Photooxidation of Glycylglycine. Two-Channel Reaction Mechanism as Studied by Time-Resolved FT EPR

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The Fourier transform electron paramagnetic resonance (FT EPR) spectroscopy has been employed as a detection technique in the investigation of the photooxidation of glycylglycine dipeptide induced by triplet-sensitized electron transfer to 9,10-anthraquinone-2,6-disulfonate in aqueous solutions at pH 6−10. Spin-polarized (CIDEP) radical species $^{+}NH_3/NH_2-CH_2-CONH-^{\bullet}CH_2$ and $^{\bullet}NH-CH_2-CONH-CH_2-COO^{-}$ were identified as transient products formed on the nanosecond time scale. The radicals have been found to originate from two different reaction channels depending on the pH. The first channel, leading to the decarboxylated radical product, occurs in the whole pH range and is ascribed to the oxidative attack of the triplet on the peptide and/or carboxylate functional groups. At pH > 8 where the peptide terminal amino group exists in deprotonated form, the second channel, electron transfer from the amino group nitrogen nonbonding electron pair followed by deprotonation, becomes possible. This reaction leads to the formation of the observed aminyl radical.

Introduction

The damage of amino acids, peptides, and proteins caused by oxidative processes attracts the attention of scientists in many research branches as those substances are of vital concern for every living organism. Especially, modifications of biological molecules induced by various reactive oxidative species are of significance.[1−3] Recent investigations of the simplest model systems as aliphatic amino acids in aqueous solutions have revealed that the mechanisms of such processes are more complex then anticipated earlier.[4−12] Thus, amino acid anions can be attacked at different sites leading to the simultaneous formation of various radicals. For example, in the case of Gly^{-} oxidation with $^{\bullet}OH$ radicals, one-electron transfer from the nitrogen lone pair occurs leading to $^{\bullet}CH_2-NH_2$ radicals production via fast decarboxylation of the $^{+\bullet}NH_2-CH_2-COO^{-}$ precursor and $NH_2-^{\bullet}CH-COO^{-}$ and $^{\bullet}NH-CH_2-COO^{-}$ radicals which can be formed by direct H-atom abstraction or deprotonation of the $^{+\bullet}NH_2-CH_2-COO^{-}$ radical, a process in competition with its decarboxylation.[4−8] Only the latter two radicals were formed when less oxidizing $O^{\bullet-}$ radicals were used as initiators.[12] Reaction with characteristic one-electron oxidants, the triplet excited benzophenone and anthraquinone derivatives, leads almost exclusively to the decarboxylated $^{\bullet}CH_2-NH_2$ radicals,[9,10] whereas protection of the glycine carboxylic group by esterification allows the primarily formed $^{+\bullet}NH_2-CH_2-COOR$ to stabilize via deprotonization into $NH_2-^{\bullet}CH-COOR$ and $^{\bullet}NH-CH_2-COOR$.[11] Rustgi and Riesz[13] could identify corresponding decarboxylated radicals ($^{\bullet}CH_2-NH_2$ and $^{\bullet}CH_2-NH_3^{+}$ for glycine) as products in the reaction of both anionic and zwitterionic amino acid forms with $SO_4^{\bullet-}$ radicals, indicating the ability of the latter to directly oxidize the amino acid carboxylate group. Identity of the various radical transients mentioned has been confirmed by time-resolved or spin-trapping EPR mesurements.[6−8,10]

Much less information is available, however, on the primary oxidation reaction mechanisms of aliphatic dipeptides. Investigations of γ-irradiated single crystals of α-glycylglycine (EPR),[14] hydroxyl radical mediated oxidation of Gly−Gly in aqueous solutions (pulse radiolysis[15−17] and spin-trapping EPR[18]) revealed formation of hydrogen atom abstraction products $H_3N^{+}-CH_2-CONH-^{\bullet}CH-COO^{-}$ and $H_2N-^{\bullet}CH-CONH-CH_2-COO^{-}$. Similar to amino acids,[5] the reaction rate constants $k(^{\bullet}OH + \text{peptide})$[17] were found to be markedly dependent on pH in the range 4−10, i.e., on the state of protonation of the terminal amino group ($pK = 8.2$ at 25°C for Gly−Gly). Decarboxylation leading to the $-CONH-^{\bullet}CH_2$ type of radicals was shown to occur if $SO_4^{\bullet-}$ radicals were used as oxidants[13] and also via intramolecular charge transfer from the photoionized aromatic group to the carboxyl group in aromatic peptide π-cation radicals.[19]

