# EPR studies on the selectivity of hydroxyl radical attack on amino acids and peptides

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Direct rapid-flow EPR experiments together with computer simulations have been used to examine the selectivity of hydroxyl radical (generated using a $Ti^{3+}/H_2O_2$ redox couple) attack on a number of aliphatic amino acids, amino acid derivatives and small peptides. For glycine, glycine derivatives and glycine peptides attack at the α-carbon position predominates under all conditions; in peptides attack at the C-terminal site is preferred over mid-chain sites, which in turn are favoured over the N-terminal position. This behaviour is rationalised in terms of the destabilising effect of the protonated α-amino group, which can exert both short- and long-range effects. With alanine peptides hydrogen atom abstraction at the side-chain methyl group predominates with free amino acid; significant levels of attack at the α-carbon position are however observed with peptides. In contrast, with valine and leucine peptides side-chain attack always predominates irrespective of whether the backbone amino group is derivatized or not; the ratio of side-chain species is also only marginally affected. The preference for attack at tertiary side-chain sites over primary side-chain methyl groups in such peptides is small. These results support the hypothesis that the selective fragmentation of large proteins as a result of exposure to hydroxyl radicals in the presence of oxygen may occur primarily as a result of attack at the α-carbon position of surface-exposed glycine and alanine residues.

It has long been known that the exposure of proteins to radical attack in the presence of oxygen can bring about multiple changes in the target molecule. These alterations include loss of structural or enzymatic activity, fragmentation, cross-linking, side-chain oxidation, unfolding and changes in hydrophobicity and conformation, altered susceptibility to proteolytic enzymes and formation of new reactive groups (such as hydroperoxides and 3,4-dihydroxyphenylalanine). These changes, and our limited knowledge about the processes that give rise to them, have been reviewed.[1–4] Of particular interest is the observation that the fragmentation of a number of proteins exposed to radiolytically-generated hydroxyl radicals in the presence of oxygen is not a random process, with discrete protein fragments observed either by polyacrylamide gel electrophoresis or HPLC.[5–8] Random damage would instead be expected to give rise to "smearing" of the parent protein band or peak rather than material with discrete molecular weights. Previous workers have suggested that the formation of these fragments, for which only approximate molecular weights have been obtained to date, arises *via* selective fragmentation of the protein structure at proline residues.[9] This hypothesis is based on the observations that these residues are relatively rare, are usually present at the ends of α-helices and hence often partially exposed on the surface, and that both 2-pyrrolidone and increased levels of glutamic acid, which is believed to arise from ring opening of the proline ring after initial attack at C-5, have been detected in oxidised peptides.[9–11] Attack at C-5 is believed to be favoured over the corresponding α-carbon position in protected proline derivatives as a result of the severe steric constraints to achieving planarity of the α-carbon radical, despite the relief of ring strain and steric interactions that occur as a result of abstraction at the α-carbon site.[12,13]

The α-carbon site of glycine residues is also believed to be a favoured site of attack in amino acid derivatives and small peptides.[12–16] Thus it has been shown that the glycine α-carbon site is the major site of hydrogen atom abstraction in several N- and C-blocked small peptides exposed to a number of radical sources including *N*-bromosuccinimide and $Bu^tO^{\bullet}$, irrespective of the order of the amino acids in the peptide. A similar selectivity has been reported in peptides and proteins subject to photoalkylation.[17–19] This preference for formation of radicals at the glycine α-carbon over other α-carbon sites has been ascribed to the increased stability of the former (secondary) species over the tertiary species observed with other amino acids as a result of unfavourable steric interactions between the side-chain substituent and the carbonyl group on the N-terminal side if the radical adopts a planar conformation.[12,13] These steric interactions, which prevent the attainment of a planar conformation, decrease the extent of overlap between the semi-occupied p orbital of the radical and the π orbitals of the amido and carbonyl substituents, and hence the stabilisation afforded to these tertiary radicals. The corresponding glycine radicals, having no side-chain, do not suffer from such unfavourable interactions, and can readily attain a planar arrangement. Studies which have compared the reactivities of various protected amino acid derivatives have shown that although protected proline derivatives react more rapidly than the corresponding glycine species the former reaction gives the C-5 species rather than the proline α-carbon radical, and that the formation of the latter radical is less rapid than the corresponding glycine species.[12–14,20] The corresponding α-carbon radicals from other amino acids have been predicted to be less stable due to these non-bonding interactions.

