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Kinetics of the interaction of sulfate and hydrogen phosphate radicals with small peptides of glycine, alanine, tyrosine and tryptophan

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The kinetics and mechanism of the oxidation of Glycine (Gly), Alanine (Ala), Tyrosine (Tyr), Tryptophan (Trp) and some di-(Gly-Gly, Ala-Ala, Gly-Ala, Gly-Trp, Trp-Gly, Gly-Tyr, Tyr-Gly), tri-(Gly-Gly-Gly, Ala-Gly-Gly) and tetrapeptides (Gly-Gly-Gly-Gly) mediated by sulfate ($SO_4^{\bullet -}$) and hydrogen phosphate ($HPO_4^{\bullet -}$) radicals was studied, employing the flash-photolysis technique. The substrates were found to react with sulfate radicals ($SO_4^{\bullet -}$, produced by photolysis of the $S_2O_8^{2-}$) faster than with hydrogen phosphate radicals ($HPO_4^{\bullet -}$, generated by photolysis of $P_2O_8^{4-}$ at pH = 7.1). The reactions of the zwitterions of the aliphatic amino acids and peptides with $SO_4^{\bullet -}$ radicals take place by electron transfer from the carboxylate moiety to the inorganic radical, whereas those of the $HPO_4^{\bullet -}$ proceed by H-abstraction from the α carbon atom. The phenoxyl radical of Tyr-Gly and Gly-Tyr are formed as intermediate species of the oxidation of these peptides by the inorganic radicals. The radical cations of Gly-Trp and Trp-Gly (at pH = 4.2) and their corresponding deprotonated forms (at pH = 7) were detected as intermediates species of the oxidation of these peptides with $SO_4^{\bullet -}$ and $HPO_4^{\bullet -}$. Reaction mechanisms which account for the observed intermediates are proposed.

Introduction

Although different theories have been proposed to explain the aging process, it is agreed that there is a correlation between aging and the accumulation of oxidatively damaged lipids, nucleic acids and proteins. The amounts of oxidatively modified proteins have been shown to increase with increasing age. Studies reveal an age-related increase in the level of protein carbonyl content, oxidized methionine, protein hydrophobicity, and cross-linked and glycated proteins.[1] Most protein damage is non-repairable, and has deleterious consequences on protein structure and function. The major fate of oxidised proteins is catabolism by proteosomal and lysosomal pathways, but some materials appear to be poorly degraded and accumulate within cells. The accumulation of such damaged material may contribute to a range of human pathologies. The oxidation of proteins by free radicals is also implicated in several human diseases, such as Alzheimer's disease,[2] atherosclerosis,[3] and diabetes.[4] Radical-mediated damage to proteins may be initiated by electron transfer, metal-ion-dependent reactions and autoxidation of lipids and sugars. Electron transfer from an amino acid is often coupled to deprotonation. Because proton-coupled electron transfer controls electron and proton flow in the proteins, and is important for catalytic substrate reactions, these mechanisms are related to many subjects of biochemical interest and are intensely debated.[5]

Protein oxidation can occur at both the protein backbone and on the amino acid side chains, with the ratio of attack dependent on the nature of the oxidants.[6] Therefore, the nature of the radicals formed on peptides and proteins depends on the reactivity of the attacking radical and on its electrophilicity ($HO^{\bullet}$, alkoxy radicals) or nucleophilicity (phenyl, C-centered radicals). The consequent protein oxidation is O_2-dependent, and involves several propagating radicals, notably alkoxyl radicals. Its products include several categories of reactive species, and a range of stable products whose chemistry is currently being elucidated.[6]

Model studies of the photodamage in proteins consider the chemistry of the oxidation of free amino acids and of the protein residues in small peptides, which may lead to formation of different reaction products through direct irradiation, dye-sensitized oxidations or reactions with radicals in complex biological assemblies.[7–9] It has been suggested that the extent of radiation-induced biological damage may be dependent on the concentration of local oxidants in the vicinity of the initial lesion.[8]

Regarding the importance of interactions of inorganic ions with molecules of biological relevance, a wide variety of reactions involving inorganic radical ions participate in different metabolic routes in the living systems. In particular, phosphate-centered radicals were identified by electron spin resonance of DNA samples irradiated by heavy ion beams.[10]

The most simple phosphate radicals are the inorganic phosphate radicals which exist in three acid–base forms (reactions (1)) with pK_a values as follows:[11]

$$H_2PO_4^{\bullet} \underset{pK_a=5.7}{\rightleftharpoons} HPO_4^{\bullet -} \underset{pK_a=8.9}{\rightleftharpoons} PO_4^{\bullet 2-} \quad (1)$$

These radicals can react with organic and inorganic substrates as do $SO_4^{\bullet -}$ or $HO^{\bullet}$ radicals, *i.e.*, they may abstract hydrogen, add to unsaturated compounds, or oxidize by electron transfer.[12] The general tendency in reactivity observed is $HO^{\bullet} > SO_4^{\bullet -} \geq H_2PO_4^{\bullet} > HPO_4^{\bullet -} > PO_4^{\bullet 2-}$. The latter trend is not, necessarily, that of their one electron oxidizing capabilities, as is the case of the $HO^{\bullet}$ radical (2.3 V *vs*. NHE in neutral solutions), which is a weaker oxidant than $SO_4^{\bullet -}$ radical (2.5–3.1 V *vs*. NHE),[13] but much more reactive in addition and hydrogen abstraction reactions.

We here investigate the reactions of $SO_4^{\bullet -}$ and $HPO_4^{\bullet -}$ with amino acids and peptides. Since these inorganic radicals are one-electron oxidants and are also able to abstract H atom from the amino acids and peptides, they are good candidates

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to generate *in vitro* several of the organic radicals involved in the biological processes mentioned above. A comparison of the kinetic behavior of the free amino acids and their peptide structure should bring some light to the role played by the peptide bonds in the radical sensitized protein oxidation.

