Radical Chemistry

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Carbon-Centered Radicals Can Transfer Hydrogen Atoms between Amino Acid Side Chains

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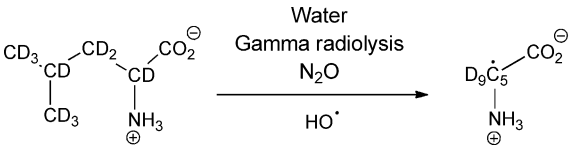
The nature and structure of radicals that can be formed in amino acids (AAs), peptides, and proteins have been extensively studied owing to their role in radiation, as well as in prebiotic chemistry and biochemistry. However, the discovery of the role of radicals in enzymatic catalysis has recently shed new light on the need for a better description of protein and AA radical reactivity. In these enzymes, tyrosyl,[1] thiyl,[2] and glycyl radicals[3] are able to transfer hydrogen atoms, either directly or by a sequential electron and proton transfer. Nevertheless, the properties of radicals from 17 other AAs, and especially of carbon-centered (C$^{\bullet}$) radicals that are involved in most protein oxidative degradations,[4] are only now beginning to be described.[5]

Herein, we show that, besides glycyl, tyrosyl, and thiyl radicals, C$^{\bullet}$ radicals from aliphatic AAs, such as leucine, can also efficiently transfer hydrogen atoms. This property of aliphatic AAs was identified by performing isotopic labeling experiments.

The initial C$^{\bullet}$ radicals were created by the action of hydroxyl radicals that were produced by the radiolysis of water. In a typical experiment, deuterated leucine was attacked by hydroxyl radicals in the presence of nondeuterated AAs. The concentrations of the deuterated and nondeuterated compounds were chosen so that most radicals (more than 90%) were initially created on deuterated leucine (Leu_D) by the reaction shown in [Eq. (1)] (see section 3 in the Supporting Information).



(1)

By using HPLC/MS, we quantified the incorporation of hydrogen atoms into Leu_D by transfer from nondeuterated donors, and the symmetric reaction of incorporation of deuterium atoms into nondeuterated compounds (see section 1 in the Supporting Information). The variation in the hydrogen content in deuterated leucine and in the deuterium content in nondeuterated leucine upon exposure to hydroxyl radicals of a Leu_D/Leu_H mixture is presented as an example in Figure 1. These two contents increase in proportion to the amount of hydroxyl radicals created by radiolysis in solution and, therefore, with the amount of C$^{\bullet}$ radicals formed on the AAs. We were careful to correct all possibilities of transfer from the solvent, such as transfer from radiolytically produced hydrogen peroxide (see section 4 in the Supporting Information). We also prevented deamination by using an N_2O atmosphere to quench hydrated electrons.

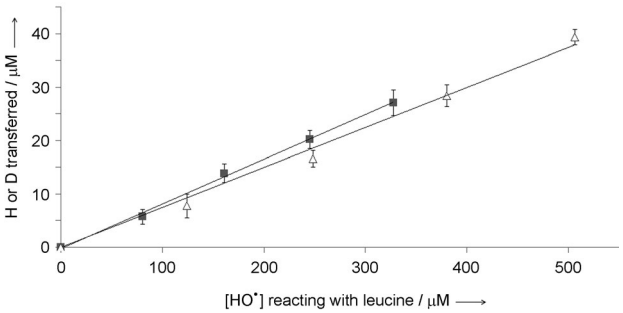
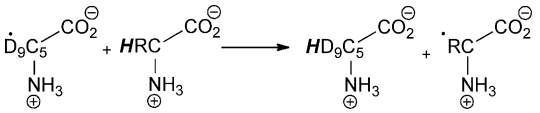
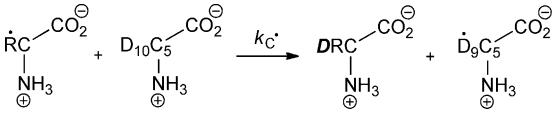


Figure 1. Hydrogen and deuterium transfers between deuterated leucine and nondeuterated leucine after reaction with an HO$^{\bullet}$ radical. ■ = deuterium transferred to nondeuterated leucine; △ = hydrogen transferred to deuterated leucine.

We can identify from Figure 1 both hydrogen and deuterium transfer between nondeuterated and deuterated leucine mediated by C$^{\bullet}$ radicals through the reactions shown in [Eq. (2)] and [Eq. (3)] where $R = C_4H_{10}$.



(2)



(3)

This transfer phenomenon seems quite general, as it was also detected for serine, asparagine, alanine, glutamine, proline, and, albeit with a low efficiency, glycine residues (Figure 2).

