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Site Reactivity in the Free Radicals Induced Damage to Leucine Residues: A Theoretical Study

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Several recent computational studies have tried to explain the observed selectivity in radical damage to proteins. In this work we use Density Functional Theory and transition state theory including tunnelling corrections, reaction path degeneracy, the effect of diffusion, and the role of free radical to get further insights on this important topic. The reaction between a leucine derivative and free radicals of biological significance, in aqueous solution, have been investigated. Both thermochemical and kinetic analyses, in both hydrophilic and hydrophobic environments, have been carried out. DPPH, $^{\bullet}OOH$, $^{\bullet}OOCH_3$, $^{\bullet}OOCH_2Cl$, $^{\bullet}OOCHCl_2$ and $^{\bullet}OOCHCH_2$ radicals do not react with the target molecule. The reactions are proposed to be kinetically controlled. The leucine gamma site was the most reactive for the reactions with $^{\bullet}N_3$, $^{\bullet}OOCCl_3$, $^{\bullet}OCH_3$, $^{\bullet}OCH_2Cl$, $^{\bullet}OCHCl_2$ and $^{\bullet}OCHCH_2$ radicals, with rate constants equal to 1.97×10^5, 3.24×10^4, 6.68×10^5, 5.98×10^6 and 8.87×10^8 M^{-1} s^{-1}, respectively. The $^{\bullet}OH$ and $^{\bullet}OCCl_3$ radicals react with leucine at the beta, gamma, and delta positions at rates close to the diffusion limit being the alpha position the less reactive one. The presented results confirm that the Bell-Evans-Polanyi principle does not apply for the reactions between amino acid residues and free radicals. Regarding the influence of the environment on the reactivity of the studied series of free radicals towards leucine residues, it is concluded that hydrophilic media slightly lower the reactivity of the studied radicals, compared to hydrophobic ones, albeit the trends in reactivity are very similar.

Introduction

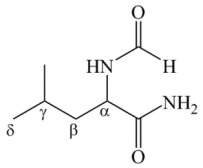
Oxidative stress (OS) has become a major concern regarding human health. It is currently associated to the development of numerous diseases such as cancer,[1-3] cardiovascular disorders,[4-6] atherosclerosis,[7-10] fetal growth restriction and preeclampsia,[11-13] and several neurological disorders including Parkinson's and Alzheimer's diseases.[14-16] OS is a chemical stress that arises as a result of an imbalance between the production and consumption of oxidative species, including reactive oxygen and nitrogen species (ROS and RNS). Most of such species are free radicals such as superoxide radical anion ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), alkoxyl ($^{\bullet}OR$), peroxyl ($^{\bullet}OOR$) and hydroperoxyl ($^{\bullet}OOH$). They are frequently able of oxidizing essential biological molecules such as fatty acids, proteins and DNA, causing cell damage.

As it is the case of many organic molecules, free alkyl side chain amino acids are highly reactive towards the $^{\bullet}OH$ radical via hydrogen transfer (HT).[17-21] Among them, alanine is the least reactive one and even in this case the HT rate constant is 7.7×10^7 M^{-1} s^{-1}. On the contrary leucine is the most reactive one with a rate constant equal to 1.3×10^9 M^{-1} s^{-1} when reacting with the $^{\bullet}OH$ radical.[22,23] However the environment of the alkyl side chain, and particularly of the alpha position, is very different in proteins, which are the real targets in biological systems, with respect to the free amino acids. This is essentially because of the zwitterionic form of the last ones. In fact it has been shown that N acetylated amino acids are less reactive than the free ones.[22]

The deactivating polar effect has been held responsible for the high barrier of the hydrogen transfer (HT) reaction from the alpha position.[24] This effect can be defined as the destabilization of the transition state caused by the interaction of two separated effects. The first one is the electron deficiency in the alpha carbon due to the electron-withdrawing character of the substituent and the second arise from the electrophilic radicals.[24]