Phosphate and sulfate radicals were generated by photolysis ($\lambda_{exc} < 300$ nm) of $H_2P_2O_8^{4-}$ and of $S_2O_8^{2-}$, reactions (2) and (5), respectively.[14]

$$H_2P_2O_8^{2-} + h\nu \rightarrow 2\,HPO_4^{\bullet -} \quad (2)$$

$$S_2O_8^{2-} + h\nu \rightarrow 2\,SO_4^{\bullet -} \quad (3)$$

Experimental

Potassium peroxodiphosphate was obtained from the electrolysis of alkaline solutions of KH_2PO_4 in the presence of KF and K_2CrO_4, as described in the literature.[15] $Na_2S_2O_8$, KH_2PO_4, K_2HPO_4 and NaOH (99%) were all from Merck. The peptides were purchased from Sigma. All commercial chemicals were used as received. Distilled water (>18 Ω cm^{-1}, <20 ppb of organic carbon) was obtained from a Millipore system. All the experiments were carried out at (25 ± 1) °C.

The pH of the samples was measured with a Metrohm-Herisau pH meter model E512.

Flash-photolysis experiments were carried out with a conventional apparatus[16] (Xenon Co. model 720C) with modified optics and electronics.[17] Two collinear quartz Xenon high-intensity pulsed flash tubes, Xenon Corp. P/N 890–1128 (FWHM ≤ 20 μs), with a continuous spectral distribution ranging from 200 to 600 nm and maximum around 450 nm were used. The analysis source was a high pressure mercury lamp (Osram HBO-100 W). The optical pathlength of the 1 cm internal diameter quartz sample cell was 20 cm. For the experiments shown in the inset of Fig. 2 a cell of 10 cm pathlength was used. The monochromator collecting the analysis beam (Bausch & Lomb, high intensity) was directed coupled to a photomultiplier (RCA 1P28), whose output was fed into a digital oscilloscope (Leader LBO-5825). Digital data were stored in a personal computer. The temperature was measured inside the reactor cell with a calibrated Digital Celsius Pt-100 Ω thermometer.

Generation of the radicals

Photolysis of $S_2O_8^{2-}$ ($\lambda_{exc} < 300$ nm) is a clean source of sulfate radical ions with high pH-independent quantum yields.[14a,b] This method was used here to generate sulfate radical ions in the conventional flash-photolysis experiments.

Phosphate radicals, generated by photolysis of peroxodiphosphate aqueous solutions, present two acid–base equilibria[13] (*vide supra*). In order to study the reactions of $HPO_4^{\bullet -}$, the pH of the samples was adjusted with mixtures of KH_2PO_4/K_2HPO_4 to 7.1 ± 0.1.[15] The ionic strength of the solutions was within the range 0.01–0.02 M.

In order to avoid thermal reactions of the peptides with the oxidants, the solutions containing $S_2O_8^{2-}$ or $P_2O_8^{4-}$ and the substrates were prepared a few minutes before their irradiation.[18] Accumulation of reaction products was precluded by doing single-shot experiments in both cases.

For the determination of the bimolecular rate constants of the reactions of the inorganic radicals with the substrates, S, lower substrate concentrations than those used for the characterization of the organic radicals were employed. Photolysis experiments performed with solutions containing the peptides in the absence of $S_2O_8^{2-}$ or $P_2O_8^{4-}$ did not yield detectable formation of transients, thus indicating that the organic radicals were formed by reaction of $SO_4^{\bullet -}$ or $HPO_4^{\bullet -}$ with the peptides.

Results

Reactions of $SO_4^{\bullet -}$ radicals with the substrates

Flash-photolysis of pH = 4.2 $S_2O_8^{2-}$ solutions showed the formation of transients absorbing in the range 300–600 nm, whose spectra were in excellent agreement with those reported in the literature[19] for $SO_4^{\bullet -}$.

The kinetic analysis of the transient traces over more than three lifetimes showed that the decay is fitted to simultaneous first- and second-order processes, the weight factor of each process strongly depending on the irradiation light intensity and peroxodisulfate concentration. Under our experimental conditions, the sulfate radical ion was observed to decay mainly by a first order process in fractions of milliseconds, as expected from the reaction between sulfate radical ions and $S_2O_8^{2-}$ ions.[14a]

Photolysis of $S_2O_8^{2-}$ in the presence of low concentrations (<10^{-4} M for the aromatic, and <10^{-3} M for the non-aromatic peptides) of the substrates showed absorption traces at $\lambda >$ 400 nm, whose spectra immediately after the flash of light agreed with those of the $SO_4^{\bullet -}$ radicals.

The traces were fitted to eqn (1).

$$A(\lambda) = a(\lambda)\exp(-bxt) + c(\lambda) \quad (1)$$

The constant b is independent of λ and linearly increases with substrate concentration, [S], as expected from reaction (4) (see Fig. 1). The small remnant absorbance [$c(\lambda)$ in eqn (1)] is associated with long-living species, mainly the organic radicals formed by reaction (4).