In this work, we report on the results obtained upon oxidation of the dipeptide glycylglycine (Gly−Gly) in aqueous solutions initiated by 9,10-anthraquinone-2,6-disulfonate in its triplet state ($AQDS_{T1}$). Investigations were carried out by means of the nanosecond laser flash photolysis with the FT EPR detection technique. The main aim was to examine the role of the peptide functional group, −CONH−, as a possible additional site for the oxidative attack. Since the most proteins are around a few hundred amino acids in length, the probability of oxidative damage via such a reaction channel might get particularly significant. The very low pK_a value of a peptide bond makes the electron transfer from the peptide nitrogen in principle possible within a very wide pH range. In addition, we are interested to identify $NH_2-^{\bullet}CH-COO^{-}$ and $^{\bullet}NH-CH_2-COO^{-}$ radicals which can be formed by direct H-atom abstraction or deprotonation of the $^{+\bullet}NH_2-CH_2-COO^{-}$ radical, a process in

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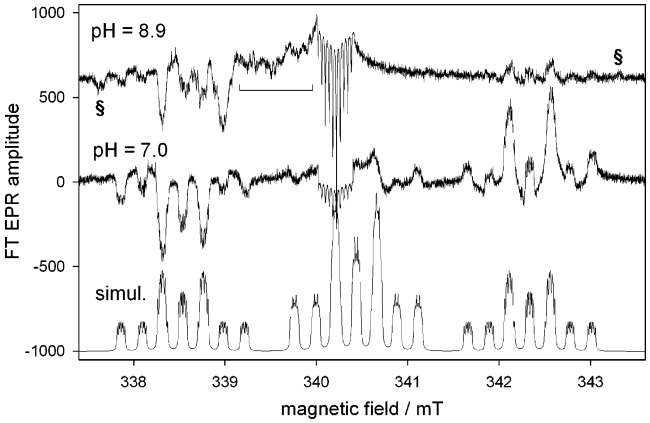


Figure 1. FT EPR spectra obtained upon laser photolysis in systems: 0.3 mM AQDS, 100 mM Gly−Gly in H_2O, pH 8.9 and 7.0, at the delay time of 96 ns at room temperature together with simulation of $^{+}NH_3-CH_2-CONH-{}^{\bullet}CH_2$ radical. Lines denoted by § belong to aminyl radical $^{\bullet}NH-CH_2-CONH-CH_2-COO^{-}$, The horizontal bracket represents part of spectrum of undetermined radical(s) R$^{\bullet}$. The *hfc* parameters of the simulated spectrum are listed in Table 1.

competition with its decarboxylation. In addition, we were interested to identify products which, similar to reactions observed with amino acid anions, should obviously be formed also upon reductive triplet quenching by the deprotonated terminal amino group of a peptide in slightly basic solutions. To our knowledge, there have been no specific investigations of this kind reported in the literature, and it was evident that the time-resolved photochemical method coupled with the FT EPR detection technique could provide interesting new results.

Experimental Section

Aqueous solutions of 0.3 mM AQDS and 100 mM Gly−Gly in the pH range 6−10 (adjusted by KOH) were irradiated by a 308 nm laser pulse (15 ns, ∼20 mJ, repetition rate: 10 Hz) from an excimer laser LPX105 (Lambda Physik). The FT EPR spectrometer used was described previously.[10] The microwave pulse power was 1 kW with a $\pi/2$ pulse length of 16 ns and excitation width of ±40 MHz. The complete spectra were therefore recorded step by step using different offsets of the magnetic field. The partial spectra were then put together by virtue of SigmaPlot graphic software. The dead time of the free induction decay (FID) used within the experiment was 100 ns. All FIDs were extrapolated into the dead time by the linear prediction singular value decomposition (LPSVD) method.[20] The number of accumulations per spectrum was 100−4000.

