With this in mind, it may be seen in Figure 1C that addition of 20 mM ascorbate to a N_2O-saturated solution of 140 μM lysozyme (Lyz) at pH 6.2, inhibits the electron transfer (410 nm) and "repairs" the Trp• radical. It may also be seen that Trp•(Lyz) undergoes a two component decay with about 15% which only disappears slowly after 5 ms. The rate constant for the fast component was found to be $8 \times 10^7\,M^{-1}\,s^{-1}$ at pH 6.2 and $4 \times 10^7\,M^{-1}\,s^{-1}$ at pH 9.0.

Kinetic Analysis of Transient Decays

The time course of the various transients depicted in Figures 1A and B can be described by the

following reactions:

$$\text{Lyz} + \text{Br}_2^{\bullet -} \rightarrow \text{Trp}^{\bullet}(\text{Lyz}) \quad (\text{rate: } k_1\,\text{M}^{-1}\,\text{s}^{-1}) \quad (1)$$

where $\text{Trp}^{\bullet}$(Lyz) represents the semioxidized Trp residues of lysozyme. Because Lyz contains 6 Trp residues, $k_1$must be assumed to be an *average* apparent rate constant since the semi-oxidized Trp residues cannot be distinguished from one another.[5,14] In a N_2o-saturated solution, the $\text{Trp}^{\bullet}$(Lyz) radical undergoes a fast second order decay that can be characterized by an apparent overall bimolecular reaction rate constant: k_2(app).

$$\text{Trp}^{\bullet}(\text{Lyz}) + \text{Trp}^{\bullet}(\text{Lyz}) \rightarrow \text{products} \quad (\text{rate: } k_2(\text{app})\,\text{M}^{-1}\,\text{s}^{-1}) \quad (2)$$

Because of the dose dependence of the second order decay (data not shown) it may be assumed that this second order decay results primarily from an *intermolecular* recombination reaction of $\text{Trp}^{\bullet}$(Lyz) radicals. On the other hand, on a corresponding time scale, an *intramolecular* "repair" reaction occurs by electron transfer from Tyr(Lyz) to $\text{Trp}^{\bullet}$(Lyz):[5,14]

$$\text{Trp}^{\bullet}(\text{Lyz}) + \text{Tyr}(\text{Lyz}) \rightarrow \text{TyrO}^{\bullet}(\text{Lyz}) + \text{Trp}(\text{Lyz}) \quad (\text{rate: } k_3) \quad (3)$$

Analysis of $\text{Trp}^{\bullet}$(Lyz) decay at 520 nm must take into account both processes and an overall equation for decay may be written:

$$-\text{d}[\text{Trp}^{\bullet}(\text{Lyz})]/\text{dt} = k_2(\text{app})\,[\text{Trp}^{\bullet}(\text{Lyz})]^2 + k_3\,[\text{Trp}^{\bullet}(\text{Lyz})]$$

Kinetic data were analyzed according to this differential equation using *Scientist* software for numerical integration. In this equation as in all related calculations, radical concentrations, such as $[\text{Trp}^{\bullet}(\text{Lyz})]$ and $[O_2^{\bullet -}]$ were determined from their initial G values. However, good fits of the data could only be obtained by assuming a fraction of the $\text{Trp}^{\bullet}$(Lyz) to be unavailable for the recombination processes. By this reaction model, the first and second order processes are parallel and not competitive. At all pHs, this "unavailable" fraction corresponded closely to the fraction of radical undergoing first order electron transfer as measured from $\text{TyrO}^{\bullet}$ radical absorbance at 410 nm. Hence the equation above was rewritten to take this assumption into account

$$-\text{d}[\text{Trp}^{\bullet}(\text{Lyz})]/\text{dt} = k_2(\text{app})\,[\text{Trp}^{\bullet}(\text{Lyz})']^2 + k_3\,[\text{Trp}^{\bullet}(\text{Lyz})''] \quad (\text{A})$$

where: $[\text{Trp}^{\bullet}(\text{Lyz})] = [\text{Trp}^{\bullet}(\text{Lyz})'] + [\text{Trp}^{\bullet}(\text{Lyz})'']$. The rate constants obtained from this model at the various pHs used are given in Table I. It may be seen that the k_2(app) decreases significantly above pH 6.2.