The regioselectivity observed in these reactions show that HT does not occurs at the alpha and beta sites in amino acids. On the contrary, the reactions are favored at distal positions from this first bond. In the particular case of leucine, the HT reaction has been proposed to take place at the γ-CH and δ-CH_3 sites.[22-26] The site selectivity observed in the reaction with the $^{\bullet}OH$ radical has been explained by the formation of reactant complexes between $^{\bullet}OH$ and the NH and C=O groups in the amino acid moiety. The stability of this complexes was assumed to represent a kinetic trap that increases the reaction barrier from the alpha position.[27] However, more recently,[28] this explanation was demonstrated to be unsatisfactory since, when entropy contributions are included, the "kinetic trap" is found to be unstable in terms of Gibbs free energy, which is the most appropriate criterion according second law of thermodynamics. In that work,[28] the oxidation of amino acids

by radicals was investigated using a realistic model (Scheme 1) and the correct thermodynamic function.
Even though this model has been widely used and accepted, please see reference[28] and references therein, we feel necessary to make clear why it is appropriate. At first sight it might seem too simple for modelling something that occurs in a protein, however what makes proteins unique is their tertiary structure with different residues located in an especial configuration for performing certain functions. For mimicking such processes, the chosen model should include all the important information and it is necessary to use hybrid methods such as QM/MM. On the other hand, proteins are not designed to be oxidized. Oxidation is an undesirable process that can involve any residue, located in any protein region. Since usual oxidants are not protein targets, no specific protein orientation or conformation is necessary for the oxidation to take place. Moreover, it is known that oxidative attacks occur randomly, and non-specifically, because of the very high reactivity and low selectivity of the OH radical (the most common radical for protein damage in biological systems). In addition, because of the lack of unsaturations in proteins backbones, the electronic effects cannot propagate further than two sigma bonds. Consequently the rest of the protein has no important effects on the oxidation processes, provided that the residue is exposed to the environment, and the used model is excellent for protein damage and repairing.



Scheme 1. N-formylleucinamide (1), molecule model for amino acids in peptides.[28]

However, there are still several aspects to explore that may be relevant to the study of the amino acids reactivity when they are in a protein environment. For example using 1 M standard state and including solvent cage effects for correcting the entropy loss in solution. Obtaining rate constants including quantum tunneling corrections, and reaction path degeneracy, when applicable, as well as the role of the diffusion limit, imposed by the solvent, for very fast reactions can also be important. This an alternative way of analyzing reactivity, probably more complete than using only reaction barriers, which has the additional advantage of being directly comparable with experimental data. Unfortunately the kinetic data available for the reactions of free radicals with amino acids residues in peptide environments are very scarce.
In addition, it is also important to note that in living organisms there is a large variety of free radicals, which might damage amino acids, while most of the previous theoretical works have been performed using $^{\bullet}$Cl and $^{\bullet}$OH. Therefore, a systematic investigation on the reactivity of diverse free radicals towards amino acids, based on kinetic considerations, seems relevant to gain deeper knowledge on the potential damage to residues under physiological conditions.
Proteins are usually in the aqueous phase and the damage is expected to occur in their residues exposed to the solvent. This is mainly because very reactive radicals, like $^{\bullet}$OH, are expected to be formed and react immediately in such environment. There is a possibility that they are in close contact with the lipid environment of the membranes. Moreover, regions containing alkyl side chain amino acids could be in a hydrophobic zone. In such situations water is too polar to account for the solvent influence and a more hydrophobic solvent would provide a more accurate model of the residue reactivity. For this reason we have also modelled the studied reactions in hydrophobic environment, using pentyl ethanoate (PE) as solvent. It was chosen because it is the largest ester for which parameters are available in the used program Gaussian 09[29]. Thus, it is the solvent closest to lipids available for the modeling.
According to the above discussion, the main goal of the present work is to provide kinetic data on the reactions of leucine residues with diverse free radicals. To this purpose we have used the N-formylleucinamide (Scheme 1), to mimic leucine in a protein environment. We have used exactly the same model proposed in reference[28] because it represents an excellent compromise between size and resemblance of leucine residue in a protein. Leucine has been chosen because of its high reactivity compared to other alkyl side chain amino acids[22, 23], which makes it a likely candidate to free radical damage. The investigated set of free radicals comprises DPPH, $^{\bullet}N_3$, $^{\bullet}NO_2$, $^{\bullet}$OR and $^{\bullet}$OOR, with R=H, CH_3, CH_2Cl, $CHCl_2$ and CCl_3. They present different intrinsic reactivity and have been chosen to represent ROS and RNS, which are relevant to biological systems.[30] The selectivity was analysed using the kinetic criterion, i.e. rate constants that depend on the Gibbs free energy of activation but that also include tunnelling corrections, reaction path degeneracy, solvent cage effects, and diffusion limits.