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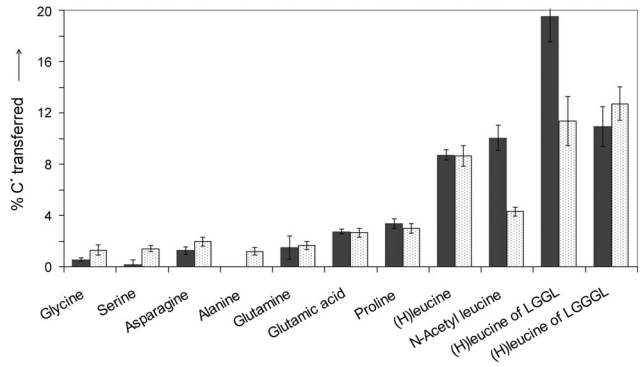
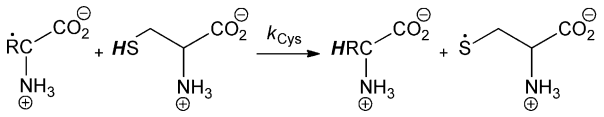


Figure 2. Proportion of the carbon-centered radicals formed by attack of an HO˙ radical that are transferred between various nondeuterated amino acids and deuterated leucine. Gray bars = radical transferred from the AA to deuterated leucine; white bars = radical transferred from deuterated leucine to the AA.

To check whether radical transfer could be detected for AAs that are incorporated into peptides, we prepared peptides with nondeuterated and deuterated leucine residues at their termini (see section 2 in the Supporting Information). The leucine residues were separated from the rest of the peptide by two (L_HGGL_D) or three (L_HGGGL_D) glycine residues. For both peptides, hydrogen and deuterium transfer was identified respectively in the L_D and L_H residues (Figure 2).

For most of these transfer reactions, the fact that the transfer efficiency is equivalent in [Eq. (2)] and [Eq. (3)] is a strong indication that the initial formation of a C˙ radical by attack of the hydroxyl radical can induce the sequence of transfers in [Eq. (2)] and [Eq. (3)], and thus can form chain reactions.[6] The transfer efficiency is clearly related to the number of hydrogen atoms that are present in the side chain of the AAs, with a regular increase when going from glycine to leucine. Such a dependence on the number of hydrogen atoms that are available for the reaction is quite common in hydrogen abstraction reactions.[7]

In a few cases we measured the kinetics of [Eq. (3)] by using a competition strategy (see section 5 in the Supporting Information). To do this, we measured the decrease in deuterium transfer upon the introduction of an alternative hydrogen transfer pathway, through the presence of free cysteine in solution [Eq. (4)].



(4)

The kinetics of the reference reaction in [Eq. (4)] are not precisely known. The repair of C˙ radicals by thiols usually occurs with kinetic constants in the range of 10^{-7}–$10^{-8}\,M^{-1}s^{-1}$,[8] but the equivalent reaction to [Eq. (4)] for alanine has a kinetic constant of only $5\times10^{6}\,M^{-1}s^{-1}$.[9] As we had no reason to suppose that [Eq. (4)] for other AAs is very different in efficiency, we used this constant for the reference reaction to provide a minimal estimate for $k_{C\cdot}$.

The various kinetic constants for [Eq. (3)] range from 10^5 to $10^6\,M^{-1}s^{-1}$ (Figure 3). Even taking into account the high kinetic isotope effect of H/D transfer reactions,[10] this order of magnitude is completely comparable, for example, to the