$$SO_4^{\bullet -} + S \rightarrow \text{Organic radicals} \quad (4)$$

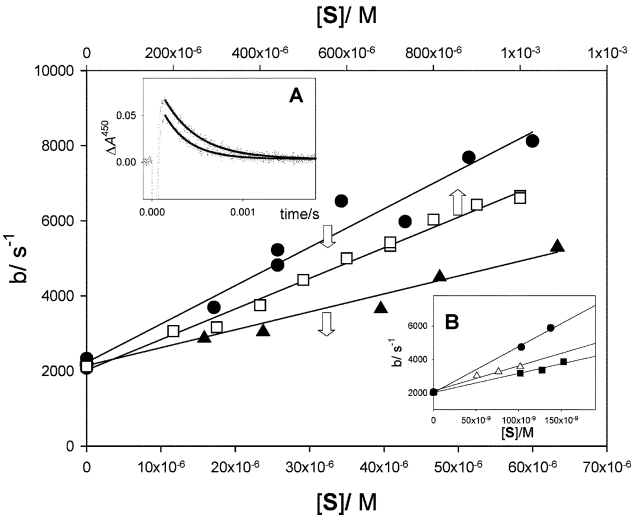


Fig. 1 Apparent rate constant b obtained for $SO_4^{\bullet -}$ radicals *vs.* [S] for Ala (□), Ala-Ala (▲) and Gly-Ala (●). Inset A: Absorbance change obtained at 450 nm for solutions containing 4.2×10^{-4} M (upper trace) and 6.5×10^{-4} M (lower trace) Gly-Ala. Inset B: *Idem* to the main Figure for Tyr-Gly (●), Trp-Gly (Δ) and Gly-Tyr (■).

The pK_as of the peptides[20] for the dissociation of the carboxyl and protonated amine groups are within 3.0–3.3 and 7.9–8.5, respectively. Thus, in the pH range from 4.0 to 7.0 the reactive species (denoted by S in reaction (4)) are the zwitterions.

The slopes of plots similar to those shown in Fig. 1 yield the bimolecular rate constants k_4 for the reactions of $SO_4^{\bullet -}$ radicals with the substrates S shown in Table 1.

In order to characterize the organic radicals formed after reaction (4) with the aromatic peptides, air-saturated 3.4×10^{-3} M $S_2O_8^{2-}$ solutions with 6×10^{-5} M Gly-Trp, Trp-Gly, Gly-Tyr or Tyr-Gly were irradiated. Under these conditions, the $SO_4^{\bullet -}$ lifetime is <2 μs.

In order to evaluate the pH dependence of the nature of the transients formed by reaction (4) with the substrate Gly-Trp,

Table 1 Values of k_4 and k_5 for several substrates

S	$k_4/M^{-1}\ s^{-1}$	$k_5/M^{-1}\ s^{-1}$
Ala	$(4.9 \pm 0.1) \times 10^6$ [a]	$(9.4 \pm 0.6) \times 10^5$
Ala-Ala	$(4.8 \pm 0.4) \times 10^7$	$(2.1 \pm 0.2) \times 10^6$
Gly	$(3.7 \pm 0.1) \times 10^6$ [a]	$(5.2 \pm 0.6) \times 10^5$
Gly-Gly	$(8.3 \pm 1) \times 10^7$	[b]
Gly-Ala	$(9.8 \pm 0.8) \times 10^7$	$(5.0 \pm 0.2) \times 10^6$
Gly-Gly-Gly	$(1.2 \pm 0.2) \times 10^8$	$(1.6 \pm 0.1) \times 10^7$
Ala-Gly-Gly	$(8.4 \pm 0.8) \times 10^7$	$(1.3 \pm 0.1) \times 10^7$
Gly-Gly-Gly-Gly	$(1.3 \pm 0.2) \times 10^8$	$(1.3 \pm 0.1) \times 10^7$
Trp	2.0×10^9 [c]	$(2.7 \pm 0.2) \times 10^8$
Gly-Trp	$(1.1 \pm 0.1) \times 10^{10}$	$(5 \pm 1) \times 10^9$
Trp-Gly	$(1.5 \pm 0.2) \times 10^{10}$	$(2.6 \pm 0.5) \times 10^9$
Tyr	5.8×10^8 [d]	4×10^8 [d]
Gly-Tyr	$(1.2 \pm 0.4) \times 10^{10}$	$(2.0 \pm 0.9) \times 10^9$
Tyr-Gly	$(2.7 \pm 0.2) \times 10^{10}$	$(2.1 \pm 0.1) \times 10^9$

[a] The values of k_4 for Ala and Gly reported in ref. 13 are 1.0×10^7 and 9.0×10^6 $M^{-1}\ s^{-1}$, respectively. [b] Not measured. [c] From ref. 31. [d] From ref. 18.

experiments with 3.4×10^{-3} M $S_2O_8^{2-}$ and 6.2×10^{-5} M Gly-Trp at various pH in the range from 4.0 to 7.0 were performed at the observation wavelength of 630 nm. The results are shown in the inset of Fig. 2.

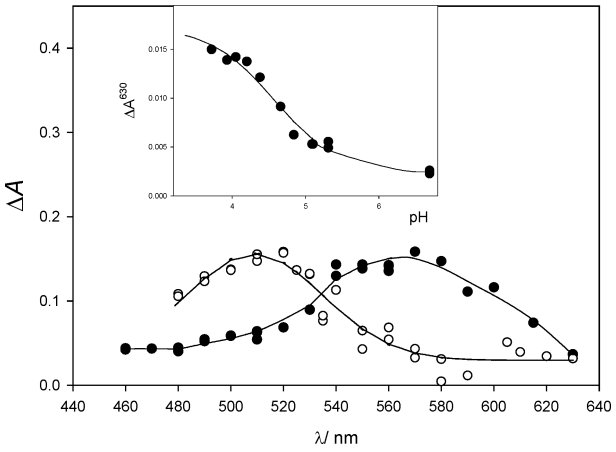


Fig. 2 Normalized absorption spectra of the transient species obtained from irradiation of 3.4×10^{-3} M $S_2O_8^{2-}$ solutions containing 6×10^{-5} M Gly-Trp taken at 200 μs after the flash of light at pH 4.0 (●); and at pH = 6.9 (○). Inset: Plot of the absorbance at 630 nm *vs.* pH obtained for 3.4×10^{-3} M $S_2O_8^{2-}$ solutions containing 6.2×10^{-5} M Gly-Trp.