Gly−Gly (Sigma, Fluka) and AQDS (Aldrich) were of purest commercially available grade and were used without further purification. Deuterium oxide (D_2O, 96.5%) was purchased from DeuChem GmbH, Leipzig. Aqueous solutions were prepared with triply distilled water (Milli-Q-system). Air dissolved in solutions was removed by bubbling with argon for around half an hour before and during the whole measurement. The sample flow rate was kept at ∼6 mL/min in order to avoid accumulation of reaction products within the irradiated area. Measurements were carried out at room temperature.

Results and Discussion

The FT EPR spectra of transient products formed upon 308 nm laser pulse irradiation of 0.3 mM AQDS and 100 mM Gly−Gly aqueous solution at pH 7.0 and 8.9, recorded after a delay time between laser pulse and microwave pulse of 96 ns, are shown in Figure 1. Narrow lines in the middle of both spectra belong to the radical anion AQDS$^{\bullet-}$. Their intensities are multiplied by a factor of 0.1 for better presentation. At both pHs, these emissive polarized spectra have been fully developed in the shown amplitude within the time of recording. This indicates a fast quenching of AQDS triplet with Gly−Gly ground-state molecules, proceeding in appreciable though pH dependent yield, via the reductive electron-transfer mechanism. The simulation parameters for AQDS$^{\bullet-}$ spectra are in a good agreement with those obtained previously using triethylamine[21] and thymine[22] as electron donors. The other lines observed at pH 7.0 belong to the carbon-centered radical $^{+}NH_3-CH_2-CONH-{}^{\bullet}CH_2$. The structure of this radical has been determined by comparing the measured spectrum with the simulated one (see Figure 1, simulation) using the *hfc* parameters listed in Table 1. In a range of pH values from 6 to 8, the radical $^{+}NH_3-CH_2-CONH-{}^{\bullet}CH_2$ exhibits almost pure radical pair (CIDEP) polarization. Practically the same radical species is observable also in the spectrum measured at pH 8.9, Figure 1, although its intensity is considerably lower. At this higher pH value, it is highly probable that the amino group of the radical is at least partly deprotonated (at pH 8.9, the terminal amino group of the parent Gly−Gly molecule is mostly (83%) in the deprotonated form) and that the spectrum belongs predominately to the neutral radical $NH_2-CH_2-CONH-{}^{\bullet}CH_2$. In Figure 1 at the periphery of $^{+}NH_3/NH_2-CH_2-CONH-{}^{\bullet}CH_2$ spectrum at pH 8.9, some additional lines are visible (symbol §). These lines originate from the radical pair CIDEP polarized aminyl radical $^{\bullet}NH-CH_2-CONH-CH_2-COO^{-}$ and the spectrum fully correlates with that measured in systems containing glycine esters NH_2-CH_2-COOR (R = CH_3 or CH_2CH_3) as electron donors[11] (for *hfc* constants cf. Table 1). To check the assignment of coupling nuclei and their hyperfine coupling constants, an experiment using D_2O as solvent has been carried out at pD 9.4. The smallest proton coupling constant A_H (−NH−) = 0.018 mT (cf. Table 1) in H_2O disappeared in D_2O (expected A_D (−ND−) ≅ 0.0028 mT is not measurable). It should be noted that in the spectrum recorded at pH 8.9 still some more lines are observable (horizontal bracket) but it is hard to attribute them to any specific radical structure because of the considerable overlapping with $^{+}NH_3/NH_2-CH_2-CONH-{}^{\bullet}CH_2$ lines and a low signal-to-noise ratio. We assume that they originate from the radical $NH_2-{}^{\bullet}CH-CONH-CH_2-COO^{-}$ formed upon deprotonation from the α-amino-C−H position of the aminium radical. This is supported by results obtained in similar experiments with glycine esters.[11]

