Electron spin resonance of spin trapped radicals in γ-irradiated polycrystalline dipeptides. Chromatographic separation of radicals

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Polycrystalline dipeptides (glycyl-glycine, glycyl-L-valine, glycyl-L-leucine, L-alanyl-glycine, and L-prolyl-L-alanine) were γ-irradiated at room temperature in the absence of air. Subsequently they were dissolved in aqueous solutions containing 2-methyl-2-nitrosopropane as the spin trap. From the esr spectra of the nitroxide radicals separated by high-performance liquid chromatography, structural assignments of the radicals were made. For glycyl peptides, H-abstraction from the α-carbon atoms of the carboxyl terminal residues and from the side-chains were observed. For L-alanyl-glycine, H-abstraction from the glycyl residue and the formation of the deamination radical could be shown to occur. For L-prolyl-L-alanine, the ring opening (deamination) reaction, decarboxylation and H-abstraction from the C-terminal α-carbon were seen.

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On a irradié aux rayons γ, à la température ambiante et en l'absence d'air, les dipeptides polycristallins suivants: (glycyl-glycine, glycyl-L-valine, glycyl-L-leucine, L-alanyl-glycine et L-prolyl-L-alanine). On les a ensuite dissous dans des solutions aqueuses contenant le capteur de spin, méthyl-2 nitroso-2 propane. A partir des spectres de rpe des radicaux nitroxydes, séparés par chromatographie en phase liquide à haute performance, on a pu déterminer les structures des radicaux. On a observé, dans le cas des peptides glycidiques, l'élimination d'un hydrogène à partir des atomes de carbone en α du residue terminal carbxyle et à partir des chaînes latérales. Dans le cas du L-alanyl-glycine, on peut montrer qu'il se produit une élimination d'hydrogène à partir du résidu glycyle et la formation d'un radical de désamination. Dans le cas du L-prolyl-L-alanine, on a observé une réaction d'ouverture de cycle (désamination), de décarboxylation et d'élimination d'un atome d'hydrogène à partir du carbone terminal en position 2.

[Traduit par le journal]

Introduction

Among spin trapping studies, 2-methyl-2-nitrosopropane (MNP) has been utilized as one of the most informative spin traps (1–5). However, it is known that when the method of spin trapping with MNP is applied to γ-radiolysis in aqueous solutions, esr spectra obtained are often too complicated to be analyzed accurately. Recently this problem has been overcome by the combination of high-performance liquid chromatography and esr spectroscopy (6–12). By this method, each spin adduct present in the sample is separated and shows its individual esr spectrum.

It has been demonstrated previously that spin trapping with MNP can be applied to the study of the effect of ionizing radiation on polycrystalline solids (13–16). The solids are γ-irradiated and subsequently dissolved in aqueous MNP solutions. By this method, free radicals produced by γ-irradiation of polycrystalline amino acids, *N*-acetyl amino acids, and dipeptides have been analyzed (14). However, when the esr spectra overlap, only major components in the spectra obtained by spin trapping have been analyzed. Since single crystal esr studies of room temperature free radicals produced in such compounds are difficult, particularly when several different radicals are present, the spin trapping method appears to be helpful. For γ-irradiated dipeptides, only glycyl-glycine has been studied by single crystal esr (17) and nine dipeptides have recently been studied by ENDOR and ELDOR of the polycrystalline powder X-irradiated at 77 K and subsequently warmed to room temperature (18).

In the present work, the method of chromatographic separation of nitroxide spin adducts was applied to polycrystalline dipeptides irradiated at room temperature in the absence of air. It should be noted that the validity of this method depends on the stability of the nitroxide spin adduct on the column during the separation.

Experimental

MNP was purchased from Aldrich Chemical Company and kept under N_2 at 5°C in the dark. Dipeptides were obtained from Sigma Chemical Company and Vega Fox Biochemicals.

Aqueous MNP solutions (5 mg/mL) were prepared by stirring in the dark for 1.5 h at 45°C (7).

Polycrystalline dipeptides samples were evacuated at 10^{-4} Torr for at least 24 h so that oxygen and moisture could not affect the production of free radicals. The evacuated samples were irradiated with ^{60}Co γ-rays at a dose rate of 6.0×10^5 rad/h to a total dose of ca. 20 Mrad at room temperature and subsequently dissolved in the aqueous MNP solutions. The range of dipeptide concentrations was 10–100 mg/mL.