Fig. 2 also shows the absorption spectrum of the transient obtained by reaction of $SO_4^{\bullet-}$ with Gly-Trp at pH = 4.2 and 6.9 taken 210 and 200 μs after the flash, respectively. The transients observed decay by a second-order kinetics with absorption maxima at 570 nm ($2k_{BR}/\varepsilon^{570} = 2.2 \times 10^5$ cm s^{-1}) and 520 nm ($2k_{BR}/\varepsilon^{520} = 2.8 \times 10^5$ cm s^{-1}), respectively.

Experiments with Trp-Gly at pH = 4.2 and 6.9 lead to formation of transients whose absorption spectra taken 155 μs after the flash of light show maxima at around 535 and 520 nm, respectively (Fig. 3). The traces at pH 4.2 and 6.9 decay with second-order kinetics and recombination rate constants of $2k_{BR}/\varepsilon^{530} = (2.9 \pm 0.3) \times 10^5$ cm s^{-1} and $2k_{BR}/\varepsilon^{520} = (3.0 \pm 0.3) \times 10^5$ cm s^{-1}, respectively.

The absorption spectrum of the transient obtained from the reaction of sulfate radicals with Gly-Tyr at pH = 4.2 taken 400 μs after the flash of light (Fig. 4) shows a peak at 400 nm and a second-order decay kinetics with $2k_{BR}/\varepsilon^{400} = (1.5 \pm 0.9) \times 10^5$ cm s^{-1} for the recombination reaction.

Reactions of $HPO_4^{\bullet-}$ radicals with the substrates

Flash-photolysis of $P_2O_8^{4-}$ solutions at pH = 7.1 showed the formation of transients absorbing in the range 300–600 nm,

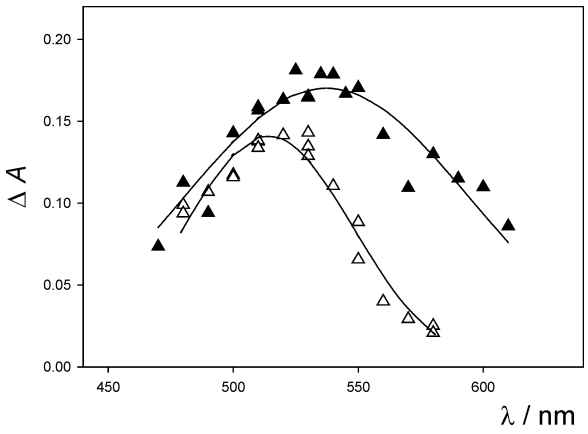


Fig. 3 Transient absorption spectra obtained from irradiation of 3.4×10^{-3} M $S_2O_8^{2-}$ solutions containing 6×10^{-5} M Trp-Gly taken at 155 μs after the flash of light at pH 4.0 (▲); and at pH = 6.9 (△).

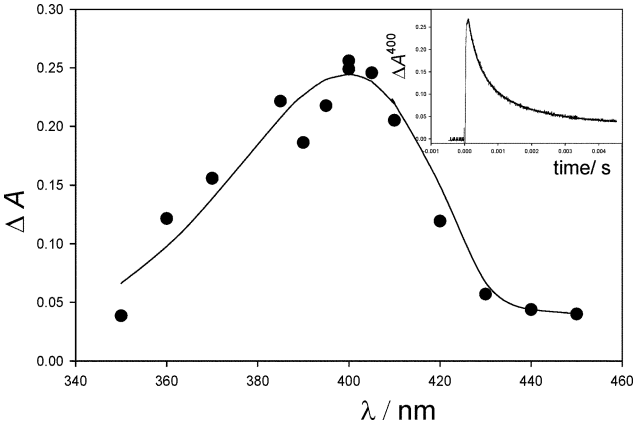


Fig. 4 Absorption spectra of the transient species obtained from irradiation of 3.4×10^{-3} M $S_2O_8^{2-}$ solutions containing 6×10^{-5} M Gly-Tyr taken at 400 μs after the flash of light at pH 4.2. Inset: Transient absorption obtained at 400 nm for the solution of the main figure.

whose spectra were in agreement with that reported in the literature for $HPO_4^{\bullet-}$.[19] The decay of the traces followed a second-order law with $2k_{BR}/\varepsilon^{510} = 5.9 \times 10^5$ cm s^{-1}, in line with the reported kinetics.[21]

Photolysis of 1×10^{-3} M $P_2O_8^{4-}$ solutions in the presence of low substrate concentrations ($<10^{-6}$ M for the aromatic, and $<10^{-3}$ M for the non-aromatic substrates) showed absorption traces at $\lambda > 400$ nm, whose spectra immediately after the flash of light were also coincident with that of the hydrogen phosphate radicals. The first order decay of the radicals in the presence of S were fitted to eqn (1). The linear plots of the apparent rate constant *b vs.* [S] are shown in Fig. 5. The rate constants k_5 for the reactions of $HPO_4^{\bullet-}$ radicals with the zwitterions of the peptides or amino acids, obtained from the slopes of plots as those shown in Fig. 5, are listed in Table 1.

$$HPO_4^{\bullet-} + S \rightarrow \text{Organic radicals} \qquad (5)$$

The absorption spectra of the organic radicals formed after reaction (5) were obtained for Gly-Trp and Tyr-Gly. To this purpose, air-saturated 1×10^{-3} M $P_2O_8^{4-}$ at pH 7.1 solutions with 4.7×10^{-5} M Gly-Trp or Tyr-Gly were irradiated. Under these conditions, the $HPO_4^{\bullet-}$ lifetime is <10 μs. The absorption spectra of the transients are shown in Fig. 6. Both the radicals obtained with Tyr-Gly and Gly-Trp decayed by second-order kinetics with $2k_{BR}/\varepsilon^{400} = (2.1 \pm 0.3) \times 10^5$ cm s^{-1} and $2k_{BR}/\varepsilon^{510} = (3.2 \pm 0.3) \times 10^5$, respectively.

