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AN EXPANDED FUNCTION FOR SUPEROXIDE DISMUTASE

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α-Hydroxyalkylperoxyl radicals were generated from the primary and secondary alcohols methanol, ethanol and 2-propanol in N_2O/O_2-saturated aqueous solutions by pulse radiolysis. These radicals *reduced* a ferric iron porphyrin complex, tetrakis-(4-N-methylpyridyl)porphine, with diffusion-controlled rate constants. The extreme sensitivity of the shift of the Soret absorption band in this reaction was used to determine, by competition kinetics, the reactivity of the peroxyl radicals with different proteins. Only native Cu,Zn-superoxide dismutase and metallothionein showed competitive behavior, with SOD exhibiting rate constants close to the dismutation rate for O_2^-. Metallothionein was slower by a factor of 30 with hydroxymethylperoxyl radicals. We propose, that SOD has unique properties of the protein surface in addition to the prosthetic copper site, having possibly evolved as a 'general-purpose radical-scavenging protein'.

KEY WORDS: Alcohol peroxyl radicals, competition kinetics, iron porphyrin complex, pulse radiolysis, rate constants.

ABBREVIATIONS:FTMP - iron(III) tetrakis-(4-N-methylpyridyl) porphine, GSH-glutathione (reduced form), MT - metallothionein, SOD Cu,Zn-superoxide dismutase.

INTRODUCTION

The ubiquity of the enzyme superoxide dismutase (SOD) and the rather low reactivity of its radical substrate, O_2^-, on first appearance contradict each other.[1-4] Should SOD solely protect the cell against detrimental effects of O_2^-, it must be due to O_2^- acting — in concert with catalytic transition metals[5-7] — as precursor of the most damaging hydroxyl radical ·OH (the 'superoxide theory of oxygen toxicity').[8-11] Evidence for specific biologically relevant target substrates of O_2^- is still scarce[12-14] and suggestions that O_2^- may participate in biological signal transduction processes are hypothetical at present.[15-17]

Considering alternative functions for SOD, reaction with other radicals is one possibility. This has been investigated earlier[18,19] by evaluating the absorption changes of the prosthetic Cu^{2+} at 680 nm. A rate constant of $8 \times 10^8\ M^{-1}\ s^{-1}$[18] has been found for the reaction of SOD with CO_2^-. For the reaction of peroxyl radicals derived from tert-butanol with SOD an upper limit of $10^8 M^{-1} s^{-1}$ was determined.[19] The low sensitivity of this absorption band and the optical transparency of most radicals, e.g. peroxyl radicals as organic analogs of $HO_2^\cdot/O_2^-$, leaves competition kinetics[20] as the logical choice to determine reaction rates. A highly sensitive reference substrate, the water-soluble ferric iron porphyrin complex, iron(III) tetrakis-(4-N-methylpy-

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ridyl)porphine (FTMP),[21,22] enabled us to limit the amount of protein substrates to sub-micromolar concentrations.

MATERIALS AND METHODS

The alcohols used in this study, methanol, ethanol and 2-propanol, were of HPLC grade purity from Baker and used as supplied. Sodium formate was purchased from Merck. FTMP was prepared from the iron-free meso-tetra(4-N-methylpyridyl)porphine tetraiodine (Strem Chemicals), according to ref.[21] and gave the theoretical molar ratio of Fe by atomic absorption spectroscopy.

Cu,Zn-SOD and lysozyme were from Boehringer Mannheim, bovine serum albumin from Fluka, carbonic anhydrase from Serva and metallothionein from Sigma. Denatured SOD was prepared from aged Sigma samples by autoclaving for 15 min at 120°C. Apo−SOD was prepared by repeated dialysis against EDTA to remove the metals, and subsequent dialysis steps to remove EDTA with NaCl and NaCl with 'Milli-Q' water.[23] The resulting protein contained 0.7% Zn and 2% Cu as compared to the native SOD and had 2% residual O_2^--dismutating activity.