In O_2-saturated solutions, it must again be assumed that we are dealing with decay kinetics representing, in addition to the processes given above, an average of individual kinetics for the reaction:

$$\text{Trp}^{\bullet}(\text{Lyz}) + O_2^{\bullet -} \rightarrow \text{products} \quad (\text{rate: } k_4(O_2^{\bullet -})\,\text{M}^{-1}\,\text{s}^{-1}) \quad (4)$$

It may be seen from Figure 1A that at 520 nm the $\text{Trp}^{\bullet}$(Lyz) decay in the presence of $O_2^{\bullet -}$ also exhibits two components of decay which differ by more than an order of magnitude. It was found that the fraction of $\text{Trp}^{\bullet}$(Lyz) involved in

TABLE I Bimolecular rate constants for the reactions $\text{Trp}^{\bullet} + \text{Trp}^{\bullet}$ and $\text{Trp}^{\bullet} + O_2^{\bullet -}$ in lysozyme

Rate constants	pH 5.8	pH 6.0	pH 6.2	pH 9.0	CTAC
$10^{-6} \times k_2$(app) ($\text{M}^{-1}\,\text{s}^{-1}$)	6.7	5.3	4.5	1.3	1.6
$10^{-7} \times k_4(O_2^{\bullet -})$ ($\text{M}^{-1}\,\text{s}^{-1}$)	18	19	9.0	6.5	8.6

Reactivities are estimated to be accurate to $\pm 10\%$. Values of k_2(app) and $k_4(O_2^{\bullet -})$ were measured in N_2O- and O_2-saturated solutions respectively. The CATC concentration used was 10 mM in bulk pH 5.8 buffer. The lysozyme concentration was 70 μM in all cases.

that second component, [Trp•(Lyz)‴] was again ~[Trp•(Lyz)″] discussed above in the model chosen to fit the N_2O data. Treating this second component in the same fashion as above, the overall rate equation in the presence of O_2 is written:

$$-d[Trp^{\bullet}(Lyz)]/dt = k_2(app)\,[Trp^{\bullet}(Lyz)']^2 + k_4(O_2^{\bullet-})\,[Trp^{\bullet}(Lyz)'][O_2^{\bullet-}] + k_5\,[Trp^{\bullet}(Lyz)'''] \quad (B)$$

While the lysozyme/O_2 system involves Trp residues in various environments within the structure of the enzyme, the O_2 data in this study could be well fitted to the model described by differential equation (B).

The results of the calculations using transient data from O_2-saturated solutions which were obtained at several pHs are included in Table I. It may be seen that the reactivity of $O_2^{\bullet-}$ with Trp•(Lyz) is markedly decreased above pH 6.2. This is the same trend in pH dependence as was observed for k_2(app). Included in the Table are data from solutions containing the positively charged detergent, CTAC, which denatures lysozyme. The data show that k_2(app) is rather higher than that found in absence of detergent while the reactivity toward $O_2^{\bullet-}$ is unchanged. It was also observed that CTAC alters the availability of both Trp and Tyr residues to $Br_2^{\bullet-}$ attack. As a result G values of 1.0 were found for initial yields of both residues. Additionally, no electron transfer could be detected; this contrasts to behavior observed in SDS micelles where a transfer of 93% was reported.[5]

An additional demonstration of $O_2^{\bullet-}$ reactivity with the Trp• radical in lysozyme is provided by an experiment in which a large excess of $O_2^{\bullet-}$ radicals is produced compared to Br_2^- by irradiating buffered solutions of 70 μM lysozyme which contain 0.1 M formate and 2 mM Br^-. As the •OH radical reacts 3 times faster with Br^-[16] than with $HCOO^-$, about 90% of •OH is scavenged by $HCOO^-$, leading to the $CO_2^{\bullet-}$ radical which in O_2-saturated solutions is converted into $O_2^{\bullet-}$.[17] Thus, immediately after the radiolytic pulse, an almost 10 fold excess of $O_2^{\bullet-}$ is produced and pseudo first order kinetics can be applied to (4). Figure 2 presents the 520 nm transient decay at pH 6.2 measured under these conditions for 0.5 mM Trp and for 70 μM lysozyme. The value of $k_4(O_2^{\bullet-})$ calculated from the first order decay is found to be $1.8 \times 10^9\,M^{-1}s^{-1}$ and $1.4 \times 10^8\,M^{-1}s^{-1}$ for Trp• and Trp•(Lyz) respectively. The value obtained with Trp•(Lyz) is in reasonable agreement with the value given in Table I, determined by second order kinetics. The value for Trp• at neutral pH has been previously reported as $2.3 \times 10^9\,M^{-1}s^{-1}$.[7]