In order to determine whether a similar selectivity of attack also applies in peptides exposed to the less selective $HO^{\bullet}$, and hence whether preferential reaction at the α-carbon position of Gly might explain the selective fragmentation of proteins, we have investigated the selectivity of $HO^{\bullet}$ attack on a number of free amino acids, amino acid derivatives and small peptides. Though a number of previous direct EPR studies have examined the reactions of reactive radicals (particularly $HO^{\bullet}$) with free amino acids and derivatives,[21–31] and a few studies have been reported for peptides, there is a lack of information on the *selectivity* of attack on such materials. Such information is difficult to obtain from EPR spin trapping studies, though this technique has been used quite extensively (reviewed in refs. 3, 32), because of uncertainties about the rates of trapping of the initial radicals, and the stability of the resulting adduct species. In this study we have examined the selectivity of $HO^{\bullet}$ attack on free amino acids, *N*-acetyl derivatives and small peptides con-

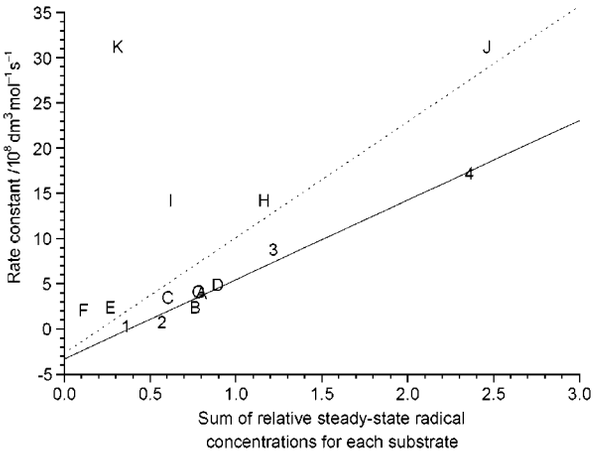


**Fig. 1** Plot of the sum of relative steady-state radical concentrations detected by rapid-flow EPR spectroscopy for each substrate (determined by double integration of the EPR spectra and comparison with a standard EtOH sample; for further details see Experimental) *versus* the rate constant (from ref. 33) for reaction of the specified amino acid, derivative or peptide with HO$^{\bullet}$. Amino acid data (solid line): (1) Gly, (2) Ala, (3) Val, (4) Leu. *N*-Acetyl derivative and peptide data (broken line): (A) *N*-Ac-Gly, (B) Gly–Gly, (C) Gly–Gly–Gly, (D) *N*-Ac-Ala, (E) Gly–Ala, (F) Ala–Gly, (G) Ala–Ala, (H) *N*-Ac-Val, (I) Gly–Val, (J) *N*-Ac-Leu, (K) Gly–Leu.

taining Gly, Ala, Val, and Leu by direct EPR with the aim of determining whether radical attack occurs primarily at side-chain or backbone sites in peptides containing these amino acids and hence whether any selectivity observed could be responsible for the selective fragmentation of proteins.

## Results and discussion

### Reaction of hydroxyl radicals with free amino acids

The radicals detected in rapid-flow experiments where HO$^{\bullet}$ was generated using a $Ti^{3+}$–EDTA/$H_2O_2$ redox couple at pH *ca.* 3 in the presence of excess substrate are summarised in Table 1. Assignment of the observed signals has been made on the basis of the observed splitting patterns and comparison of the hyperfine coupling constants with data from previous studies, where available. Assignments, and the relative concentrations of individual species, were confirmed by computer simulation. It should be noted that the relative intensities (steady state levels) of the radicals reported in Table 1 reflect both the rate constants for reaction of HO$^{\bullet}$ with these substrates and the rate of decay of the radicals so formed. It is assumed that the various radicals produced have similar lifetimes.

As expected, reaction of HO$^{\bullet}$ with free Gly gave relatively weak spectra from the C-2 (α-carbon) radical formed by hydrogen abstraction, due to the deactivating effect of the protonated amino group and the lack of other, readily abstractable, hydrogens. Substitution of Gly with Ala, Val or Leu resulted in increased overall radical yields due to the availability of increasing numbers of non-deactivated C–H sites. The sum of the relative radical concentrations determined for each substrate is plotted in Fig. 1 against the rate constant for reaction of HO$^{\bullet}$ with the zwitterion form of the substrate determined in previous pulse-radiolysis studies.[33]

In the case of Val hydrogen abstraction from both the C-3 and C-4 sites was observed, with the latter favoured by a ratio of *ca.* 2:1. This is in line with previous estimates for HO$^{\bullet}$ attack on valine[21,29] and 4-methylpentanoic acid (isovaleric acid)[22] but is significantly different to another report for valine which gave a value of 1.1:1.[22] The discrepancy between these values is probably due to the high concentration of $H_2O_2$ (0.1 M) used in the latter study (but not the former or present studies where concentrations of 0.01 and 0.012 M were employed respectively), as it is known that excess $H_2O_2$ can readily oxidise tertiary radicals[34] thereby resulting in misleading ratios. Thus there is some selectivity for the tertiary site, despite the (6:1) statistical weighting in favour of the primary C–H bonds.