Computational details

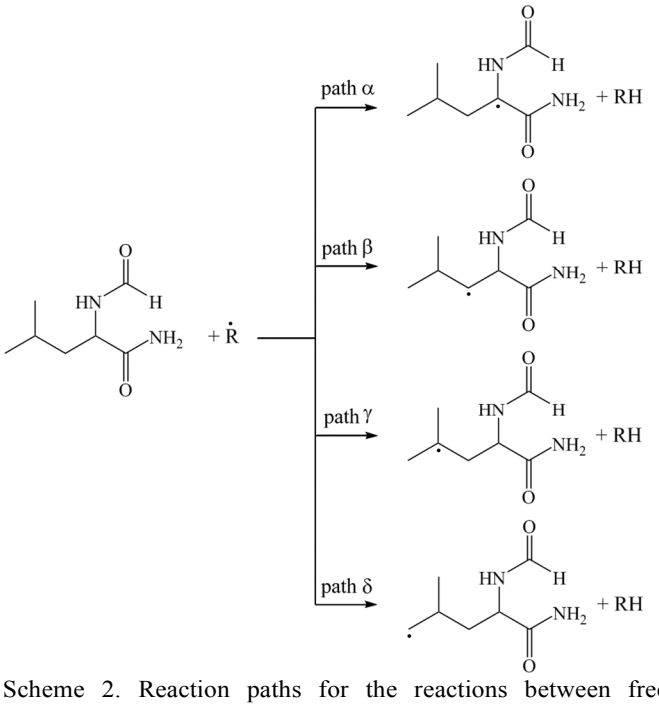
The computational methodology used in this manuscript is a protocol developed by our group and it is described in detail in previous publications, particularly in reference31. However we repeat here the main details so the reader can see them without additional reading. All electronic calculations were performed with the Gaussian 09 package of programs. Geometry optimizations and frequency calculations were carried out using the M05-2X[32] and the 6-311+G(d,p) basis set, in conjunction with the SMD continuum model[33] using water and pentyl ethanoate as solvents. The M05-2X functional has been recommended for kinetic calculations by its developers,[32] and it has been successfully used by authors other than the developers to that purpose. [34-38] It is also among the best performing functional for calculating reaction energies involving free radicals.[39] SMD is considered a universal solvation model, due to its applicability to any charged or uncharged solute in any solvent or liquid medium for which a few key descriptors are known.[33] SMD has no adjustable parameters as is the case of more commonly used IEF-PCM, which sometimes lead to artificial inconsistencies. It is important to mention that, according to the developers, SMD can be successfully used for optimization and frequency calculations in solution,[40] while other continuum solvation models like COSMO have been disqualified for thermodynamic corrections in solution.[41]
Unrestricted calculations were used for open shell systems. Local minima and transition states were identified by the number of imaginary frequencies (0 and 1, respectively). Relative energies are calculated with respect to the isolated reactants. Thermodynamic corrections at 298.15 K were included in the calculation of relative energies, which correspond to 1M standard state. In addition the solvent cage effects have been included according to the corrections proposed by Okuno,[42] taking into account the free volume theory.[43] This correction is important because the cage effects

of the solvent reduce the entropy loss associated with any chemical reaction with molecularity equal or larger than two.
The rate constants (k) were calculated using the Conventional Transition State Theory (TST).[44-46] The Gibbs free energy of activation ($\Delta G^{\neq}$) used in these calculations are equivalent to the barriers calculated in reference 28. Tunnelling corrections were included and calculated using the Zero Curvature Tunnelling corrections (ZCT).[47] For the calculated rate constants that are close to, or within, the diffusion-limited regime the apparent rate constant (k_{app}) cannot be directly obtained from TST calculations. In the present work the Collins-Kimball theory[48] is used to that purpose, in conjunction with the steady-state Smoluchowski[49] and the Stokes–Einstein[50,51] approaches.
The methodology used in this work has been previously proven to accurately reproduce experimental rate constants in solution.[31]

Results and discussion

Aqueous media

The reaction paths included in the present study are shown in Scheme 2. Regarding the thermochemical viability of these reactions (Table 1), it was found that for $^{\bullet}$DPPH, $^{\bullet}NO_2$, $^{\bullet}OCHCH_2$, $^{\bullet}OOCH_3$, and $^{\bullet}OOCHCH_2$ radicals the reactions are endergonic regardless of the reaction site. Thus, these radicals are not able of directly damaging leucine in biological systems. For the reactions involving $^{\bullet}OOH$ and $^{\bullet}OOCH_2Cl$ it was found that the HT reactions at the α sites are slightly endergonic (< 1.5 kcal/mol), which suggests that this reaction path might contribute, to some extent, to the leucine damage. This is also the case for path γ in the reactions involving $^{\bullet}N_3$.



Scheme 2. Reaction paths for the reactions between free radicals and leucine.