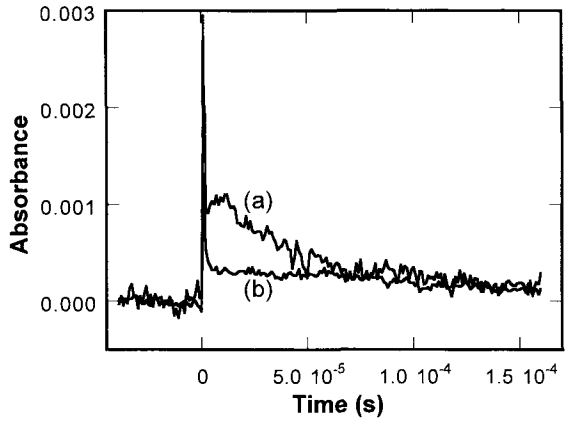

FIGURE 2 Decay of the Trp• radical at 520 nm in (a) free Trp (0.5 mM) and (b) 70 μM lysozyme in O_2-saturated solution. The solution was buffered to pH 5.8 with 10 mM phosphate and contained 2 mM KBr and 0.1 M formate. Radiolytic dose was ~30 Gy. The time scale for (b) should be multiplied by 2.5.

The decay of Trp•(Lyz) in O_2-saturated solution was also examined in the presence of superoxide dismutase (SOD) which reacts competitively with $O_2^{\bullet-}$ and adds further confirmation to the reaction mechanism proposed above. As was shown to be the case in studies of free Trp, addition of 10 μM SOD to an O_2-saturated lysozyme solution inhibits (4) and restores the intramolecular Tyr → Trp• electron transfer reaction (data not shown).

DISCUSSION

The data presented above demonstrate that semi-oxidized Trp residues can be used as probe of the reaction of $O_2^{\bullet -}$ with proteins. To our knowledge, this is the first kinetic study of $O_2^{\bullet -}$ reaction with a protein devoid of prosthetic groups such as hemes. This is significant because, in hemoproteins, the transient spectral modifications induced by $O_2^{\bullet -}$ reflect changes in the redox and/or coordination states of the heme and not necessarily changes induced at the apoprotein level.[18,19]

In previous pulse radiolysis studies of electron transfer in lysozyme,[14] very low radiolytic doses were used to obtain a total radical concentration of about 1 μM. Such conditions were chosen to minimize second order recombination reactions of the $Trp^{\bullet}$ and/or $TyrO^{\bullet}$ radicals. In this study, we choose instead to use higher doses (~30 Gy) to favor the study of *intermolecular* recombination reactions. Under these conditions, the reaction of $O_2^{\bullet -}$ with the $Trp^{\bullet}$ radical and its effect on the intramolecular $Tyr \rightarrow Trp^{\bullet}$ electron transfer reaction can be conveniently monitored, although the electron transfer yield is reduced because of the competition with $Trp^{\bullet}$(Lyz) recombination. This approach has allowed us to demonstrate that the Trp residues responsible for the intermolecular and for the intramolecular reaction paths are not identical (see below).

The pH-independent G value of 3.6 for the formation of the $Trp^{\bullet}$ radical in a N_2O-saturated solution of 70 μM lysozyme suggests that at least 3 and possibly 4 Trp residues of the 6 Trp residues in lysozyme are susceptible to the $Br_2^{\bullet -}$ attack. The much lower yield of $TyrO^{\bullet}$ radical, formed under our experimental conditions as compared to $Trp^{\bullet}$(Lyz) radical yield, is understandable since lysozyme contains only 3 Tyr residues. Additionally, the reaction rate constant of exposed Tyr with $Br_2^{\bullet -}$ can be expected to be smaller than that of exposed Trp by more than an order of magnitude at pH 6 and almost the same factor at pH 9.[12]

It is interesting to note that Trp 62, 63, 123 and 108 are exposed to solvent[20] and/or are susceptible to reaction with halide radical anions[14] with Trp 62 being the most exposed to the solvent.[19] However, it has also been shown that Trp 108 located within the active site can be oxidized by iodine[12] suggesting that it too should be accessible to small radical species. As a consequence, it may be suggested that, statistically, all four semi-oxidized Trp residues should be able to react with the negatively charged $O_2^{\bullet -}$.[4] Considering that most of the Trp residues should be accessible, the fraction of $Trp^{\bullet}$(Lyz) remaining after the completion of the fast component of the "repair" reaction with ascorbate (Figure 1C) and $O_2^{\bullet -}$ (Figure 1A) is greater than would be anticipated. This behavior may be explained by some steric hindrance for the access of ascorbate to a $Trp^{\bullet}$(Lyz) radical which has become less accessible due to Br_2^{-} oxidation. Such interpretation supports the hypothesis of Weinstein *et al.*[14] that there is a strong pH dependent reorganizational effect of radical reaction on lysozyme conformation. For example, the oxidation of the Trp residues may lead to substantial structural changes, introducing steric hindrance and/or electrostatic repulsion by negatively charged residues[4] which limit the effectiveness of electron transfer and/or the reaction with $O_2^{\bullet -}$. Such large structural changes are produced in the oxidation of the Trp 108 indole ring by iodine. This oxidation induces conformational changes large enough to inhibit lysozyme activity.[12] Based on the published three-dimensional structure of the enzyme,[20] it might be suggested that Trp 108 would be the least susceptible to the ascorbate reaction after $Br_2^{\bullet -}$ attack.