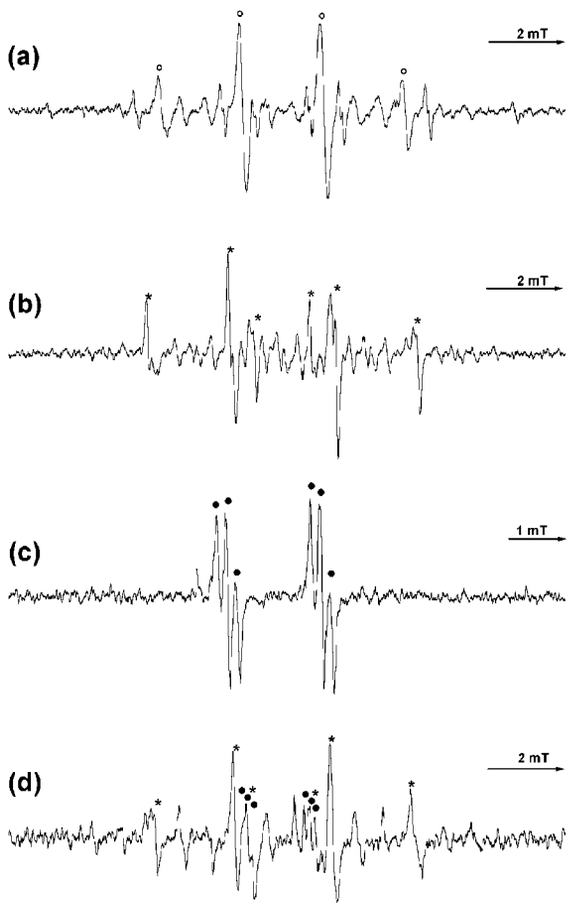


**Fig. 2** EPR spectra observed on reaction of amino acids, derivatives and peptides with $H_2O_2$ and $Ti^{3+}$–EDTA in a rapid-flow system at pH 3. (a) EPR spectrum observed with leucine; signals marked [○] are attributed to the radical formed by hydrogen abstraction at C(5), the remaining signals are assigned to the corresponding C(4) radical. (b) EPR spectrum observed with *N*-acetylvaline; signals attributed to radicals formed on hydrogen abstraction from the Val side chain. Features marked [*] are assigned to radicals formed at C(4); remaining signals are assigned to the radicals formed at C(3). (c) EPR spectrum observed with Ala–Gly; signals marked [●] are assigned to radicals formed on hydrogen abstraction from C(2) of Gly. This species gives additional small *a*(H) couplings which are not fully resolved, but cause distortion of the observed line shapes. The remaining features are attributed to the formation of radicals from hydrogen abstraction at C(3) of Ala. (d) EPR spectrum observed with Val–Gly. Signals marked [●] are attributed to the radical formed at C(2) on Gly by hydrogen abstraction; this species gives additional small *a*(H) couplings which are not fully resolved, but cause distortion of the observed line shapes. Features marked [*] are assigned to the radical formed on hydrogen abstraction from the valine side chain at C(4); the remaining lines are attributed to the corresponding valine C(3) radical.

With Leu a somewhat similar ratio is observed for the selectivity of attack at C-4 *versus* C-5, but in this case no signals from radicals arising from attack at the C-3 methylene group could be discerned [Fig. 2(a)]. This is attributed to the long range deactivating effect of the protonated amino group, with sites further removed from this group being least deactivated. The C-4 radical from Leu has not been observed in previous studies,[22,29,30] with the selective formation of the C-5 (γ) radical ascribed in one of these studies to the formation of a complex of the substrate with $Ti^{3+}$ (*i.e.* site-specific oxidation), rather than attack by "free" HO$^{\bullet}$.[31] The discrepancy between the present report and these previous studies may be due to the large number of lines from this species which results in a low overall