In the case of $^{\bullet}OOCHCl_2$ radical the HT reaction was exergonic at the α site, while at the other sites significantly positive Gibbs free energies of reaction (ΔG) are predicted. For the $^{\bullet}OOCCl_3$ radical the HT reaction is thermochemical viable at two different sites, α and γ, with $\Delta G\alpha < \Delta G\gamma$. The HT reactions for the rest of the studied radicals ($^{\bullet}Cl$, $^{\bullet}OH$, $^{\bullet}OCH_3$, $^{\bullet}OCH_2Cl$, $^{\bullet}OCHCl_2$, and $^{\bullet}OCCl_3$) were all exergonic, with the largest exergonicity systematically corresponding to path α. Among these radicals, $^{\bullet}OH$ is the one leading to the most negative ΔG values. It seems worthwhile to call attention to the fact that, according to the above discussed results, analyses based only on thermochemical considerations might lead to conclude that the α site is the most reactive in leucine residues.
For the kinetic study we have not included the reaction paths found as endergonic because, even if they take place at significant rates, they would be reversible and, therefore, the formed products will not be observed. However, they might still represent significant channels if their products rapidly react further. This would be particularly important if these later stages are sufficiently exergonic to provide a driving force, and if their barriers of reactions are low. In addition, slightly endergonic processes can be important when there are no exergonic competing paths. This is the case of path α in the reactions involving $^{\bullet}OOH$, $^{\bullet}OOCH_3$ and $^{\bullet}OOCH_2Cl$. That is why they have also been included in the kinetic study.

Table 1. Free energies (ΔG, kcal/mol) of the HT reactions, at 298.15K.

Radical	path α	path β	path γ	path δ
$^{\bullet}$DPPH	10.25	19.16	15.08	21.39
$^{\bullet}N_3$	-4.79	4.12	0.03	6.34
$^{\bullet}NO_2$	3.48	12.39	8.31	14.62
$^{\bullet}Cl$	-16.02	-7.11	-11.19	-4.88
$^{\bullet}OH$	-31.88	-22.97	-27.05	-20.74
$^{\bullet}OCH_3$	-16.25	-7.34	-11.42	-5.11
$^{\bullet}OCH_2Cl$	-17.23	-8.32	-12.41	-6.09
$^{\bullet}OCHCl_2$	-22.47	-13.56	-17.64	-11.33
$^{\bullet}OCCl_3$	-25.06	-16.15	-20.23	-13.92
$^{\bullet}OCHCH_2$	4.43	13.34	9.25	15.57
$^{\bullet}OOH$	1.35	10.26	6.18	12.49
$^{\bullet}OOCH_3$	3.08	11.99	7.90	14.22
$^{\bullet}OOCH_2Cl$	0.80	9.71	5.62	11.94
$^{\bullet}OOCHCl_2$	-3.61	5.30	1.22	7.53
$^{\bullet}OOCCl_3$	-5.97	2.94	-1.14	5.17
$^{\bullet}OOCHCH_2$	1.69	10.60	6.51	12.83

The kinetic data is reported in Table 2. For the peroxyl and alcoxyl series of radicals, their reactivity increases with the halogenation degree. As expected there is an inverse relationship between reactivity and selectivity. For the reactions involving $^{\bullet}Cl$ all the reaction paths have similar contributions to the overall reactivity. For $^{\bullet}OH$ there are two main reaction paths (γ and β, in that order), while the other two (δ and α) also have significant contributions (higher than 10%). For $^{\bullet}OCCl_3$ the situation is similar, but with the most important paths being α and δ, followed by γ, with small but not negligible contributions from path β. Moving forward in the series, the relative importance of the HT from the γ site increases. The exceptions are those radicals for which this reaction path was ruled out, based on thermochemical considerations ($^{\bullet}OOCH_2Cl$, $^{\bullet}OOH$, $^{\bullet}OOCHCH_2$, and $^{\bullet}OOCH_3$).
It seems important to note that for those reactions with exergonic HT from sites other than α, this is never the main reaction channel when the analysis is based on the kinetic results. This is a clear example of reactions controlled by kinetics, for which performing only thermochemical-based analyses would not be enough to properly describe site reactivity, i.e. the Bell-Evans-Polanyi principle does not apply. Therefore more simple studies, such as those based on bond dissociation energies of even on reaction energies would not be

appropriate. The crucial role of kinetics studies on the reactions between amino acids and free radicals has also been pointed out previously by Chan et al.[28] Our results are in agreement with the data reported in that work and confirm their proposal.