The sample solution (2.0 mL) was loaded on an IEX-210SC column (Toyo Soda, 3/8 in. id × 60 cm) which was attached to the chromatograph (Waters, Model 6000A). A Pyrex flow cell, which was fixed in the esr sample cavity, was connected to the exit of the column with ca. 0.25 mm id Teflon tubing. In order to obtain higher resolution, gradient elution was adopted. Eluents

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were: 0.3 *M* phosphate buffer (pH 6.8) and 0.3 *M* NaOH–Na_2HPO_4 buffer (pH 10.8). Other chromatographic conditions were: pressure, ca. 200 psi; flow rate, 0.3 mL/min; temperature, ca. 25°C. The eluate was collected in a fraction collector after passing through the flow cell. During the separation, the magnetic field was fixed at one position and a high amplitude of 10 G modulation was applied to cover a wide range of signal. The collected fractions which showed the chromatographic radical peaks were measured by esr (Varian, E-9, X-band) in the standard aqueous flat cell.

Results and discussion

In Figs. 1, 2 and 3, the esr spectra obtained from γ-irradiated polycrystalline glycyl dipeptides (glycyl-glycine (Gly-Gly), glycyl-L-valine (Gly-L-Val), and glycyl-L-leucine (Gly-L-Leu)) are shown. Figure 1 displays the original esr spectrum of γ-irradiated Gly-Gly, which was dissolved in an aqueous MNP solution. When this sample solution was loaded on the column, only one peak appeared in the chromatogram. From the fraction which gave the peak, the same spectrum as the original one was obtained. The spectrum changed with pH in acidic solution as shown in the same figure whereas it was not altered in alkaline solution. This suggests that

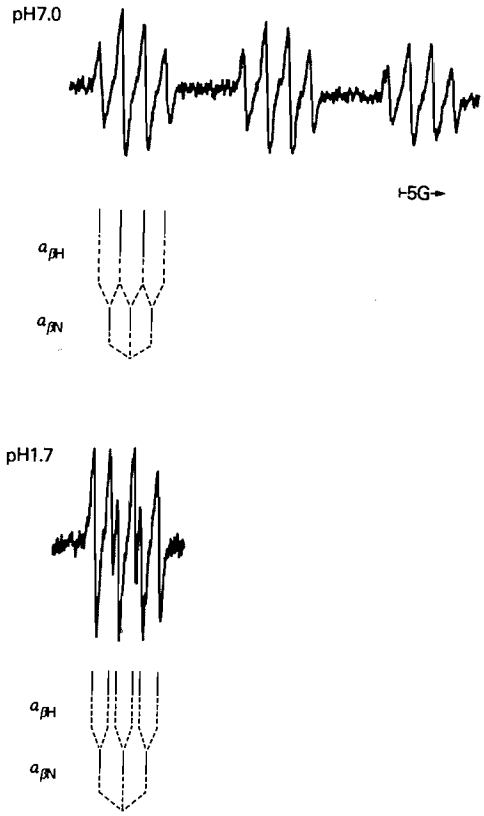


FIG. 1. Electron spin resonance spectra and stick diagram of polycrystalline Gly-Gly γ-irradiated at room temperature and subsequently dissolved in aqueous MNP solutions.

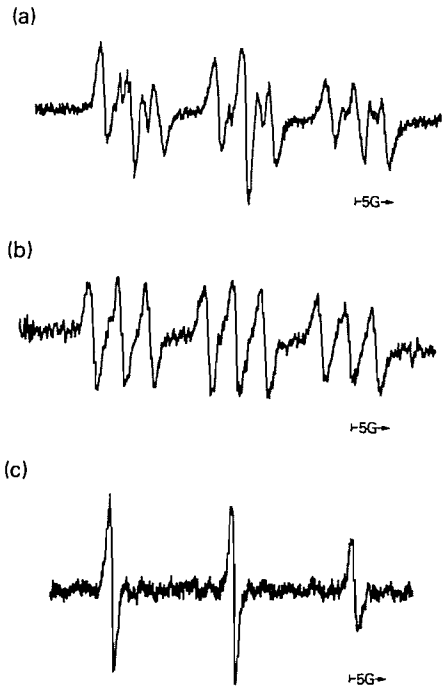
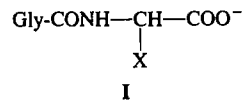


FIG. 2. (*a*) Electron spin resonance spectrum of polycrystalline Gly-L-Val γ-irradiated at room temperature and subsequently dissolved in aqueous MNP solutions. (*b*), (*c*), Electron spin resonance spectra obtained from the same sample by chromatographic separation. The spin adduct whose esr spectrum is shown in (*b*) was eluted faster than the other.

the nitroxide group is close to the carboxyl group. The stick diagram in the same figure shows that the spin adduct has a β-H and a β-N. Consequently the spectrum is assigned to structure **I**



I

where X = ^{t}Bu—N(O•)— The parent radical of the spin adduct is formed by H-abstraction from the C-terminal carbon. The hyperfine coupling constants (hfcc) measured in the spectrum are listed in Table 1. This spin adduct has been previously identified (12, 17) and the parent radical of **I** has been found in the single crystal studies of Gly-Gly (19).