TABLE 1: Hyperfine Coupling Constants *A* [mT] and *g* Factors for Radicals Derived from Gly−Gly

$^{+}NH_3CH_2C(=O)N(\bullet CH_2)H$		$^{\bullet}NHCH_2CONHCH_2COO-$	
A_N (−NH−)	0.234	A_N ($^{\bullet}$NH−)	1.356
A_N ($^{+}NH_3$−)	0.032	A_H ($^{\bullet}$NH−)	2.267
A_H (−$^{\bullet}CH_2$)	1.898	$A_{H\beta1}$ (−CH_2−)	4.336
A_H (−CH_2−)	0.442	$A_{H\beta2}$ (−CH_2−)	4.383
A_H (−NH−)	0.018		
g value	2.00278	*g* value	2.00442

Figure 2 shows the pH dependence of the relative yields of two main radicals ($^{\bullet}NH-CH_2-CONH-CH_2-COO^{-}$ (●) and $^{+}NH_3/NH_2-CH_2-CONH-{}^{\bullet}CH_2$ (×)) present in the system 96 ns after the laser pulse. The yields have been normalized taking the yield of $^{\bullet}NH-CH_2-CONH-CH_2-COO^{-}$ at pH = 10.1 as the unit (100%). The sigmoidal curve delineated in Figure 2 displays the fraction of deprotonated terminal amino group (pK = 8.2) in the overall [Gly−Gly] vs pH. In the insert of Figure

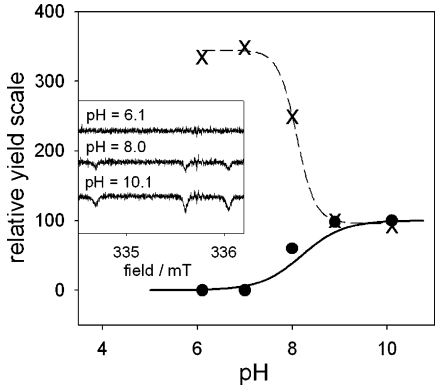
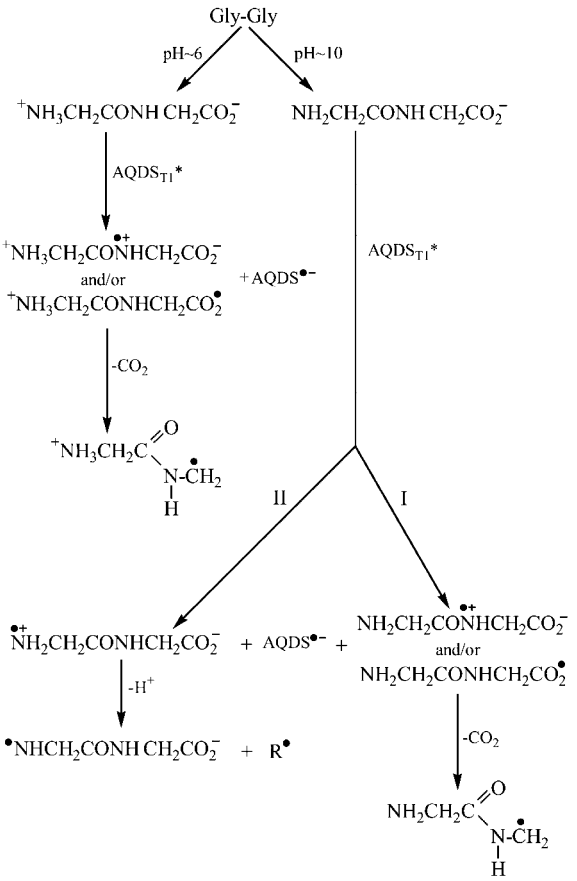


Figure 2. pH dependence of relative yields of radicals: $^{\bullet}NH-CH_2-CONH-CH_2-COO^-$ (●) and $^+NH_3/NH_2-CH_2-CONH-^{\bullet}CH_2$ (×) measured in the system: 0.3 mM AQDS, 100 mM Gly−Gly in H_2O, at the delay time of 96 ns at room temperature. The solid sigmoidal curve displays the fraction (%) of deprotonated terminal amino group ($pK = 8.2$) in the overall Gly−Gly vs pH. Inset demonstrates intensity tendency of selected lines from $^{\bullet}NH-CH_2-CONH-CH_2-COO^-$ spectrum against pH.