The total rate constants for the reactions of 1 with $^{\bullet}$Cl, $^{\bullet}$OH, $^{\bullet}OCCl_3$, and $^{\bullet}OCHCl_2$ were found to be within, or close to, the diffusion limited regime. This indicates that these radicals are particularly damaging, via HT, for leucine residues in proteins. A second subset including $^{\bullet}OCH_2Cl$, $^{\bullet}OCH_3$, $^{\bullet}N_3$, $^{\bullet}OOCCl_3$, and $^{\bullet}OOCHCl_2$ are also predicted to be dangerous to the proteins integrity, but to a lower extent (at least via HT), with rate constants ranging from 10^3 to 10^7 M^{-1} s^{-1}. On the contrary the reactions of $^{\bullet}OOCH_2Cl$, $^{\bullet}OOH$, $^{\bullet}OOCHCH_2$, and $^{\bullet}OOCH_3$ are so slow (< 100 M^{-1} s^{-1}) that they are not supposed to represent a risk for leucine residues. Based on the calculated kinetic data, the trend in reactivity (in decreasing order) of the studied radicals, towards leucine residues in aqueous solution, is proposed to be $^{\bullet}$Cl, $^{\bullet}$OH, $^{\bullet}OCCl_3$, $^{\bullet}OCHCH_2$, $^{\bullet}OCH_2Cl$, $^{\bullet}OCH_3$, $^{\bullet}N_3$, $^{\bullet}OOCCl_3$, $^{\bullet}OOCHCH_2$, $^{\bullet}OOCH_2Cl$, $^{\bullet}OOH$, $^{\bullet}OOCHCH_2$, and $^{\bullet}OOCH_3$.

Table 2. Gibbs energies of activation ($\Delta G^{\neq}$, kcal/mol), enthalpies of activation ($\Delta H^{\neq}$, kcal/mol), tunnelling corrections (κ), diffusion rate constants (k_D, M^{-1} s^{-1}), apparent rate constants (k_{app}, M^{-1} s^{-1}), and branching ratios (Γ, %), at 298.15 K.

Path	$\Delta G^{\neq}$	$\Delta H^{\neq}$	κ	k_D	k_{app}	Γ
			$^{\bullet}N_3$			
α	16.76	10.63	20.19	2.63×10^{9}	6.50×10^{1}	0.03
γ	10.88	5.43	2.97	2.63×10^{9}	1.96×10^{5}	99.97
Total					1.97×10^{5}	
			$^{\bullet}$Cl			
α	0.00	-3.91	1.00	3.56×10^{9}	3.56×10^{9}	24.20
β	0.00	-3.94	1.00	3.55×10^{9}	3.55×10^{9}	24.13
γ	0.00	-4.04	1.00	4.04×10^{9}	4.04×10^{9}	27.46
δ	0.00	-3.75	1.00	3.51×10^{9}	3.51×10^{9}	23.98
Total					1.47×10^{10}	
			$^{\bullet}$OH			
α	4.93	-0.76	1.00	2.94×10^{9}	9.92×10^{8}	11.73
β	3.79	-1.48	1.00	2.95×10^{9}	2.58×10^{9}	30.50
γ	3.28	-1.76	1.00	3.01×10^{9}	2.68×10^{9}	31.68
δ	5.07	0.25	1.00	2.94×10^{9}	2.21×10^{9}	26.09
Total					8.46×10^{9}	
			$^{\bullet}OCH_3$			
α	12.17	5.55	11.39	2.59×10^{9}	8.53×10^{4}	12.76
β	14.06	7.18	16.06	2.57×10^{9}	9.92×10^{3}	1.48
γ	10.47	3.80	4.08	2.60×10^{9}	5.37×10^{5}	80.36
δ	13.62	6.59	9.31	2.56×10^{9}	3.60×10^{4}	5.39
Total					6.68×10^{5}	
			$^{\bullet}OCH_2Cl$			
α	9.38	2.59	3.05	2.49×10^{9}	2.50×10^{6}	41.86
β	12.80	5.31	7.59	2.49×10^{9}	3.93×10^{4}	0.66
γ	8.92	1.38	1.93	2.49×10^{9}	3.44×10^{6}	57.48
δ	16.58	10.12	3.46	2.49×10^{9}	9.07×10^{1}	0.00
Total					5.98×10^{6}	
			$^{\bullet}OCHCl_2$			
α	6.80	-0.63	1.00	2.48×10^{9}	6.25×10^{7}	7.05
β	8.79	1.23	1.81	2.45×10^{9}	8.11×10^{6}	0.91
γ	5.07	-2.08	1.00	2.51×10^{9}	8.04×10^{8}	90.64
δ	9.09	0.88	1.54	2.46×10^{9}	1.24×10^{7}	1.40
Total					8.87×10^{8}	
			$^{\bullet}OCCl_3$			
α	0.62	-5.93	1.00	2.48×10^{9}	2.48×10^{9}	38.84
β	6.41	-0.54	1.00	2.48x109	2.24×10^{8}	3.52
γ	4.22	-3.73	1.00	2.48×10^{9}	1.66×10^{9}	26.02
δ	4.83	-1.49	1.00	2.48×10^{9}	2.02×10^{9}	31.62
Total					6.38×10^{9}	
			$^{\bullet}$OOH			
α	20.77	13.83	67.50	2.74×10^{9}	2.49×10^{-1}	100
			$^{\bullet}OOCH_3$			
α	21.84	14.70	97.11	2.57×10^{9}	5.90×10^{-2}	100
			$^{\bullet}OOCH_2Cl$			
α	20.05	11.35	39.45	2.46×10^{9}	4.92×10^{-1}	100
			$^{\bullet}OOCHCl_2$			
α	16.07	7.62	16.20	2.33×10^{9}	1.67×10^{2}	4.20
γ	13.52	5.41	5.19	2.33×10^{9}	3.98×10^{3}	95.97
Total					4.15×10^{3}	
			$^{\bullet}OOCCl_3$			
α	16.37	8.27	12.60	2.23×10^{9}	7.82×10^{1}	0.24
γ	12.03	5.57	3.41	2.27×10^{9}	3.23×10^{4}	99.76
Total					3.24×10^{4}	
			$^{\bullet}OOCHCH_2$			
α	20.79	8.89	23.06	2.46×10^{9}	8.25×10^{-2}	100