In Fig. 2(*a*), the original esr spectrum of γ-irradiated Gly-L-Val which was dissolved in the spin trap aqueous solution is shown. Overlapping lines are observed in the spectrum. By chromatographic separation, two different esr spectra were obtained as shown in Fig. 2 (*b*) and (*c*), respectively. The spectrum in Fig. 2 (*b*) consists of triplet split into a further triplet with a 1:1:1 intensity ratio. The

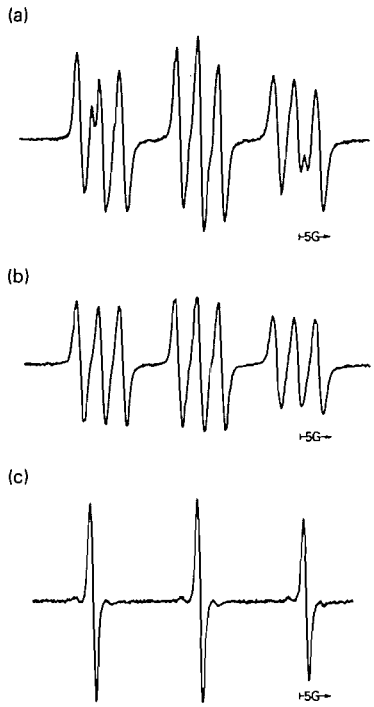


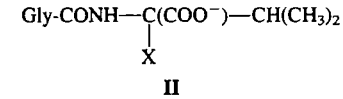
FIG. 3. (*a*), Electron spin resonance spectrum of polycrystalline Gly-L-Leu γ-irradiated at room temperature and subsequently dissolved in aqueous MNP solutions. (*b*), (*c*), Electron spin resonance spectra obtained from the same sample by chromatographic separation. The spin adduct whose esr spectrum is shown in (*b*) was eluted faster than the other.

TABLE 1. Hyperfine coupling constants of the separated spin adducts*

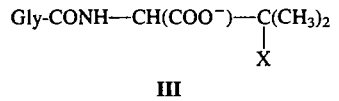
	Hyperfine coupling constants (G)				
Spin adduct	N	β-H	β-N	γ-H	γ-N
I	15.5	2.2	2.4		
II	15.4		3.8		
III	16.5				
IV	15.5		3.4		
V	16.7				
VI	15.7	2.6	2.6	0.5	0.5
VII	15.4	2.4	2.4		
VIII	15.8	2.0	2.3		
IX	15.3		3.3		
X	15.4	3.6		0.6	0.6

*All the values were obtained in neutral solutions.

primary splitting is due to a N nucleus in the nitroxide group and the secondary splitting is attributed to the β-N in the peptide group. The unusually broad lines imply that the spin adduct might contain γ-nuclei with different characteristics. Consequently the spectrum is assigned to the spin adduct formed by H-abstraction from the C-terminal α-carbon (**II**). The spectrum was unchanged with pH in alkaline solutions. The other spectrum shown in Fig. 2 (*c*) has only three lines, implying that the spin adduct does not have any β-nuclei. The only possible structure is



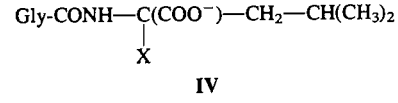
II



III

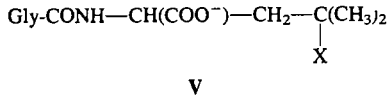
The untrapped free radical of **III** is produced by H-abstraction from the side-chain carbon of the C-terminal carbon. The hfcc of spin adduct **II** and **III** are shown in Table 1. The spin adduct **III** could not be found in a previous work (14).

Similar to the results obtained with Gly-L-Val, in the analogous experiment with Gly-L-Leu, a triple–triplet and a simple triplet were detected as shown in Fig. 3 (*b*) and (*c*), respectively. According to the assignment made for the spectrum in Fig. 2 (*b*), the spectrum in Fig. 3 (*b*) is assigned to structure **IV**. The parent radical of the spin adduct **IV** is formed by H-abstraction from the C-terminal α-carbon. The spectrum was unchanged with pH in alkaline



IV

solutions. The triplet in Fig. 3 (*c*) is also consistent with structure **V**. The hfcc of the spin adducts **IV** and **V** are summarized in Table 1.