**Table 1** Parameters of the radicals observed on reaction of certain amino acids, *N*-acetyl derivatives and peptides with HO$^{\bullet}$ at pH 3

| Substrate | Site of radical formation | Relative intensity | Hyperfine coupling constant/mT[a] | | |
|---|---|---|---|---|---|
| | | | α-H | β-H | Other |
| Glycine | Gly C(2) | 100 | 1.19 (1H) | 0.62 (2H) | 0.58 (1N) |
| Alanine | Ala C(3) | 100 | 2.22 (2H) | 2.61 (1H) | 0.35 (1N) |
| Valine | Val C(3) | 33 | | 2.37 (6H) | 0.71 (1N) |
| | | | | 0.65 (1H) | |
| | Val C(4) | 67 | 2.21 (2H) | 2.97 (1H) | |
| Leucine | Leu C(4) | 24 | | 2.34 (6H) | |
| | | | | 1.95 (1H) | |
| | | | | 1.23 (1H) | |
| | Leu C(5) | 76 | 2.17 (2H) | 2.23 (1H) | |
| *N*-Acetylglycine | Gly C(2)[b] | 78 | 1.64 (1H) | | 0.22 (2 C–H) |
| | | | | | 0.22 (1 N–H) |
| | *N*-Acetyl group | 22 | 2.12 (2H) | | 0.24 (1H) |
| | | | | | 0.24 (1N) |
| *N*-Formylglycine | Gly C(2)[b] | 68 | 1.67 (1H) | | 0.29 (1 C–H) |
| | | | | | 0.18 (1 N–H) |
| | *N*-Formyl group | 32 | | | 2.36 (1N) |
| *N*-*tert*-BOC-glycine | Gly C(2) | 30 | 1.59 (1H) | | 0.31 (1 N–H) |
| | | | | | 0.17 (1N) |
| | *N*-*tert*-BOC group | 70 | 2.21 (2H) | | 0.13 (6 C–H) |
| Gly–Gly | C-terminal Gly[b] | 92 | 1.68 (1H) | | 0.29 (2 C–H) |
| | | | | | 0.18 (1 N–H) |
| | N-terminal Gly | 8 | 1.83 (1H) | | 0.64 (1 N–H) |
| | | | | | 0.47 (1N) |
| Gly–Gly–Gly | C-terminal Gly | 65 | 1.68 (1H) | | 0.28 (2 C–H) |
| | | | | | 0.19 (1N) |
| | Middle Gly | 35 | 1.58 (1H) | | 0.30 (2 C–H) |
| | | | | | 0.13 (1N) |
| *N*-Acetylalanine | Ala C(2) | 45 | | 1.81 (3H) | 0.20 (1N) |
| | Ala C(3) | 46 | 2.25 (2H) | 2.61 (1H) | 0.32 (1N) |
| | *N*-Acetyl group | 9 | 2.01 (1H) | | 0.21 (1 N–H) |
| | | | | | 0.21 (1N) |
| Gly–Ala | Ala C(2) | 84 | | 1.89 (3H) | 0.25 (2 C–H) |
| | | | | | 0.10 (1N) |
| | Ala C(3) | 16 | 2.21 (2H) | 2.93 (1H) | 0.32 (1N) |
| *N*-Acetyl-Gly–Ala | Ala C(2) | 100 | | 1.75 (3H) | 0.21 (2 C–H) |
| | | | | | 0.10 (1N) |
| Ala–Gly | Gly | 85 | 1.69 (1H) | | 0.16 (1N)[c] |
| | Ala C(3) | 15 | 2.24 (2H) | 2.56 (1H) | 0.32 (1N) |
| *N*-Acetyl-Ala–Gly | Gly | 100 | 1.65 (1H) | | 0.18 (1N)[c] |
| Ala–Ala | Ala C(2) | 67 | | 1.87 (3H) | 0.14 (1N) |
| | Ala C(3) | 36 | 2.21 (2H) | 2.91 (1H) | 0.33 (1N) |
| *N*-Acetyl-Ala–Ala | Ala C(2) | 69 | | 1.83 (1H) | 0.10 (1N) |
| | Ala C(3) | 31 | 2.24 (2H) | 2.90 (1H) | 0.34 (1N) |
| Gly–Gly–Ala | Ala C(2) | 78 | | 1.84 (1H) | 0.27 (2 C–H) |
| | | | | | 0.13 (1N) |
| | Ala C(3) | 22 | 2.21 (2H) | 2.89 (1H) | 0.32 (1N) |
| Gly–Ala–Gly | C-terminal Gly | 100 | 1.68 (1H) | | 0.16 (1N)[c] |
| *N*-Acetylvaline | Val C(3) | 33 | | 2.35 (6H) | 0.57 (1N) |
| | | | | 0.97 (1H) | |
| | Val C(4) | 67 | 2.20 (2H) | 2.78 (1H) | 0.11 (1H) |
| Gly–Val | Val C(3) | 29 | | 2.33 (6H) | 0.35 (1N) |
| | | | | 0.94 (1H) | |
| | Val C(4) | 71 | 2.19 (2H) | 2.98 (1H) | |
| *N*-Acetyl-Gly–Val | Val C(3) | 52 | | 2.35 (6H) | 0.57 (1N) |
| | | | | 0.99 (1H) | |
| | Val C(4) | 48 | 2.19 (2H) | 2.84 (1H) | |
| Val–Gly | Val C(3) | 25 | | 2.37 (6H) | 0.74 (1N) |
| | | | | 0.65 (1H) | |
| | Val C(4) | 46 | 2.21 (2H) | 2.63 (1H) | |
| | Gly | 28 | 1.66 (1H) | | 0.23 (1N)[c] |
| *N*-Acetyl-Val–Gly | Val C(3) | 56 | | 2.36 (6H) | 0.60 (1N) |
| | | | | 1.00 (1H) | |
| | Val C(4) | 44 | 2.21 (2H) | 2.87 (1H) | |
| Ala–Val | Val C(3) | 19 | | 2.33 (6H) | 0.58 (1N) |
| | | | | 1.00 (1H) | |
| | Val C(4) | 81 | 2.19 (2H) | 2.86 (1H) | 0.10 (1H) |
| Val–Ala | Val C(3) | 27 | | 2.37 (6H) | 0.64 (1N) |
| | | | | 0.76 (1H) | |
| | Val C(4) | 44 | 2.20 (2H) | 2.70 (1H) | |
| | Ala C(2) | 22 | | 1.88 (3H) | 0.11 (1N) |
| | Ala C(3) | 7 | 2.23 (2H) | 2.77 (1H) | 0.32 (1N) |