The transition states geometry are shown in Figure S1. (supplementary information) It can be observed that for all the cases under analysis, the gamma transition state is the earliest one. Thus, according to the Hammond postulate this route should be the fastest HT reaction. This is in agreement with the kinetic data in those cases where path γ is thermochemicaly viable. In the particular case of $^{\bullet}$OH, all transition states present H bond interactions, which are expected to lower the reaction barriers contributing to the high reactivity of this radical.

In general the interaction distances are larger for the TSs corresponding to HT from α sites, which indicates weaker interactions and thus justify why the barriers for this channels are higher, i.e. the reactions are slower. This structural features support the lower reactivity of α sites, and represent another factor contributing to the protection of the integrity of the peptide backbone, in addition to the deactivating polar effect previously proposed.[24,28] This effect indicates that the α sites are deactivated with respect to the remote sites, while the H bonding interactions in the transition states suggest a relative activation of the remote sites compared to the α ones.

Lipid media

To account for the reactivity of Leucine residues in the hydrophobic zones of proteins, the study of the reaction between leucine and the free radicals was also carried out in non-polar media. The transition states geometry are shown in Figure S2. (supplementary information) The corresponding thermochemical data is shown in Table 3. It was found that the reactions with radicals $^{\bullet}NO_2$, $^{\bullet}OCHCH_2$, $^{\bullet}OOH$, $^{\bullet}OOCH_3$, $^{\bullet}OOCH_2Cl$, $^{\bullet}OOCHCl_2$ and $^{\bullet}OOCHCH_2$, via HT, are all endergonic. Radical $^{\bullet}OOCCl_3$ has only one exergonic route, that involves the alpha position, while for the $^{\bullet}N_3$ radical, two routes (α and γ) are thermochemical viable. On the other hand, all the modelled reactions were found to be exergonic for radicals $^{\bullet}$Cl, $^{\bullet}$OH, $^{\bullet}OCH_3$, $^{\bullet}OCH_2Cl$, $^{\bullet}OCHCl_2$ and $^{\bullet}OCCl_3$. In general, as the electrophilicity of the radical increases, so does the exergonicity of the reactions.

Table 3. Free energies (ΔG, kcal/mol) of the HT reactions in lipid media, at 298.15K.

Radical	path α	path β	path γ	path δ
$^{\bullet}N_3$	-2.55	5.31	-0.47	5.86
$^{\bullet}NO_2$	10.01	17.87	12.08	18.41
$^{\bullet}$Cl	-13.21	-5.35	-11.13	-4.80
$^{\bullet}$OH	-27.55	-19.69	-25.47	-19.14
$^{\bullet}OCH_3$	-12.07	-4.21	-9.99	-3.66