Solutions were prepared with 'Milli-Q' water and pH was adjusted to 8.5–9.5 with NaOH. A gradual drop of 0.3–0.5 pH−units during the experiments did not affect transient spectra or kinetics. CO_2^- and O_2^- were produced in 0.01 M solutions of sodium formate saturated with N_2O and O_2, respectively. The alcohol peroxyl radicals were generated in solutions which were separately saturated with either N_2O or O_2 and mixed at various ratios prior to delivering the pulse.The radiolysis was performed using a Febetron 705 accelerator, generating 1.7 MeV electron pulses. A description of the peripheral set-up and data processing has been published earlier.[24]

Rate constants of radicals with FTMP were determined from kinetic evaluation of

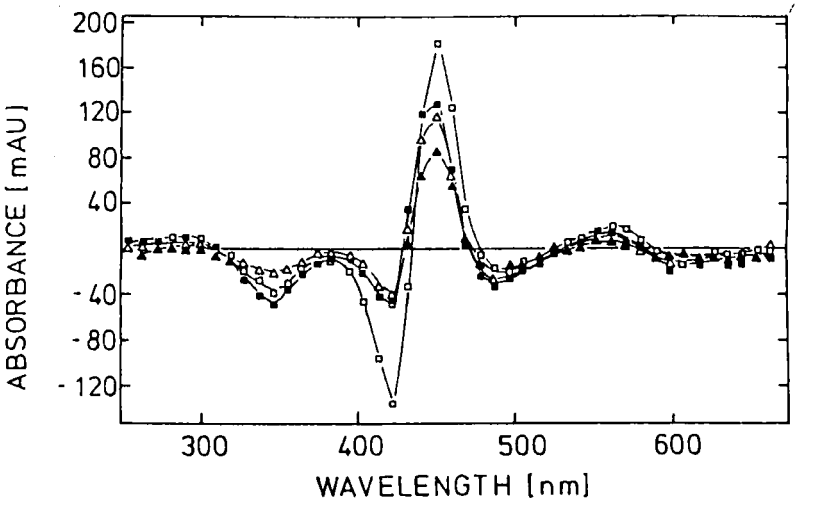

FIGURE 1. Transient spectra of reduction of Fe(III)-tetrakis-(4-N-methylpyridyl)porphine. Dose-normalized uncorrected spectra in solutions containing 3.2–3.6 μM FTMP, pH adjusted to ~ 8.5 with NaOH; average pulse doses 5.5 Gy; concentration of radicals ~ 3.2 μM. Observation time for maximal absorption change after the pulse was 0.35 ms for CO_2^- (□) and 1 ms for the other radicals: O_2^- (■), $CH_2(OH)OO^{\cdot}$ (△), $^{\cdot}CH_2OH$ (▲).

satellite absorption bands at 497 and 562 nm, allowing substrate concentrations high enough for pseudo-first order conditions at pulse doses of 2–6 Gy. For competition experiments the observation of the shift of the Soret band at 424 nm was employed. Control data in the absence of competitors were obtained by extrapolation, as only experiments between 20–80% O_2 could be evaluated for peroxyl radical reactions (see below).

RESULTS

The only reported reaction of the ferric iron porphyrin complex with radicals is its reduction to the ferrous iron form.[25,26] In the difference spectra this is reflected as absorbance increase at 452 nm and as bleaching at 424 nm (Fig. 1). The wavelength maxima and molar absorptivities of the pure Fe(III) and FE(II) porphyrin complexes are $\varepsilon_{424} = 165\,mM^{-1}cm^{-1}$ and $\varepsilon_{445} = 160\,mM^{-1}cm^{-1}$, respectively.[22] Surprisingly, as seen from the similarity of the transient spectra, alkyl as well as peroxyl radicals reduce the ferric iron to the ferrous state. Such a reaction is most likely due to a strongly positive oxidation potential of FTMP[22] and the fact that α-hydroxyalkyl-peroxyl radicals specifically can be further oxidized to carbonyl compounds (Scheme I).

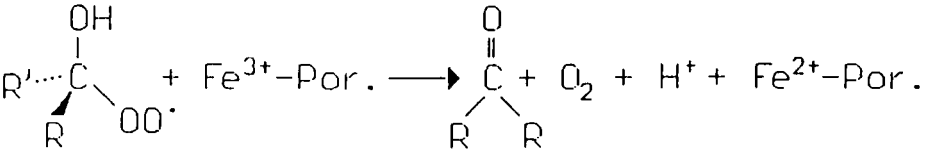

Table I lists all relevant rate constants for FTMP, some of which were determined previously.[25] As can be seen, there is a little difference between the alkyl and corresponding peroxyl radicals. To ensure that the diffusion-controlled attachment of O_2 at the initially formed alkyl radicals[27] occurs nearly quantitatively, the ratio of O_2 to N_2O should range between 1:4 and 4:1. The upper limit of O_2 is to minimize the amount of O_2^- being formed from direct attack of e_{aq}^-. For 0.01–0.1 M alcoholic solutions containing 7–20 μM FTMP and irradiated with pulse doses of ~ 2–6 Gy, we calculated an initial peroxyl radical concentration of ~ 1–3.5 μM.