**Table 1** (*Contd*)

| Substrate | Site of radical formation | Relative intensity | Hyperfine coupling constant/mT[a] | | |
|---|---|---|---|---|---|
| | | | α-H | β-H | Other |
| Gly–Gly–Val | Val C(3) | 35 | | 2.36 (6H) | 0.74 (1N) |
| | | | | 0.63 (1H) | |
| | Val C(4) | 65 | 2.20 (2H) | 2.97 (1H) | |
| Val–Gly–Gly | Val C(3) | 28 | | 2.38 (6H) | 0.75 (1N) |
| | | | | 0.63 (1H) | |
| | Val C(4) | 50 | 2.22 (2H) | 2.66 (1H) | |
| | C-terminal Gly | 22 | 1.66 (1H) | | 0.23 (1N)[c] |
| *N*-Acetylleucine | Leu C(4) | 33 | | 2.32 (6H) | 0.10 (1H) |
| | | | | 1.94 (1H) | |
| | | | | 1.26 (1H) | |
| | Leu C(5) | 67 | 2.19 (2H) | 2.46 (1H) | |
| Gly–Leu | Leu C(4) | 34 | | 2.31 (6H) | |
| | | | | 2.01 (1H) | |
| | | | | 1.24 (1H) | |
| | Leu C(5) | 66 | 2.19 (2H) | 2.47 (1H) | |
| Leu–Gly | Leu C(4) | 40 | | 2.34 (6H) | |
| | | | | 1.76 (1H) | |
| | | | | 1.29 (1H) | |
| | Leu C(5) | 50 | 2.15 (2H) | 2.23 (1H) | |
| | Gly | 10 | 1.69 (1H) | | 0.14 (1N)[c] |
| Ala–Leu | Leu C(4) | 43 | | 2.32 (6H) | |
| | | | | 1.96 (1H) | |
| | | | | 1.36 (1H) | |
| | Leu C(5) | 57 | 2.18 (2H) | 2.45 (1H) | |
| Leu–Ala | Leu C(4) | 28 | | 2.37 (6H) | |
| | | | | 1.73 (1H) | |
| | | | | 1.26 (1H) | |
| | Leu C(5) | 53 | 2.17 (2H) | 2.19 (1H) | |
| | Ala C(2) | 18 | | 1.93 (3H) | 0.19 (1N) |
| Gly–Gly–Leu | Leu C(4) | 43 | | 2.31 (6H) | |
| | | | | 1.93 (1H) | |
| | | | | 1.27 (1H) | |
| | Leu C(5) | 57 | 2.18 (2H) | 2.47 (1H) | |
| Gly–Leu–Gly | Leu C(4) | 29 | | 2.33 (6H) | |
| | | | | 1.92 (1H) | |
| | | | | 1.37 (1H) | |
| | Leu C(5) | 47 | 2.18 (2H) | 2.40 (1H) | |
| | C-terminal Gly | 24 | 1.69 (1H) | | 0.17 (1N)[c] |
| Leu–Gly–Gly | Leu C(4) | 6 | | 2.36 (6H) | |
| | | | | 1.76 (1H) | |
| | | | | 1.30 (1H) | |
| | Leu C(5) | 86 | 2.18 (2H) | 2.18 (1H) | |
| | C-terminal Gly | 8 | 1.79 (1H) | | 0.16 (1N)[c] |
| Gly–Ala–Leu | Leu C(4) | 25 | | 2.31 (6H) | |
| | | | | 1.85 (1H) | |
| | | | | 1.29 (1H) | |
| | Leu C(5) | 56 | 2.19 (2H) | 2.46 (1H) | |
| | Ala C(2) | 13 | | 1.87 (3H) | 0.14 (1N) |
| | | | | | 0.23 (2 C–H) |
| | Ala C(3) | 6 | 2.38 (2H) | 2.58 (1H) | 0.42 (1N) |