$^{\bullet}OCH_2Cl$	-12.41	-4.55	-10.33	-4.00
$^{\bullet}OCHCl_2$	-16.26	-8.40	-14.18	-7.85
$^{\bullet}OCCl_3$	-19.38	-11.52	-17.30	-10.97
$^{\bullet}OCHCH_2$	7.92	15.78	9.99	16.32
$^{\bullet}OOH$	5.89	13.75	7.97	14.30
$^{\bullet}OOCH_3$	9.17	17.03	11.25	17.58
$^{\bullet}OOCH_2Cl$	5.63	13.49	7.71	14.04
$^{\bullet}OOCHCl_2$	2.18	10.04	4.26	10.59
$^{\bullet}OOCCl_3$	-0.37	7.49	1.71	8.04
$^{\bullet}OOCHCH_2$	6.89	14.76	8.97	15.30

The kinetic study was carried out only for the thermochemical viable reaction paths (Table 4). This is because, as the results for

Table 4. Gibbs energies of activation ($\Delta G^{\neq}$, kcal/mol), enthalpies of activation ($\Delta H^{\neq}$, kcal/mol), tunnelling corrections (κ), diffusion rate constants (k_D, M^{-1} s^{-1}), apparent rate constants (k_{app}, M^{-1} s^{-1}), and branching ratios (Γ, %) in lipid media, at 298.15 K.

Path	$\Delta G^{\neq}$	$\Delta H^{\neq}$	κ	k_D	k_{app}	Γ
				$^{\bullet}N_3$		
α	15.86	8.74	8.89	2.86×10^9	1.31×10^2	0.1
γ	11.39	5.45	3.87	2.86×10^9	1.08×10^5	99.9
Total					1.08×10^5	
				$^{\bullet}Cl$		
α	0.00	-2.84	1.00	3.71×10^9	3.69×10^9	25.3
β	0.00	-3.30	1.00	3.43×10^9	3.43×10^9	23.4
γ	0.73	-2.52	1.00	4.04×10^9	4.03×10^9	27.6
δ	0.00	-2.85	1.00	3.47×10^9	3.47×10^9	23.8
Total					1.46×10^{10}	
				$^{\bullet}OH$		
α	3.00	-3.43	1.00	3.22×10^9	2.97×10^9	24.0
β	2.80	-3.54	1.00	3.19×10^9	3.10×10^9	25.0
γ	2.04	-3.81	1.00	3.28×10^9	3.22×10^9	26.0
δ	3.47	-2.92	1.00	3.20×10^9	3.10×10^9	25.0
Total					1.24×10^{10}	
				$^{\bullet}OCH_3$		
α	12.40	5.29	6.83	2.71×10^9	3.47×10^4	41.6
β	13.54	6.23	9.41	2.68×10^9	1.39×10^4	16.7
γ	12.32	5.01	5.08	2.70×10^9	2.93×10^4	35.1
δ	14.83	7.27	11.08	2.67×10^9	5.52×10^3	6.6
Total					8.35×10^4	
				$^{\bullet}OCH_2Cl$		
α	10.79	2.79	1.92	2.65×10^9	1.48×10^5	12.4
β	12.58	5.33	8.07	2.58×10^9	5.98×10^4	5.0
γ	9.83	2.61	2.26	2.64×10^9	8.70×10^5	73.2
δ	12.52	4.35	4.52	2.58×10^9	1.11×10^5	9.3
Total					1.19×10^6	
				$^{\bullet}OCHCl_2$		
α	8.46	0.65	1.32	2.58×10^9	5.17×10^6	1.9
β	7.18	-4.44	1.00	2.51×10^9	6.56×10^7	24.6
γ	6.30	-1.89	1.00	2.51×10^9	1.42×10^8	53.4
δ	8.02	0.32	1.10	2.50×10^9	5.34×10^7	20.0
Total					2.66×10^8	
				$^{\bullet}OCCl_3$		
α	1.47	-6.06	1.00	2.59×10^9	2.58×10^9	91.3
β	9.29	0.64	1.29	2.50×10^9	2.46×10^6	0.1
γ	6.29	-1.13	1.00	2.60×10^9	1.45×10^8	5.1
δ	7.76	0.81	1.34	2.55×10^9	9.87×10^7	3.5
Total					2.83×10^9	
				$^{\bullet}OOCCl_3$		
α	18.55	18.55	18.55	2.32×10^9	1.17	100.0