V

Considering the results obtained in the γ-radiolysis and subsequent spin trapping of three glycyl dipeptides, the following facts were revealed: (*1*), The deamination radicals which are usually observed in the analogous experiments with amino acids are not detected. (*2*), The decarboxylation radical is not observed. (*3*), The major products are the spin adducts whose parent radicals are generated by H-abstraction from the C-terminal α-carbon and the carbon of the side-chain on the C-terminal residue. In previous papers (20, 21), it has been demonstrated that the deamination radicals, which are produced by the reaction of the hydrated electron with these three dipeptides and the decarboxylation radicals, which are generated by the

reaction of $SO_4^{\cdot -}$ can be efficiently spin trapped with MNP.

From γ-irradiated polycrystalline L-Ala-Gly which was dissolved in an aqueous MNP solution, two kinds of esr spectra were detected by the method of chromatographic separation, as shown in Fig. 4 (*b*) and (*c*), respectively. The spin adduct giving the spectrum in Fig. 4 (*b*) was eluted very fast compared to the other one. As exhibited by the expanded spectrum of the $M_I = 0$ component of Fig. 4 (*b*) and by the stick diagram in the same figure, the primary N triplet is split into doublets due to a β-H which are further split into sextets with an intensity ratio of 1:4:7:7:4:1 by three equivalent γ-hydrogens and a γ-N. Consequently the spectrum can be assigned to the spin adduct which is produced by deamination, **VI**.

$$CH_3—\underset{\displaystyle X}{\overset{|}{CH}}—CONH\text{-}Gly$$

VI

The other spectrum shown in Fig. 4 (*c*) consists of a triplet which is further split into four lines due to a β-H and a β-N. The structure of the spin adduct is

$$L\text{-}Ala\text{-}CONH—CH(COO^-)—X$$

VII

The hfcc are listed in Table 1. Both the spin adduct **VI** and **VII** have been detected previously (14). In the same paper (14), the spin adduct produced by decarboxylation has been found.

Figure 5 (*a*) shows the original esr spectrum of γ-irradiated polycrystalline L-Pro-L-Ala dissolved in an aqueous MNP solution. The major component in the spectrum consists of a triplet further split into four lines. This has been analyzed previously (14) as the decarboxylation radical, **VIII**.

$$L\text{-}Pro\text{-}CONH—CH(CH_3)—X$$

VIII

Using chromatographic separation, two other spectra could be obtained from the same sample solution, as shown in Fig. 5 (*b*) and (*c*), respectively. The triple triplet in Fig. 5 (*b*) is attributed to a N nucleus in the nitroxide group and to a β-N nucleus

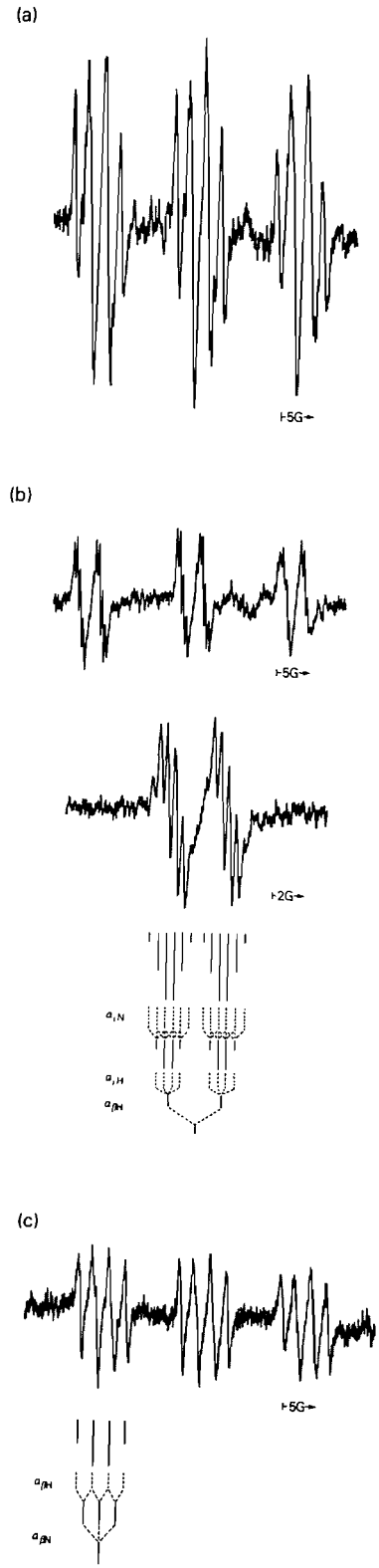


FIG. 4. (*a*), Electron spin resonance spectrum of polycrystalline L-Ala-Gly γ-irradiated at room temperature and subsequently dissolved in aqueous MNP solutions. (*b*) Electron spin resonance spectrum of the separated spin adduct and the expansion of the $M_I = 0$ component with the stick diagram. (*c*) Electron spin resonance spectrum of the separated spin adduct, which was eluted more slowly than that whose esr spectrum is shown in (*b*).