[a] ±0.02 mT. [b] The *a*(N) splitting is too small to be resolved. [c] Further small *a*(H) splittings are not resolved but cause a distortion in the signals observed.

signal intensity, and/or the known very ready oxidation of such tertiary radicals by either excess $H_2O_2$[34] or $Ti^{4+}$[35] generated from the $Ti^{3+}/H_2O_2$ couple. A direct comparison of the reactivity of Val *versus* Leu side-chains was also carried out using equimolar ratios of the two amino acids. This resulted in the detection of signals from all four of the above species, with the ratio of the radicals Leu (C-5 and C-4) : Val (C-4 and C-3) being *ca.* 66 : 34 as calculated from computer simulation of the experimental spectra. This ratio of Leu-derived to Val-derived species of 1.9 : 1 is very close to that expected on the basis of the known overall rate constants for reaction of $HO^{\bullet}$ with the zwitterion forms of these two amino acids (*cf.* rate constants of $1.7 \times 10^9$ and $0.86 \times 10^9$ $dm^3$ $mol^{-1}$ $s^{-1}$ for Leu and Val respectively[33]).

### Reaction of hydroxyl radicals with amino acid derivatives and peptides

Derivatisation of the protonated amino group at the N-terminus results in an increased yield of substrate derived radicals in all cases (*cf.* Fig. 1). Thus there was a 2.5-fold increase in the steady-state radical concentration from the *N*-Ac derivative of Gly compared to that from the free amino acid. This increase cannot be solely ascribed to reaction at the *N*-Ac function [to give a radical with partial structure $^{\bullet}CH_2C(O)$-NHR] as this species represents a relatively small % of the total radical yield (*ca.* 22%); this increased yield is ascribed to the capto-dative stabilisation of the C-2 (α-carbon) radical by the neighbouring amide and carboxyl groups, and is in accord with previous kinetic data.[33] Other amino derivatisation groups (*e.g.* formyl and ′BOC) also gave radicals arising from hydrogen abstraction; with the ′BOC group, with nine available C–H bonds, hydrogen abstraction at this site became a major process (representing *ca.* 70% of the total, steady-state, radical concentration), though the overall yield of radicals arising from the C-2 (α-carbon) position was still significantly greater than that obtained with the parent amino acid. The relative yield of

radicals resulting from attack at such sites decreased with increasing availability of alternative side-chain sites; thus the yield of *N*-Ac-derived radicals [*i.e.* species of the type $^{\bullet}CH_2C(O)NHR$] decreased from 22% for *N*-Ac-Gly to 9% for *N*-Ac-Ala and undetectable amounts for *N*-Ac-Val and Leu [*cf.* Fig. 2(b)]. This can be readily rationalised as being due to the electron-withdrawing effect of the amide function.

As expected, much higher yields of C-2 (α-carbon) radicals were obtained with the *N*-Ac derivatives compared to the free amino acids. Thus *ca.* 45% of the steady-state yield of radicals detected with *N*-Ac-Ala are due to this species, and 46% to the C-3 derived species, compared to the 100% yield of the C-3 radical detected with Ala itself. However no C-2 (α-carbon) radicals were detected with *N*-Ac-Val [Fig. 2(b)] or Leu despite the fact that this position might be expected to be a favourable site of hydrogen atom abstraction given the potential stabilisation offered by the neighbouring functional groups. A similar effect has been observed previously with other protected amino acids, with attack at C-2 disfavoured compared to side-chain sites. This has been ascribed[12] to the constraints imposed by the side-chain group, and its unfavourable steric interaction with the N-terminal acyl group, when the C-2 radical tries to adopt a planar conformation which would maximise delocalisation on to the neighbouring functional groups. Little, or no, attack is also observed on the C-3 site of Leu; this may be due to steric crowding, the secondary nature of these potential radicals and the low statistical favourability of attack at these C–H bonds (compared to, for example, the two methyl groups). The ratio of attack at C-4 *versus* C-5 for the Leu derivative is similar to that observed with the free amino acid, suggesting that these sites are only marginally affected by derivatisation of groups present on the backbone. This is again consistent with previous kinetic data with the rate constants for H-atom abstraction by $HO^{\bullet}$ from these two substrates varying by only a factor of *ca.* 2.[33]