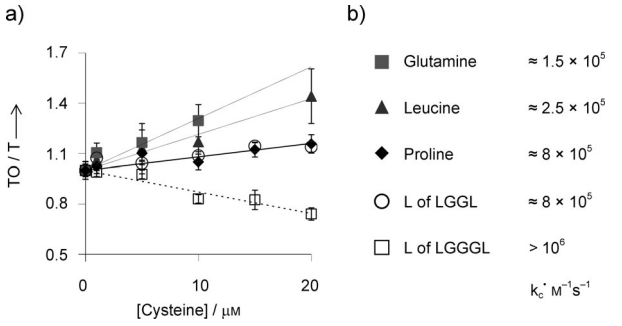


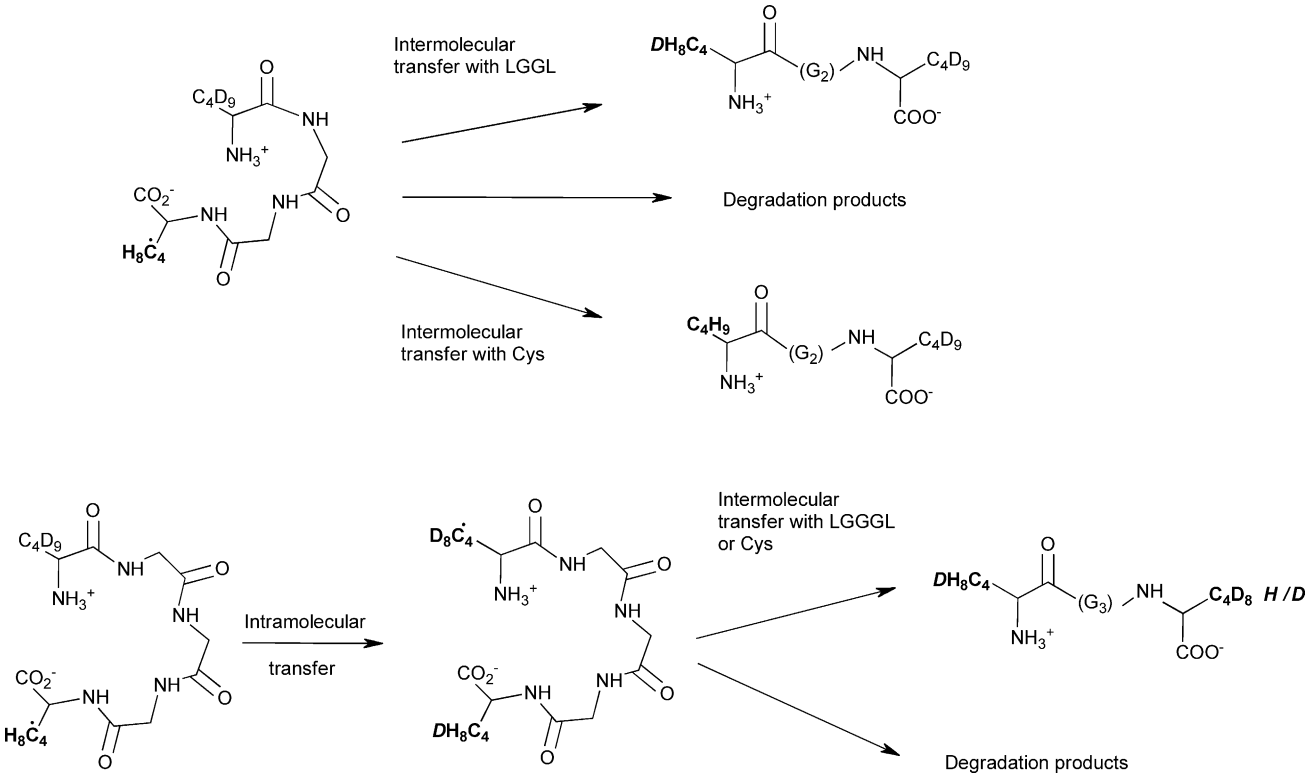
Figure 3. a) Ratio of the deuterium transfer from deuterated leucine measured in the absence of cysteine (T0) to that in the presence of cysteine (T), as a function of cysteine concentration. b) Kinetic constant $k_{C\cdot}$ of [Eq. (3)] evaluated by competition.

kinetics of hydrogen abstraction from alanine by methyl radicals ($(1.2\pm0.4)\times10^5\,M^{-1}s^{-1}$).[11] As expected from their lower (calculated) stability,[12] the C˙ radicals that form in proline are more reactive than those that form in leucine and glutamine. But this higher rate of deuterium transfer is not associated with more efficient labeling, which suggests that the transfer efficiency presented in Figure 2 is not directly related to the kinetics of the transfer reaction, but rather measures the capacity of the various AAs to sustain radical reactions without fragmentation or recombination.

The same competition method was applied to radical transfer in peptides. The reaction in [Eq. (3)] is only marginally faster for LGGL relative to the transfer to leucine. In contrast, no competition was detected for LGGGL. An increase in the efficiency of deuterium transfer was even measured upon the introduction of cysteine. We can rationalize these observations by assuming that the transfer occurs intermolecularly for LGGL and intramolecularly for LGGGL. When intermolecular processes are faster than intramolecular ones, cysteine acts directly as a competitor of the labeling step and, thus, favors the formation of unlabeled products (Scheme 1, top). When intramolecular processes are faster than intermolecular ones, cysteine reacts with radicals after their internal transfer. This late reaction of cysteine increases the labeling by preventing the degradation of labeled peptides after they have undergone a first hydrogen abstraction (Scheme 1 bottom). The existence of this protection reaction is supported by the finding that hydrogen labeling at deuterated sites increases upon the introduction of cysteine (Figure S3 in the Supporting Information).

Our results do not give direct clues about the origin of these different behaviors of LGGL and LGGGL. However, the introduction of an additional glycine residue is expected to increase the degrees of freedom of the peptide,[13] and to influence the kinetics of formation of short loops.[14]

To get a more detailed picture of the regioselectivity of hydrogen transfer to leucine, we conducted ^{1}H NMR experi-



Scheme 1. Impact of cysteine competition on intermolecular (top) and intramolecular (bottom) radical transfer processes.

ments on H/D labeling sites (Table 1, see also section 6 in the Supporting Information). The labeling of Leu_D resulting from hydrogen transfer from *N*-acetyl leucine and proline suggests

Table 1: Relative reactivity of leucine sites with respect to hydrogen transfer.