The bimolecular rate constants given in Table I indicate that at all pHs $k_4(O_2^{\bullet -})$ is more than an order of magnitude greater than k_2(app) characterizing the intermolecular $Trp^{\bullet} + Trp^{\bullet}$ radical recombination. Interestingly, the lower values for both $k_4(O_2^{\bullet -})$ and k_2(app) are found above pH 6.0. Around pH 6 the fastest rate for the

intramolecular electron transfer was also reported[14] and was attributed to a strong conformational reorganization. Because the electron transfer rate depends on both the orientation and solvation[21] of donor and acceptor, it may be thought such change in the lysozyme structure may lead to less exposed Trp residues decreasing both $k_4(O_2^{\bullet-})$ and k_2(app).

As deduced from the crystal structure of lysozyme,[19] Trp 28 and 111 are in contact with Tyr 23. At first glance, they could therefore be considered as potential candidates for the intramolecular electron transfer. However, both the reaction of $O_2^{\bullet-}$ with Trp$^{\bullet}$(Lyz) and the parallel inhibition of the intramolecular electron transfer by $O_2^{\bullet-}$ or by ascorbate strongly support that any one of the Trp residues more or less exposed to the radical attack are involved in the intramolecular electron transfer which is observed on the 50 ms time scale. As suggested in Weinstein *et al.*[14], the experimentally observed first order kinetics may result from the participation of only one Trp and one Tyr or one semi-oxidized Trp residue reacting with any one of the 3 Tyr residues. It has been reported that the TyrO$^{\bullet}$ radical also reacts with $O_2^{\bullet-}$.[22] However, we did not detect appreciable reaction of $O_2^{\bullet-}$ with TyrO$^{\bullet}$ in lysozyme.

The inhibition of the Trp$^{\bullet}$(Lyz) + Tyr reaction by $O_2^{\bullet-}$, shown in Figure 1B, illustrates an intermolecular pathway to radical repair alternative to that involving intramolecular electron transfer. In related studies on intramolecular electron transfer reactions in several proteins, it was found that the amount of Trp$^{\bullet}$ lost did not correspond to the amount of additional TyrO$^{\bullet}$ formed.[5] Furthermore, in some proteins, such as pepsin, the participation of two concurrent first order processes could not be ruled out involving, for instance, *intermolecular* electron transfer between Trp and Tyr residues on different molecules.[5] Here, with lysozyme, the independence of the transfer rate with lysozyme concentration (data not shown) seems to rule out such intermolecular first order processes. Interestingly, Weinstein *et al.* in their detailed study on the Trp + Tyr reaction in lysozyme estimated a 50% discrepancy between the first order Trp$^{\bullet}$ loss and the TyrO$^{\bullet}$ formation.[14] Thus, first order reactions other than TyrO$^{\bullet}$ formation, insensitive to O_2 probably take place in lysozyme. This may explain why, even in the presence of O_2, a parallel first order decay of the Trp$^{\bullet}$ contributes to the transient kinetics.

In conclusion, the reaction of $O_2^{\bullet-}$ with reactive radical intermediates of aminoacids in proteins may have important consequences for the turnover of proteins involved in metabolic reactions that involve both the formation of protein radicals and $O_2^{\bullet-}$. In the case of Trp residues such reactions should be taken into account in all enzymic systems presented in the introduction. It would also be interesting to study the role of O_2 in the intraprotein electron transfer between Tyr and Trp which has recently been reported in the DNA photolyase of *Anacystis nidulans* during a native biological reaction.[23] Furthermore, this reaction must also be considered in cells undergoing a photo-oxidative stress induced by the solar UV or visible light.[24] In such cells, Trp residues of proteins can be directly photo-oxidized by UV light or indirectly oxidized through light absorption by endogenous or exogenous chromophores leading to type 1 photodynamic reactions in which $O_2^{\bullet-}$ is produced in conjunction with Trp$^{\bullet}$.[25]

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