With small peptides such as Gly–Gly, attack occurs preferentially at the C-terminal C-2 (α-carbon) site over the N-terminal site with the ratio of attack at these two positions being *ca.* 92 : 8; the radicals formed at these sites can be readily distinguished on the basis of the size of the coupling to the nitrogen atom—in the case of the C-terminal position this is too small to be resolved, whereas the N-terminal C-2 (α-carbon) radical gives rise to a relatively large coupling (of 0.47 mT). This selectivity for the C-terminal site is in accord with an earlier study,[30] though in this previous work only this radical was detected and not the N-terminal species. With Gly–Gly–Gly, differentiation between the radicals formed at the C-terminal α-carbon position and the mid-chain position is more difficult, with two species detected in a ratio of 65 : 35, with both of these species possessing small nitrogen couplings (0.13 and 0.19 mT respectively). On the basis of previous product studies[36] using $Bu^{t}O^{\bullet}$ as the attacking radical, we have assigned the former, more intense, signal to the C-terminal species. Thus the positional selectivity for the C-terminal C-2 (α-carbon) position appears to be retained even when other non-deactivated α-carbon sites are available.

The positional selectivity for the C-terminal α-carbon site over the N-terminal position is retained for all the other dipeptides tested, though in cases where there are side-chain sites available, reaction at these positions can predominate. Thus with small Ala peptides some side-chain derived radicals were detected [Fig. 2(c)], unlike in a previous study,[30] though attack at the α-carbon site predominated in most cases.

With Val or Leu containing peptides the situation is more extreme with little backbone attack observed in the presence of these large side-chains. Thus even with Val–Gly [Fig. 2(d)], where the Val side-chain sites would be expected to be deactivated to some extent by the protonated N-terminal amino group, attack at the C-3 and C-4 sites on Val predominates with only *ca.* 28% attack at the C-terminal Gly α-carbon site. Reversing the order of these residues results in exclusive attack on the Val side-chain. Similar behaviour is observed with the analogous Leu peptides, though the larger Leu side-chain has a more marked effect—thus only *ca.* 10% attack at the C-terminal Gly α-carbon site is observed in Leu–Gly. The accessibility of the side-chain sites does however appear to have marginal effects, with the percentage of attack at the Leu side-chain sites in the series Leu–Gly–Gly, Gly–Leu–Gly and Gly–Gly–Leu varying in the order 86, 76 and 100%, with attack at the C-terminal Gly α-carbon site accounting for the remainder in the first two cases. In none of these cases was any attack at the Val or Leu α-carbon sites detected.

In more complex peptides where a choice of side-chain sites is available a somewhat similar pattern is observed. Thus whilst with Ala–Val exclusive attack on the Val side-chain was observed, with Val–Ala attack at both Val side-chain sites, and the Ala C-2 (α-carbon) and C-3 sites was detected in the ratio 71 : 22 : 7; similar behaviour was detected with Ala–Leu and Leu–Ala, though no attack on the Ala side-chain (*i.e.* C-3) was observed in the latter case. These differences are suggested to arise, at least in part, from the deactivating effect of the N-terminal protonated amino group on the corresponding side-chain sites. There is also a very noticeable selectivity for attack at the terminal methyl groups of the Leu and Val side-chains over the Ala side-chain methyl group, which is far greater than might be expected on a purely statistical basis (*i.e.* double the number of available C–H bonds for Val and Leu). This is particularly noticeable with the di-peptides Val–Ala and Leu–Ala, and the tri-peptide Gly–Ala–Leu, with the ratio in the latter case being nearly 9 : 1 for Leu C-5 over Ala C-3; the accessibility of the respective sites may play a significant role in this selectivity.

## Conclusions

The rapid-flow EPR experiments reported above have allowed, for the first time, a number of new amino acid derived radicals to be identified, and the relative ratios of these species in a number of amino acid derivatives and small peptides to be determined. The results obtained confirm that electronic and steric factors can play an important role in determining the relative rates of attack at different sites within peptides. In particular it is very noticeable that attack of $HO^{\bullet}$ on peptides at backbone α-carbon sites only occurs to any significant extent when there is either no side-chain (*i.e.* Gly residues) or only a methyl group present (Ala). With larger side-chains, *e.g.* Val or Leu, attack is only observed at side-chain sites and not at the corresponding α-carbon. This is probably due, as suggested previously,[12,14,20,37] to the inability of the α-carbon radicals formed at such sites to achieve maximal delocalisation of the unpaired electron due to their inability to readily achieve a planar conformation as a result of unfavourable steric interactions with backbone groups. This selectivity for Gly and Ala sites would also be expected to hold when other (untested) side-chains are present, as the side-chains that have been studied are the least favoured for $HO^{\bullet}$ attack due to the absence of heteroatom, or aromatic, radical-stabilising functional groups. This would suggest that fragmentation of larger peptides and proteins, *via* the formation and subsequent reaction of backbone peroxyl radicals, is likely to occur mainly at surface accessible Gly and, possibly to a lesser extent, Ala residues, unless there are efficient side-chain to backbone rearrangement reactions which can convert initial side-chain damage into backbone cleavage. At present there are few examples of such processes known (reviewed in ref. 3).