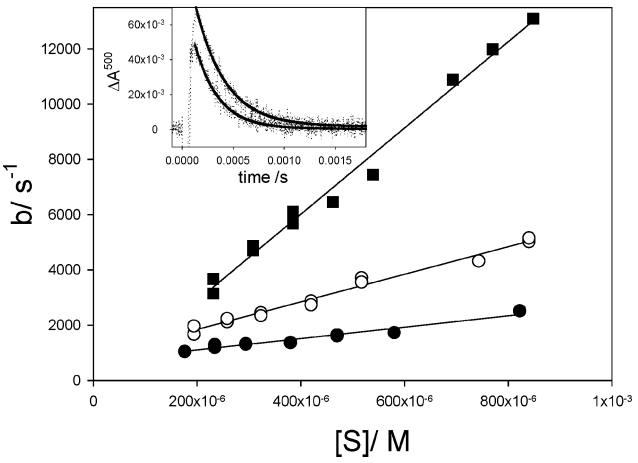


Fig. 5 Apparent rate constant b obtained for $HPO_4^{\bullet -}$ radicals *vs.* [S] for Gly-Gly-Gly (■), Gly-Ala (○) and Ala-Ala (●). The plots were not extrapolated to [S] = 0 because $HPO_4^{\bullet -}$ radicals decay by second-order kinetics in the absence of substrates (see text). Inset: Traces obtained at 500 nm with 1×10^{-3} M $P_2O_8^{4-}$ solutions containing: 2.3×10^{-4} M (upper trace) and 3.1×10^{-4} M (lower trace) Gly-Gly-Gly. The solid lines show the fittings to eqn. (1).

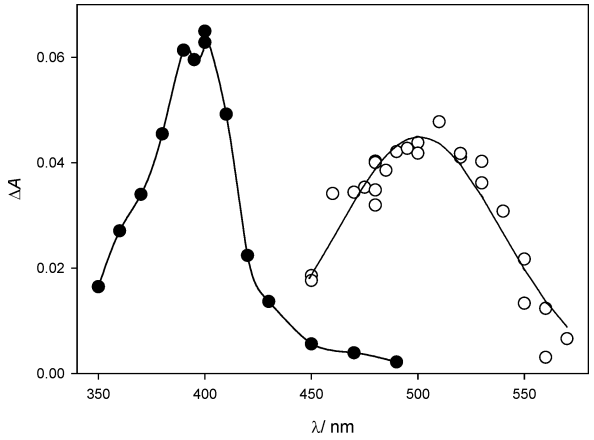


Fig. 6 Absorption spectra of the transient species taken 700 μs after the flash of light as obtained from irradiation of 1×10^{-3} M $P_2O_8^{4-}$ (pH = 7.1) solutions containing 4.7×10^{-5} M: Tyr-Gly (●) and Gly-Trp (○).

Discussion

The analysis of the data shown in Table 1 leads to the following general conclusions:

(i) The values of rate constants k_4 are higher than those of k_5 for all the substrates, as expected from the general trend reported for the reactivity of the inorganic radicals: $HO^{\bullet} > SO_4^{\bullet -} \geq H_2PO_4^{\bullet} > HPO_4^{\bullet -} > PO_4^{\bullet 2-}$.[11]

(ii) Peptides are much more reactive than their parent free amino acids. The enhancing effect of the peptidic bond is observed when comparing the values of k_4 and k_5 obtained for the amino acids with those corresponding to the dipeptides.

(iii) Aromatic substituents confer a higher reactivity to the free amino acids and peptides. In fact, the more photooxidable amino acids Trp and Tyr react with $SO_4^{\bullet -}$ or $HPO_4^{\bullet -}$ with diffusion-controlled rate constants, much faster than those containing only Gly and/or Ala.

We will therefore discuss separately the behavior of free amino acids and peptides according to their aliphatic or aromatic nature.

Aliphatic amino acids and peptides

Ala, Gly and their peptides are included in this group of compounds.

The zwitterions of Ala and Gly will be considered members of the family of substituted acetates, X–CHY–COO^-, where X, Y = CH_3, H, Br, Cl, F, NH_3^+ and $NHCOCH_3$. Hydroxyl radicals have been reported to react with substituted acetates by hydrogen abstraction from the substituted methylene (–CYXH–) as also reported for Gly,[22] reaction (6). In fact, Hammett correlations between the logarithm of the reported rate constants, k, for the reactions of the substituted acetates and the inductive electron transfer σ parameter, σ_I,[23] yields a straight line also including the reactions of the zwitterions of Gly and Ala, as shown in Fig. 7. The observed straight line with negative slope is in agreement with the attack of the electrophilic $HO^{\bullet}$ radical to the –CYXH– group of the acetates. The reported rate constants for the reaction of the dipeptide Gly-Gly with $HO^{\bullet}$ radicals, also fits to the correlation (Fig. 7). This behavior means that the formation of a peptide bond transforms the $-NH_3^+$ group of the free amino acid into a group with a lesser electron withdrawing effect, R–CO–NH–, therefore increasing the reactivity of the dipeptide toward $HO^{\bullet}$ radicals.