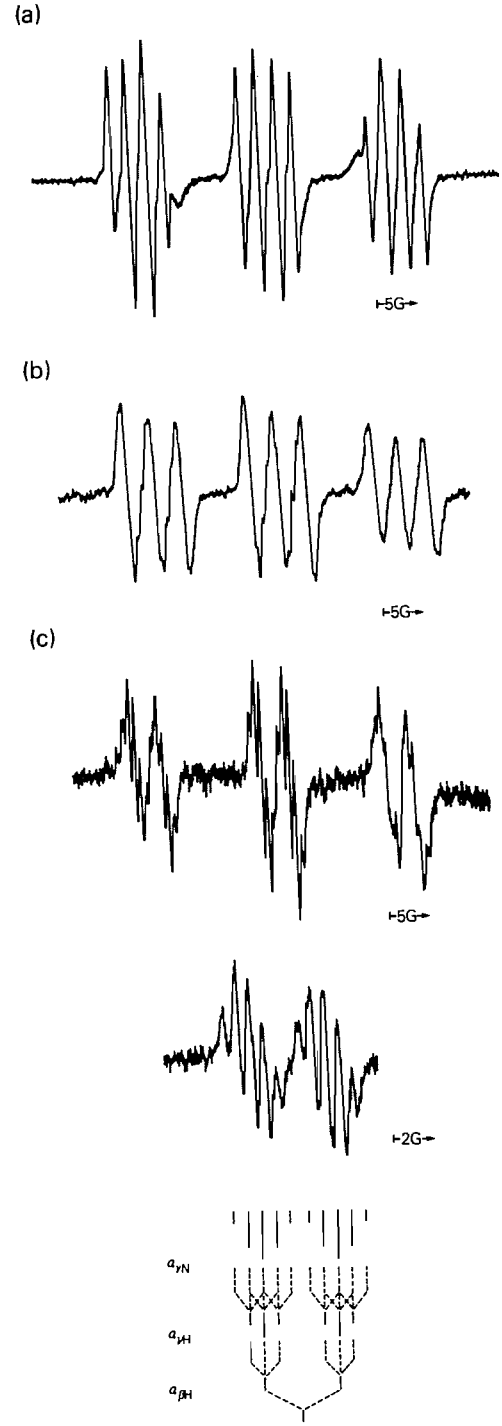
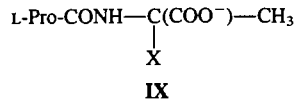


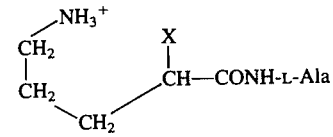
FIG. 5. (*a*) Electron spin resonance spectrum of polycrystalline L-Pro-L-Ala γ-irradiated and subsequently dissolved in aqueous MNP solutions. (*b*) Electron spin resonance spectrum of the separated spin adduct, which was eluted faster than the other. (*c*) Electron spin resonance spectrum of the separated spin adduct and the expansion of the $M_I = 0$ component with the stick diagram.

in the peptide group. The structure of the spin adduct is



IX

This spin adduct has not been found previously. The fine structure due to γ-hydrogens could not be observed even when a low modulation amplitude was used. In Fig. 5 (*c*), the other spectrum is shown with the expansion of the $M_I = 0$ component and its stick diagram. The triple doublet is split into five lines with an intensity ratio of 1:3:4:3:1. This spectrum is due to the ring opening (deamination) radical which has been identified (14);



X

The hfccs of spin adducts **VIII**, **IX**, and **X** are listed in Table 1.

In experiments with γ-irradiated polycrystalline L-Ala-Gly and L-Pro-L-Ala, the deamination radicals and the decarboxylation radical could be detected in addition to the spin adducts produced by H-abstruction. These results are quite different from those obtained in the experiments of γ-irradiated polycrystalline glycyl dipeptides (Gly-Gly, Gly-L-Val, and Gly-L-Leu).

The present work indicates that spin trapping combined with high-performance liquid chromatography is a useful method, apart from single crystal esr, for studying the room-temperature free radicals in γ-irradiated polycrystalline peptides. Further work remains to be done to establish quantitative methods for determining free radical distributions in γ-irradiated solids.

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