AA pair	α	β	γ	δ
$Leu_D/AcLeu_H$	< 5	20	37	42
Leu_D/Pro_H	10	6	32	50

a preferential localization of C· radicals on the γ and δ sites of leucine. The relative deactivation of the α site with respect to hydrogen transfer may seem surprising, considering the traditional view that radicals are stabilized at this position because of a captodative effect.[15,16] However, many studies confirm that the side chains are at least as active in radical reactions at the α position.[5,17–21] as a result of electronic deactivation of the α position in the radical formation step.[22]

Part of the regioselectivity may also be determined by the nature of the hydrogen donor in the radical repair step: whereas the γ and δ positions are preferred when the radical is repaired by proline, significant β labeling occurs also upon repair by *N*-acetyl leucine.

Molecular dynamics simulations show that the probability of finding hydrogen from *N*-acetyl leucine in the vicinity of leucine increases when going from the α to the δ position (Figures S4 and S5 in the Supporting Information). This calculation suggests that the formation of transient hydrophobic patches in solution controls the hydrogen transfer process.

Our experimental results demonstrate that, in solution, C· radicals can migrate between the side chains of AAs and intramolecularly in peptides. Such mobility was initially hypothesized based on theoretical calculations[15] and was used to explain some fragmentation schemes in peptide mass spectrometry.[23,24] Conversion between S· and C· radicals was even detected in peptides,[25,26] but this is, to our knowledge, the first observation of a real migration of C· radicals.

The question arises about the biological relevance of these findings, and especially about the possible involvement of hydrogen transfer between alkyl chains in radical enzymology. From a thermodynamic point of view, glycyl, tyrosyl, and cysteinyl radicals are formed with comparable gas-phase bond dissociation energies (BDE) of about 80 kcal mol^{-1},[27,28] whereas C–H bonds on secondary carbon atoms have BDEs of more than 96 kcal mol^{-1}.[29] These energy differences seemed to constitute a firm boundary between energetic species, such as side-chain C· radicals, which can only be formed by accident, and more stabilized radicals that are amenable to catalysis. Schoneich and co-workers were the first to break this boundary by demonstrating that cysteinyl radicals can activate β C–H bonds.[8,26] Radical transfer from a tyrosyl radical to a leucine residue was then detected in the gas phase.[24] We, in turn, measured a small but significant hydrogen transfer from a leucine residue to a glycyl radical (Figure 2), which confirms that an interplay between "stabilized" and "unstabilized" radicals is possible.

A kinetic point of view does not reveal differences in reactivity between these two types of species: the H· radical self-exchange rate of $1.8 \times 10^6\ \text{M}^{-1}\,\text{s}^{-1}$ for tyrosine[30] is only marginally more efficient than the Leu_D/Leu_H transfer analyzed here. Therefore, a hydrophobic cluster can provide hydrogen transfer pathways that are comparable to the tyrosine chains in radical enzymes.[31] It is noteworthy that hydrophobic clusters are encountered in the vicinity of various metallic sites in proteins (for instance, cytochrome P450 and methane monooxygenases) that are sufficiently reactive to initiate the formation of C· radicals.[32,33]

Our results are also relevant to the impact of oxidative stress on proteins. The formation of C radicals is the initial step of the irreversible protein carbonylation that occurs in various diseases and aging.[34] Protein carbonylation is a highly selective process that forms hot spots of modifications.[35] We suggest that the local propensity of protein side chains to undergo carbonylation depends on the competition between O_2 binding and radical transfer. Assuming an intracellular O_2 concentration of about 50 μM[36] and a diffusion-limited binding of O_2 to the radical site, the lifetime of C· radicals is estimated to be 20 μs (see section 8 in the Supporting Information). Depending on the uncertainty in the *k* values in Figure 1, we estimate that the distance that a radical could move during the same time is between 1–2 nm (see section 8 in the Supporting Information). The fact that radicals probably have enough time to diffuse some distance in proteins will favor the clustering of defects on the side chains that are the least efficient in transfer, which favors the appearance of so-called carbonylation hot spots. This hypothesis also raises the possibility that the radical sites formed in proteins migrate until they are finally deactivated by better hydrogen-donating groups, such as cysteine and tyrosine, thus, enhancing the role of these specific amino acids as intramolecular antioxidants.

Of all cell constituents, lipids were the first in which the propagation of radical damage was extensively described.[37] More recently, the long-distance transmission of damage in DNA[38–40] and from DNA to proteins[41,42] has been demonstrated. Our results suggest a need to re-evaluate the possibility of long-distance radical chemistry in proteins that occurs by atom transfer and, in turn, of defect transfer from proteins to DNA.

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