$$HO^{\bullet} + X\text{–}CHYCOO^- \rightarrow H_2O + X\text{–}C^{\bullet}YCOO^- \qquad (6)$$

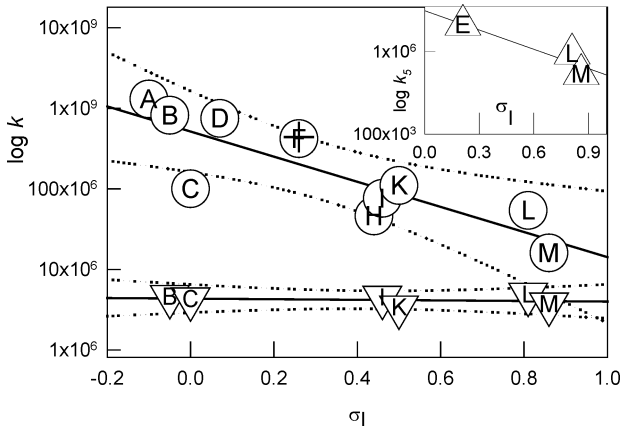


Fig. 7 Logarithmic plots of the $\log k$ *vs.* the Hammett parameter σ_I, taken from refs. 23 and 24*a*. Circles stand for the reactions of hydroxyl radicals and triangles for the reactions of sulfate radical ions with: (A) methylpropanoate, (B) propanoate, (C) acetate, (D) deprotonated Ala zwitterion, (E) Ala-Ala zwitterion, (F) acetylglycine zwitterion, (G) Gly-Gly zwitterion, (H) bromoacetate, (I) chloroacetate, (K) fluoroacetate, (L) Ala zwitterion and (M) Gly zwitterion. The solid line represents the linear correlation and dotted curves show the corresponding 99% confidence interval. Reaction rate constants not measured here were taken from ref. 13. Inset: Plots of the $\log k_5$ *vs.* σ_I for the reactions of the free amino acids Ala and Gly and the dipeptide Ala-Ala.

For the plots shown in Fig. 7, the field effect contribution of the $-NH_3^+$ group of the zwitterions of the amino acids were considered equal to those of $-N(CH_3)_3^+$ groups, as supported by their very similar σ_M parameter. For compounds with two substituting groups on the methylene group, the σ_I values for each group were added, in agreement with the additive effect of the field effects.[24a] For the reaction of the Gly-Gly zwitterion with $HO^{\bullet}$ radicals (cross in Fig. 7), the inductive effect of the substituent $^+NH_3CH_2CONH$– was taken equal to that of the CH_3CONH– group due to the poor field contribution expected for the terminal NH_3^+ group, which is separated by a large number of bonds from the reacting center.

A similar plot for the reactions of $SO_4^{\bullet -}$ radicals with the same family of compounds (Fig. 7), yields a straight line with almost zero slope, which can be interpreted by a substituent independent reaction rate. In fact, sulfate radicals are known to react by an electron transfer mechanism with the carboxylate group of acetate and halogenated acetates, yielding carboxy radicals, as shown in reaction (7). The carboxy radicals undergo further decarboxylation to the X–CH_2 radicals, reaction (8).[25]

Gly and Ala zwitterions react with $SO_4^{\bullet-}$ radicals with rates similar to other substituted acetates, which makes us propose that the electron transfer-decarboxylation leading to a reducing α-amino radical[26] is a plausible mechanism. However, the dipeptides Ala-Ala and Gly-Gly react faster than their parent amino acids and their rate constants do not fit to the plot shown in Fig. 7, thus suggesting that the presence of the peptide bond leads to an additional reaction channel different from that shown in reactions (7) and (8). The observed rates are of the order of those reported for the electron transfer reaction of amines to sulfate radicals[13] and therefore an alternative electron transfer mechanism involving the amide group could take place, reaction (9).

$$SO_4^{\bullet-} + XCYHCOO^- \rightarrow SO_4^{2-} + XCYHCOO^{\bullet} \quad (7)$$

$$XCYHCOO^{\bullet} \rightarrow {}^{\bullet}CYHX + CO_2 \quad (8)$$

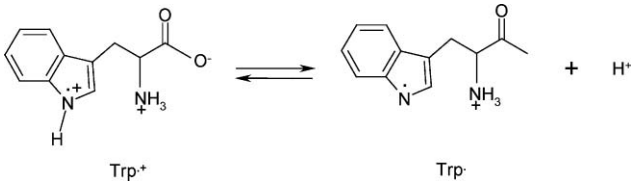
$$SO_4^{\bullet-} + {}^{+}H_3NCHXCONHCHYCOO^- \rightarrow SO_4^{2-} + [{}^{+}H_3NCHXCONHCHYCOO^-]^{\bullet+} \quad (9)$$

Phosphate radicals are much less reactive than sulfate radicals, and the reaction of $HPO_4^{\bullet-}$ radicals with aliphatic acids and Gly was reported to proceed by a reaction mechanism involving H-abstraction from the α carbon atom.[27] The charge transfer reaction between the inorganic radical and the carboxylate group was proposed to be of lesser significance. In fact, Hammett plots of the $\log k$ *vs.* σ_I for the reactions of phosphate radicals with the free amino acids Ala and Gly and Ala-Ala yield a straight line (inset of Fig. 7), as expected for an H-abstraction from the α carbon, in coincidence with the reaction channel observed for $HO^{\bullet}$ radicals.

Aromatic amino acids and peptides

Aromatic side chains have a decisive effect on the reactivity and reaction channels of free amino acids and peptides structures. In fact, the nature of the observed reaction intermediates strongly depends of the reactivity of the aromatic moiety.