the aqueous phase show, the reaction paths with low but positive Gibbs free energies of reaction, lead to very low rate constants. The γ site was found to be responsible for almost all the reactivity of leucine towards the $^{\bullet}N_3$ radical. This site was also identified as the main channel of reaction for radical $^{\bullet}OCHCl_2$, but in this case β and δ routes also have significant contributions to the overall reactivity. For the reactions with $^{\bullet}OH$ and $^{\bullet}Cl$, the rate constants for all the reaction paths are diffusion limited, i.e., there is no significant site selectivity. In the case of chlorine the are no reaction barriers (they were found to be negative, albeit the transition states can be located). Since according to TST the lower limit to reaction barriers is zero, which is the value used in this case. The α site was identified as the main reaction channel for the leucine reaction with $^{\bullet}OCCl_3$, while both α and γ sites represent significant reaction channels for the reactions involving $^{\bullet}OCH_2Cl$ and $^{\bullet}OCH_3$. Regarding the overall reactivity of the studied radicals towards leucine, in lipid media, the trend was found to be $^{\bullet}Cl$ > $^{\bullet}OH$ > $^{\bullet}OCCl_3$ > $^{\bullet}OCHCH_2$ > $^{\bullet}OCH_2Cl$ > $^{\bullet}N_3$ > $^{\bullet}OCH_3$ > $^{\bullet}OOCCl_3$.

It is almost identical to the one found for aqueous solution. The only exception correspond to radicals $^{\bullet}OCH_3$ and $^{\bullet}N_3$. In lipid media the latter was found to be more reactive, while in aqueous solution their relative reactivity is inverted. On the other hand, the similarities are numerous. The total rate constants for the reactions involving $^{\bullet}Cl$, $^{\bullet}OH$, $^{\bullet}OCCl_3$, and $^{\bullet}OCHCl_2$ are within, or close to, the diffusion limited regime. It has found that the Cl and $^{\bullet}OH$ radicals are the most reactive ones, the alkoxyl radicals are more reactive than the peroxyl radicals with similar halogenation degree, and the reactivity increases with the halogenation degree.

In addition, for all the reactions that are not diffusion-controlled, the rate in constants in lipid media are lower (2.3 to 8 times) than in aqueous solution. Thus it is proposed that hydrophobic environments slightly reduce the reactivity of the studied radicals towards leucine residues, compared to hydrophilic ones. The only exception is the reaction with $^{\bullet}OOCCl_3$ because in aqueous solution the γ channel is exergonic, with a rate constant equal to 3.24×10^4 $M^{-1}s^{-1}$, while in the hydrophobic phase it is endergonic, i.e., thermodynamically forbidden. In addition, channels that are exergonic in aqueous solution also become endergonic in hydrophobic media for the reactions of peroxyl radicals less reactive than $^{\bullet}OOCCl_3$.

Conclusions

The study of the HT reactions between leucine derivative 1 and a large series of free radicals, in aqueous solution was carried out using the Density Functional Theory. For $^{\bullet}OCHCH_2$, $^{\bullet}NO_2$, and $^{\bullet}$DPPH all the reaction paths were found to be significantly endergonic, which indicate that they do not to react with leucine.

Based on kinetic considerations it can be concluded that $^{\bullet}Cl$, $^{\bullet}OH$, $^{\bullet}OCCl_3$, and $^{\bullet}OCHCl_2$ are particularly damaging, via HT, for leucine residues in proteins, since the rate constants of such reactions are within, or close to, the diffusion limited regime. $^{\bullet}OCH_2Cl$, $^{\bullet}OCH_3$, $^{\bullet}N_3$, $^{\bullet}OOCCl_3$, and $^{\bullet}OOCHCl_2$ are also predicted to be dangerous to the proteins alkyl side chain integrity, but to a lower extent because their reactions are slower with rate constants ranging from 10^3 to 10^7 M^{-1} s^{-1}. On

the contrary, the reactions of $^{\bullet}OOCH_2Cl$, $^{\bullet}OOH$, $^{\bullet}OOCHCH_2$, and $^{\bullet}OOCH_3$ are so slow (< 100 M-1 s-1) that they are not expected to represent a risk for leucine residues. This is an important conclusion because alkylperoxyl and hydroperoxyl radicals are among most abundant ones in living systems.

In general γ sites were found to be the more susceptible to radical damage, except for the reactions involving $^{\bullet}OOCH_2Cl$, $^{\bullet}OOH$, $^{\bullet}OOCHCH_2$, and $^{\bullet}OOCH_3$, for which this reaction path was ruled out, based on thermochemical considerations. On the other hand thermochemical calculations show that the Gibbs energies of reaction are lower for the α sites. These results confirm the crucial role of kinetics studies on the reactions between amino acids and free radicals.

Regarding the influence of the environment on the reactivity of the studied series of free radicals towards leucine residues it can be concluded that hydrophobic media slightly lower the reactivity of the studied radicals towards, compared to hydrophilic ones, albeit the trends in reactivity are very similar.

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