TABLE I
Rate constants of alcohol-derived alkyl and peroxyl radicals with iron(III) tetrakis-(4-N-methylpyridyl)porphine (FTMP).

Substrate	Rate Constant ($\times 10^9\,M^{-1}s^{-1}$)	
	·ROH	R(OH)OO·
methanol (MeOH)	0.85+	0.90
ethanol (EtOH)	0.27	0.42
2-propanol (iPOH)	1.70	0.68

All rate constants were measured in slightly alkaline unbuffered solutions, pH adjusted to 8.5–9.5 with NaOH; ·ROH radicals were generated in N_2O-saturated solutions of 0.01 M alcohol, R(OH)OO· in mixtures of solutions, separately saturated with either N_2O or O_2. Values given are averages from multiple experiments at different concentration ranges, taken as slopes from linear $kxc = f(c)$ plots. +a value of $0.9 \times 10^9\,M^{-1}s^{-1}$ was previously determined at pH 8.1.[25]

TABLE II

Rate constants of proteins and models components with different types of radicals. Values from competition studies, using FTMP as reference substrate.

Substrate	Rate Constant ($\times 10^9 M^{-1}s^{-1}$)				
	$Me(OH)OO^{\cdot}$	$Et(OH)OO^{\cdot}$	$iP(OH)OO^{\cdot}$	O_2^-	CO_2^-
Cu,Zn-SOD	2.40	0.75	1.09	2.70*	23.7
Metallo-thionein	0.08	-	-	(5.5×10^{-4})	-
Glutathione	%	-	-	(6.7×10^{-4})	-
Cysteine	0.1	-	-	(15×10^{-9})	-
FTMP	0.90	0.42	0.68	0.67+	16.0

The values in brackets are literature values for the reaction of O_2^- with metallothionein,[28] glutathione[29] and cysteine.[30] All competition kinetics were determined at FTMP concentrations of 7.5–8.6 μM and at the peak absorption increase of the difference spectra at 452 nm. Other conditions were as in Table I.

*O_2^- reaction with SOD was also determined by the pulse radiolysis method[31] and gave $2.8 \times 10^9 M^{-1}s^{-1}$.

%An apparent rate constant of $1.7 \times 10^9 M^{-1}s^{-1}$ is considered far too high, as GSH strongly affects the spectrum of Fe(III)-FTMP and slows its reduction to Fe(II)-FTMP.

+As reported in the literature,[25] the rate constant with O_2^- is strongly dependent on ionic strength, i.e. from $0.38 \times 10^9 M^{-1}s^{-1}$ in 0.1 M formate solutions to $2 \times 10^9 M^{-1}s^{-1}$ in the t-BuOH/O_2-system (the listed value was determined in 0.01 M formate solution).

–not determined.

Denatured SOD (rate constant with O_2^- $5.3 \times 10^7 M^{-1}s^{-1}$) in the absence of EDTA accelerates dramatically the decay of the peroxyl radicals; with 0.1 mM EDTA we obtain a competition rate constant of $9 \times 10^9 M^{-1}s^{-1}$, if based on the molecular weight and concentration of native SOD. Apo-SOD (rate constant with O_2^- $1.2 \times 10^7 M^{-1}s^{-1}$) as well as the small globular proteins bovine serum albumin, carbonic anhydrase and lysozyme all affected spectrum and kinetics of FTMP to such an extent, that competition evaluation was not feasible.

Based on the rate constants of Table I, a method of competition kinetics was employed in order to investigate the reactivities of the peroxyl radicals with various proteins. Aside from holo- and apo-SOD, which were studied with the peroxyl radicals listed in Table I and with CO_2^- and O_2^-, the other studies were limited to hydroxymethylperoxyl radicals. Table II lists the results together with pertinent literature data.[28-30] Most prominent is the fact, that only holo-SOD and metallothionein showed competitive behavior with FTMP. For GSH, apo- and denatured SOD and the other globular proteins lysozyme, carbonic anhydrase and bovine serum albumin, competition kinetics could not be evaluated. In these cases concentration-dependent changes of the spectrum of FTMP and/or its reduction kinetics occurred (data now shown).

DISCUSSION

Using the shift of the Soret band of the ferric iron porphyrin as an extremely sensitive indicator of univalent reduction, we obtained surprisingly high rate constants for the scavenging of alcohol peroxyl radicals by native Cu,Zn-SOD, close to the catalytic dismutation rate. The first question obviously is, how these data relate to the function of the enzyme.