The one-electron charge transfer from Trp to $Br_2^{\bullet-}$, $(SCN)_2^{\bullet-}$ and $N_3^{\bullet}$ leads to the formation of the radical cation $Trp^{\bullet+}$, which deprotonates to yield $Trp^{\bullet}$ ($pK_a = 4.2$–4.3),[28,29] reaction (10). Both $Trp^{\bullet+}$ ($\lambda_{max} = 510$ nm) and $Trp^{\bullet}$ decay by second-order kinetics with recombination rate constants $2k_{BR} = 5 \times 10^8$ M^{-1} s^{-1} at λ_{max}.[28,30] Formation of $Trp^{\bullet+}$ was also proposed as the product of reaction (4).[31]



(10)

According to the data shown in Table 1, Trp reacts with $SO_4^{\bullet-}$ and $HPO_4^{\bullet-}$ faster than Gly. Hence, reactions (4) and (5) should involve the interaction of the Trp moieties of Trp-Gly and Gly-Trp. These reactions are expected to take place by the charge transfer route yielding the corresponding radical cations.

If these radicals deprotonate with pK_as similar to that of $Trp^{\bullet+}$, at pH = 4.2 mixtures of the corresponding radical cations (Gly-$Trp^{\bullet+}$ or $Trp^{\bullet+}$-Gly) and of their deprotonated forms (Gly-$Trp^{\bullet}$ or $Trp^{\bullet}$-Gly) should be obtained. The absorption spectra of the radical cations and those of their corresponding undissociated forms are also expected to be similar to those of $Trp^{\bullet+}$ and $Trp^{\bullet}$, respectively. Thus, the transients obtained by reaction (4) in experiments at pH = 4.2 (see Fig. 2 and 3) should contain contributions of both acid–base forms, while those determined at pH = 6.9 are assigned to the neutral radicals Gly-$Trp^{\bullet}$ and $Trp^{\bullet}$-Gly. If the absorption coefficient of Gly-$Trp^{\bullet}$ at 560 nm is assumed to be similar to that of $Trp^{\bullet}$ at the same wavelength, 3000 M^{-1} cm^{-1}, from the experimental decay rate constant (see above), an estimation of $2k_{BR} = 6.6 \times 10^8$ M^{-1} s^{-1} is obtained in good agreement with the value reported for $Trp^{\bullet}$.

The experiments shown in the inset of Fig. 2 allow the determination of the dissociation pK_a of the radical cation of Gly-$Trp^{\bullet+}$. If the contribution of Gly-$Trp^{\bullet+}$ and Gly-$Trp^{\bullet}$ to the absorbance at 630 nm is considered and assuming the equilibrium condition between both radicals, eqn (2) is obtained.

$$\Delta A^{630} = \frac{a}{\left(1 + \frac{K_a}{[H^+]}\right)} + \frac{b}{\left(1 + \frac{[H^+]}{K_a}\right)} \quad (2)$$

In eqn (2), $a = \varepsilon^{630}{}_{RC} C_o l$ and $b = \varepsilon^{630}{}_{NR} C_o l$. The amounts $\varepsilon^{630}{}_{RC}$ and $\varepsilon^{630}{}_{NR}$ are the absorption coefficients of Gly-$Trp^{\bullet+}$ and Gly-$Trp^{\bullet}$ at 630 nm, respectively; C_o is the analytical concentration of the organic radicals, l is the optical pathlength of 10 cm, and K_a is the dissociation constant of Gly-$Trp^{\bullet+}$. The first and second terms in eqn (2) yield the absorbance contributions of Gly-$Trp^{\bullet+}$ and Gly-$Trp^{\bullet}$, respectively. Constant $b = 2.4 \times 10^{-3}$ is calculated from the value of ΔA^{630} obtained for the experiments at pH > 6 (see the inset of Fig. 2), since in this pH range $\Delta A^{630} \approx \varepsilon^{630}{}_{NR} C_o l$. The data were fitted to eqn (2) with $[H^+] = 10^{-pH}/\gamma$ and the activity coefficient $\gamma = 0.90$. Taking $b = 2.4 \times 10^{-3}$, the best fitting of the data (solid line in the inset of Fig. 2) was obtained for $a = 0.017 \pm 0.001$ and $K_a = (3.1 \pm 0.4) \times 10^{-5}$. The latter value yields 4.5 ± 0.1 for the pK_a for dissociation of Gly-$Trp^{\bullet+}$. This value is very close to that determined for Trp.[28,29]

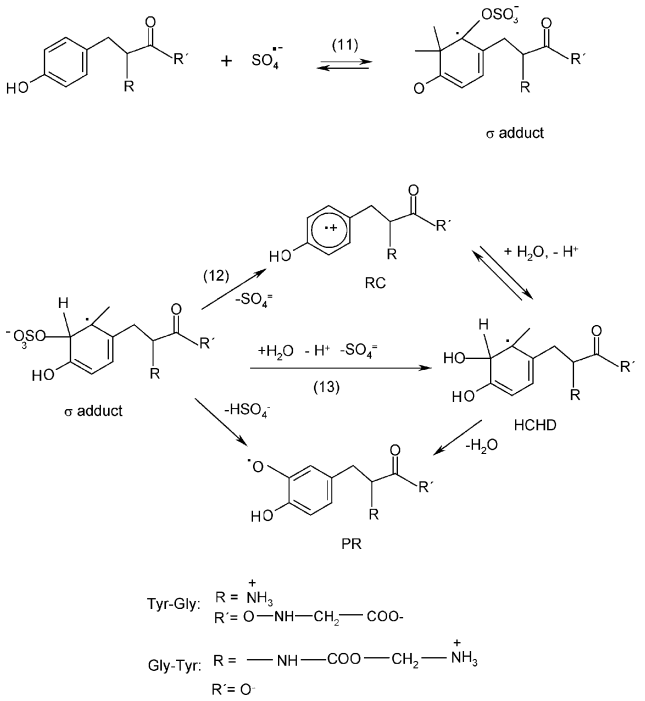
The spectrum obtained for the radical generated after reaction (5) with Gly-Trp at pH = 7.1 is also coincident with that of Gly-$Trp^{\bullet}$, the reaction mechanism being similar to that proposed for the reaction of Gly-Trp with the sulfate radical.