SCHEME 1



2 some low field lines of the $^{\bullet}NH-CH_2-CONH-CH_2-COO^-$ radical are shown to demonstrate their intensity tendency against pH.

Scheme 1 shows the proposed pathways of Gly−Gly photooxidation mediated by the AQDS triplet in aqueous solution for two pH values. The AQDS triplet is generated via 308 nm laser excitation of the AQDS ground state into the excited singlet state followed by intersystem crossing resulting in a spin-polarized triplet state of AQDS ($AQDS_{T1}$*). At pH ∼ 6, the terminal amino group of Gly−Gly is completely protonated. Therefore, an electron transfer from this group is blocked. On the other hand, the lone electron pair at the peptide nitrogen is accessible for electron transfer to $AQDS_{T1}$* (although the oxidation is more difficult because of electron withdrawal effect of carbonyl group), providing formation of the intermediate radical cation $^+NH_3-CH_2-CO-^+{}^{\bullet}NH-CH_2-COO^-$ and radical anion $AQDS^{\bullet-}$. Electron transfer to $AQDS_{T1}$* from the carboxylate group could in principle also take place leading to the intermediate $^+NH_3-CH_2-CONH-CH_2-COO^{\bullet}$. We cannot say which of those two processes is more probable to occur as neither of the supposed Gly−Gly derived intermediate radicals could be experimentally detected. They are most probably very short-lived species due to the fast decarboxylation into the identical successor radical $^+NH_3-CH_2-CONH-^{\bullet}CH_2$, which has been unambiguously identified (Figure 1). The deprotonated terminal amino group at pH ∼ 10 opens the second reaction channel (pathway II) for the oxidation of Gly−Gly. It leads to the formation of the corresponding nitrogen-centered aminium radical, a species which also seems to be too short-lived for detection on the nanosecond time scale. In the basic pH range, it is reasonable to propose that aminium radicals suffer fast proton elimination forming the observed aminyl radical and, to a much lower extent, some further radical products $R^{\bullet}$ (for example $NH_2-^{\bullet}CH-CONH-CH_2-COO^-$ radicals). An important feature arising from the results obtained at pH ∼ 10 is that the reaction channel leading to $NH_2-CH_2-CONH-^{\bullet}CH_2$ (pathway I) is occurring as well, in competition with channel II. Formation of the two main products at pH above 9 (aminyl and decarboxylated C-centered radicals) in about equal yields indicates similar efficiency of the two reaction channels. However, a significant decrease of the radical anion $AQDS^{\bullet-}$ FT EPR intensity observed by going to lower pH region where oxidation of terminal amino group is prevented indicates considerable decrease of the overall triplet quenching rate constant with Gly−Gly in the zwitterionic form.

Conclusions

Excited triplets of 9,10-anthraquinone-2,6-disulfonate react with glycylglycine in aqueous solution via a reductive quenching mechanism resulting in the formation of the triplet polarized AQDS radical anions in high yields. Transfer of an electron from Gly−Gly in anionic form can thereby occur from all three functional groups (NH_2-, $-CONH-$, and COO^-). Oxidative attack at the amino group leads to the formation of N-centered aminyl radicals which have been identified by their FT EPR spectrum. Parallel to this process a formation of the decarboxylated $^+NH_3/NH_2-CH_2-CONH-^{\bullet}CH_2$ radicals occurred. Their formation mechanism can be associated with the electron transfer from the one or from both other functional groups to the anthraquinone triplet. The resulting primary radical species could not be experimentally observed because of their very fast CO_2 elimination and transformation into $^+NH_3/NH_2-CH_2-CONH-^{\bullet}CH_2$ radicals. The latter were the only products observed upon photooxidation of glycylglycine in its zwitterionic form.

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