The fact that we obtain nearly identical rate constants with O_2^-, either by direct pulse-radiolytic assay[31] or by competition with FTMP, first of all demonstrates the

validity of the latter method. It also shows that the reaction with O_2^- exclusively occurs at the copper site, yet with the distinction that it is a catalytic dismutation reaction whereas competition results only reflect undefined scavenging of radicals.

In contrast to the results with O_2^-, the rate constant with CO_2^- was determined by Cabelli and Bielski[18] to be $8 \times 10^8 M^{-1}s^{-1}$ from changes of the 680 nm absorption of the prosthetic Cu^{2+}, whereas we found it to be 30 times higher in the competition experiments. If we consider the higher rate constant to represent the sum of all reactions at the enzyme surface, then the slower process is the intramolecular electron transfer to the prosthetic copper. In this case the reaction should be apparent as a first-order reaction independent on neither $[CO_2^-]_0$ nor [SOD].[32] However, as the slower reaction has been reported to be a second-order process with almost stoichiometric reduction of both Cu^{+2} atoms by the CO_2^- radicals, the discrepancy between the two rate constants thus far remains unresolved.

Metallothionein, the only other protein which competitively scavenged hydroxymethylperoxyl radicals, has originally been proposed as radical scavenging protein by Thornalley and Vašak.[28] While the overall rate constant of this protein with $^{\cdot}OH$ of $10^{12}M^{-1}s^{-1}$ is not surprising in view of the high reactivity of this radical, the rate constants with O_2^- ($5 \times 10^5 M^{-1}s^{-1}$ [28]) and $CH_2(OH)OO^{\cdot}$ ($8 \times 10^7 M^{-1}s^{-1}$, this work) are rather low as compared to SOD. Assuming that, except for O_2^-, other radicals such as CO_2^- or $R(OH)OO^{\cdot}$ react on the protein surface of SOD, the small size of metallothionein (6600 Da vs. 32600 Da for Cu,Zn-SOD) at least partially explains its lower reactivity. Furthermore, the total number of thiol groups in metallothionein, as suggested by Thornalley and Vašak,[28] may not be the controlling factor of its reactivity with radicals, since it cannot be assumed that the various thiol groups are equally accessible.

Cysteine as the predominant amino acid in metallothionein, in free form barely reacts with O_2^- ($k \leqslant 15 M^{-1}s^{-1}$ [30]) and only slightly faster than the protein with $CH_2(OH)OO^{\cdot}$ ($1 \times 10^8 M^{-1}s^{-1}$). Glutathione reacts only marginally faster with O_2^- than metallothionein ($6.7 \times 10^5 M^{-1}s^{-1}$ [29]), which contains a similar ratio of thiol groups.[33] Unfortunately, the reactivity of GSH with $CH_2(OH)OO^{\cdot}$ could not be determined by the FTMP competition assay (see Table II), but is certainly much lower than that of SOD. For each of the examined substrates, the difference in the rate constants is much smaller for $CH_2(OH)OO^{\cdot}$ radicals than for O_2^-. As higher reactivity is equivalent to lower selectivity, this again proves that *organic* oxygen radicals, i.e. alkoxyl and particularly peroxyl radicals, are indeed highly reactive and relevant in biological systems.[34-36]

The applicability of FTMP as competitor seems to be limited because of its tendency to interact with proteins, resulting in spectral and kinetic changes. In the case of human serum albumin, such interaction has been demonstrated by fluorescence spectroscopy.[37] However, these limitations should not discredit the results with SOD and metallothionein. Rather, it is interesting to note that the mere removal of the prosthetic metals in Cu,Zn-SOD evidently modifies the surface properties of the apo-protein in such a way that competition studies with FTMP are no longer feasible. In the case of denatured SOD, liberation of low molecular weight Cu complexes, deduced from the effect of EDTA in these experiments, is probably the cause of the considerable spectral and kinetic alterations (data not shown).

Both the competition experiments with FTMP and previous kinetic modelling studies of the attack of hydroxyalkyl radical at cytochrome *c*[32] demonstrated that radical scavenging properties reside on the protein surfaces. Taking the alcohol-

derived peroxyl radicals as models for fatty acid-derived peroxyl radicals occurring during lipid peroxidation, the probability of SOD to react with the latter species with similar rate constants, e.g. during ischemia/reperfusion episodes, might be taken into consideration. Thus, the extremely high rate constants with different types of radicals, in conjunction with the 'non-competitive' behavior of apo-SOD, are suggestive of a much wider role for the native enzyme: it is intriguing to consider it an evolutionary derived 'general purpose radical-scavenging protein' and certainly worthwhile to find further corroborative evidence for this proposal.

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