Attack at large side-chains also appears to be strongly favoured over Ala residues even when both sites are primary in nature; this may be for steric reasons. The selectivity for reaction at such residues lends further support to the use of oxidised products of such amino acid side-chains (*e.g.* the hydroxylated products arising from the corresponding peroxyl radicals and

hydroperoxides[38–42]) as markers of oxidative damage to proteins in biological systems (reviewed in refs. 3, 4). The overall selectivity of $HO^{\bullet}$ attack between the Leu and Val side-chains in an equimolar mixture of the free amino acids, as determined by these direct EPR studies, is in reasonable agreement with the rate constants for $HO^{\bullet}$ attack determined previously (*i.e.* with Leu favoured over Val by a factor of *ca.* 2). Furthermore there is also a reasonable correlation between these EPR data and the end products of such oxidations (carried out in the presence of $O_2$) with the yields of the alcohols determined from product analysis after reduction, derivatisation and separation by HPLC. Thus the ratio of hydroxylated Val C-4 : Val C-3, determined by product analysis, is 2.1 : 1[38] which compares well with the relative yield of the corresponding radicals detected in this work (of *ca.* 2 : 1). The corresponding Leu products however correlate less well, with the product ratio Leu C-5 : Leu C-4 alcohols being reported as 0.51 : 1[39] whereas the ratio of the corresponding radicals detected in this work is *ca.* 3.2 : 1. The reason for this discrepancy is not absolutely certain, but previous studies have shown that the Leu C-5 derived radical can undergo alternative reactions which do not give rise to alcohols (or species which are reduced back to this material under the analytical conditions employed).[39] These alternative processes give methyl-proline derivatives as a result of aldehyde formation and internal cyclisation with the free amine group. The formation of these materials probably accounts for the lower ratio of C-5 : C-4 derived alcohols with Leu compared to Val (where such cyclisations do not occur) in these product studies and hence the discrepancy with the ratio of radicals reported in the current study.

## Experimental

Experiments were carried out using a Bruker EMX spectrometer equipped with 100 kHz modulation and either a standard rectangular (ER4102ST) or cylindrical (ER4103TM) cavity. The rapid flow system consisted of a glass two-stream cell with integral mixing chamber (Wilmad), with the flow maintained using either a Harvard Apparatus 22 syringe pump at a rate of 60 $cm^3\ min^{-1}$ or a Watson–Marlow 503-S peristaltic pump at a flow rate of 135 $cm^3\ min^{-1}$. The pH of the reaction effluent was measured using a Hanna Instruments H9321 pH meter and kept $\geqslant 3$ (*i.e.* above the $pK_a$ of the carboxylic acid function, which in the free amino acids is *ca.* 2.3; the di- and tripeptides have slightly higher $pK_a$ values). Solutions were prepared using water which had been passed through a four-stage Milli Q system equipped with a 0.2 μm-pore-size final filter and subsequently purged with oxygen-free nitrogen. One reaction stream contained $TiCl_3$ ($4 \times 10^{-3}$ mol $dm^{-3}$) together with EDTA ($6 \times 10^{-3}$ mol $dm^{-3}$), the second contained $H_2O_2$ ($12.5 \times 10^{-3}$ mol $dm^{-3}$) and the substrate ($5 \times 10^{-2}$ mol $dm^{-3}$); the concentrations stated are those after mixing. pH control was achieved using 2.5 M NaOH. All chemicals were commercial samples of high purity and used without further purification with the exception of the *N*-acetyl peptide derivatives which were prepared from the parent peptide using ethanoic anhydride–ethanoic acid using standard acetylation procedures.

Hyperfine coupling constants were measured directly from the field scan and confirmed by computer simulation of the experimental data using the program WINSIM which allows the simulation of multiple radical species, their relative concentrations, and the correlation coefficient between the simulated and experimental data;[43] the latter values were usually greater than 0.95, with the few exceptions due to base-line drift in the experimental spectra. Relative steady-state radical concentrations for different substrates were obtained by double integration of the radical signals using the Bruker software WINEPR, with each sample standardised against an EtOH spectrum run immediately after the sample using identical experimental and spectrometer conditions.


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