The observation of second-order decays for both the undissociated radicals Gly-$Trp^{\bullet}$ and $Trp^{\bullet}$-Gly obtained at pH = 6.9 and for their mixtures with the corresponding radical cations at pH = 4.2 (see above), indicate that these radicals do not appreciably react with molecular oxygen. These results are in line with the very low oxygen uptake observed in steady-state experiments of Trp autoxidation, where $Trp^{\bullet}$ is the main species present in the system[32] and with the upper limit of 5×10^6 M^{-1} s^{-1},[33] reported for the rate constant of the reaction of the latter radicals with molecular oxygen.

It is noteworthy that in biological systems $Trp^{\bullet}$ radical lifetimes span a wide range:[34] in short peptide chains, they live only ~400 ns, but in DNA photolyase (~10 ms) and a ribonucleotide reductase (RNR) mutant (49 s), they persist much longer, thereby facilitating spectroscopic characterization. In a structurally defined protein environment this radical has a lifetime of several minutes. These observations imply that in these systems it must either have its reactivity modified by the protein environment and/or be inaccessible to oxygen.[33]

The similar reactivity of sulfate and hydrogen phosphate radicals with substituted benzenes was shown.[35] Reactions (4) and (5) with Tyr-Gly or Gly-Tyr are expected to proceed through the interaction of the inorganic radicals with the aromatic amino acid Tyr. Both $SO_4^{\bullet-}$ and $HPO_4^{\bullet-}$ despite being powerful one-electron oxidants do not react with the substituted benzenes by electron transfer, but by addition to yield the corresponding cyclohexadienyl radicals (sigma adducts), as shown in reaction (11) of Scheme 1 for the interaction of $SO_4^{\bullet-}$ radicals with Tyr-Gly and Gly-Tyr.

The spectra shown in Fig. 4 and 6 for the interaction of Gly-Tyr with $SO_4^{\bullet-}$ and Tyr-Gly with $HPO_4^{\bullet-}$, respectively, are coincident with those reported in ref. 36 for the phenoxyl radical of Tyr. In fact, sulfate and phosphate radicals are known to react with phenols to yield phenoxyl radicals.[37,38] Taking $\varepsilon_{(400\ nm)} = 2000$ M^{-1} cm^{-1} as for the phenoxyl radicals of other phenols, values of $2k_{BR}$ of the order of 10^9 M^{-1} s^{-1} are obtained, as those reported for phenoxyl radicals of other phenols.[38]



Scheme 1 Scheme proposed for the reactions of Tyr-Gly and Gly-Tyr with sulfate radicals.

Since the sulfate and phosphate ions are much better leaving groups than OH^-, a short lifetime is also expected for these adducts[35] as for those of the sulfate radicals, in agreement with the lack of observation of these species in our experiments.

Scheme 1 considers sulfate or phosphate loss yielding the radical cation of the aromatics (reaction (12)) as proposed for the sulfate and phosphate adducts[35] and formation of the hydroxycyclohexadienyl (HCHD) radical (reaction (13)) as also suggested for the sulfate radical cation.[24b] Sulfite or phosphite elimination yielding phenoxyl radicals was previously reported[35,37] in agreement with a similar pathway reported by Merga *et al.*[24b] for the sulfate radical. This mechanism explains the detection of the phenoxyl radicals generated by reactions (4) and (5) with S = Tyr-Gly and Gly-Tyr.

The reported rate constants k_4 and k_5 for Tyr (see Table 1) are of the order of those for other 4-substituted phenols, *i.e.*, k_4 in the range from 1×10^9 to 6×10^9 M^{-1} s^{-1} (for *p*-chlorophenol and *p*-cresol, respectively) and $k_5 = 5.3 \times 10^8$ and 5.9×10^8 M^{-1} s^{-1} for phenol and *p*-trifluoromethylphenol with phosphate radicals.[39] However, the observed rate constants for the Tyr-Gly and Gly-Tyr dipeptides are more than one order of magnitude higher than the corresponding constants for the free amino acid Tyr. The observed difference cannot be due to the electron withdrawing ability of the $–CH_2–CH(NH_3^+)–CO–NH–R$ or $–CH_2–CH(COO^-)–NH–COR$ groups and therefore, the participation of additional reaction channels involving the peptide bond cannot be neglected. The involvement of the peptide bond due to spin delocalization has also been proposed in tyrosyl radical-based electron transfer reactions.[40]

Our experimental results clearly indicate that the presence of the –NH–CO– bonds in the peptides significantly increase the reactivity of the amino acid residues with $SO_4^{\bullet -}$ and $HPO_4^{\bullet -}$ radicals. This effect seems to be negligible for $HO^\bullet$ radicals, since its rate constant for the reaction with Gly-Tyr ($k = 6 \times 10^9$ $M^{-1}s^{-1}$) is lower than that with the free amino acid Tyr (1.3×10^{10} $M^{-1}s^{-1}$)[13] and a similar observation was discussed before for Gly and Ala free amino acids and peptides.

Sulfate and phosphate radicals are known to participate in electron transfer reactions, while $HO^\bullet$ radicals are much more reactive but do not undergo electron transfer reactions even with small inorganic ions. We therefore infer that the peptide bond might show some specific contribution to the overall reactivity of the peptide when an electron transfer reaction channel is involved.

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