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Modelling the Chemical Repair of Protein Carbon-Centered Radicals Formed via Oxidative Damage with Dihydrolipoic Acid

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Abstract

The chemical repair of radical-damaged leucine residues by dihidrolipoic acid (DHLA) in solution has been studied using density functional theory. Because of the low electron affinity of carbon-centered radicals, hydrogen-atom transfer (HAT) reactions were the only ones studied. DHLA was found to repair the radical-damaged leucine residue by HAT from the thiol groups. The calculated rate constants are in the diffusion-controlled regime, which indicates that these reactions are fast enough to be considered a possible repair process for an initially damaged protein with a leucine lateral chain.

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1. Introduction

Proteins carry out numerous different roles in cells and tissues, and are involved in virtually all life processes in biological organisms. They are the most abundant macromolecules in cells and the number of different functional proteins in cells and tissues is much greater than that of other macromolecules.[1,2] Many proteins function as enzymes, and it has been shown that aging is associated with a decrease in their activity.[3,4,5,6,7,8]

Due to their abundance and great tendency to react (with high reaction rate constants) with many biochemical species, proteins are a major target for biological oxidants. Protein damage is therefore a major consequence of their oxidation, reaction that can occur on both the side chains and the backbone.[9] The extent of the attack to a particular site in a protein depends on multiple factors. In some cases, the damage is limited to specific residues, whereas with other attacking species (*e.g.*, hydroxyl radical) the damage is widespread and non-specific. Moreover, damaged proteins may contain very reactive chemical groups that could facilitate the secondary damage by other biomolecules.[10,11]

As most protein damage is non-repairable, oxidation can have harmful effects such as fragmentation, aggregation, unfolding, altered interaction with other proteins, *etc*. As a result of free radical exposure, many changes can occur in proteins, including amino acid modification, loss (or sometimes gain) of function (*e.g.*, enzymatic, structural or signaling),[12] fragmentation, aggregation, changes in absorption and fluorescence spectra,[13] or increase in proteolytic susceptibility.[14] All these modifications can be used as markers of protein damage by free radicals.

The accumulation of oxidized proteins is associated with a number of diseases, including amyotrophic lateral sclerosis, Alzheimer´s disease, respiratory distress syndrome, muscular dystrophy, cataractogenesis, rheumatoid arthritis, progeria, and Werner´s syndrome. There is also reason to believe that the oxidative modification of proteins is related to atherosclerosis, diabetes, Parkinson´s disease, essential hypertension, cystic fibrosis, and ulcerative colitis.[15]

A lot of information exists about antioxidants and the role on their free radical scavenging activity. At the present time, much interest has focused on lipid and DNA oxidation and their protection by antioxidants, while comparatively little is known about oxidative protein damage and their modification by antioxidants. Hoey and Butler[16] have investigated the radical transfer reaction between tryptophan radicals and various natural and synthetic antioxidants. They have extended their study to repair tryptophan and tyrosine radicals in lysozyme, their model enzyme system. Hoey and Butler have shown that α-tocopherol, uric acid and ascorbic acid are capable of reacting with tryptophan radicals; however, the oxidized proteins are repaired very inefficiently by α-tocopherol, while uric and ascorbic acids are much better antioxidants. Domazou *et al.* have measured the rate constants (*k*) for the reaction between ascorbic acid and radicals generated in proteins.[17] The *k* values measured for protein reactions with tryptophanyl and tyrosyl radicals are (2.2-18) x 10^7 $M^{-1}s^{-1}$ and (4-290) x 10^5 $M^{-1}s^{-1}$, respectively. An earlier study reported *k* values for the reduction of aromatic amino acids and protein radicals in the absence of O_2 by antioxidants such as urate, ascorbate, glutathione (GSH), flavonoids, vitamin E and its analogue, Trolox.[18]

Several studies have been designed to test the possibility that protein radicals generated by reactive oxygen species can oxidize molecules with thiol groups such as GSH.[19-21] Studies in vitro of the GSH oxidation of lysozyme-tryptophan carbon radicals generated by nanosecond pulse radiolysis and flash photolysis lead to rate constants of (1.05 $\pm$ 0.05) x 10^5 $M^{-1}s^{-1}$.[19,20] A rate constant of 2 x 10^6 $M^{-1}s^{-1}$ for the radical repair of tyrosyl by GSH was recently estimated in the absence of ascorbate.[21] However, no previous research in this area has addressed the mechanistic study of the repair of damaged amino acids in proteins.

Focusing on N–formyl–leucinamide (see Figure 1) as a model of leucine in a protein previously used to study protein damage, please see Ref. 22 and references therein, this paper deals with the chemical repair of the different carbon-centred radicals derived from its lateral chain (shown in Figure 2). Even though this model has been widely used and accepted, we would like to emphasize why it is adequate. At first, it might seem too simple to model something that occurs in a protein. What makes proteins unique is their tertiary and quaternary structure, which determines their 3D shape and is essential for performing certain functions. For mimicking such

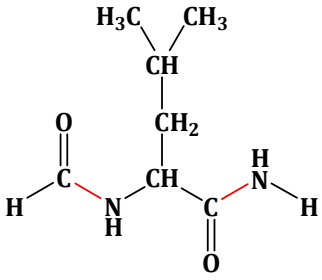


Figure 1. N–formyl–leucinamide

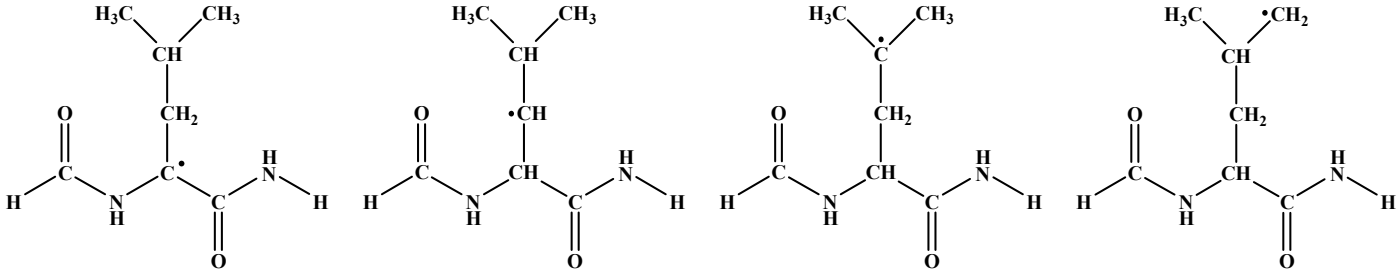
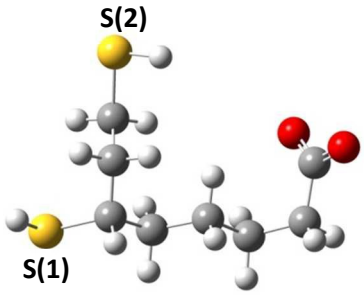


Figure 2. Model radicals of N–formyl–leucinamide to be repaired



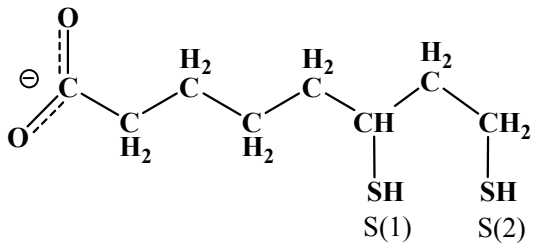


Figure 3. Dihydrolipoate

processes (*e.g.*, protein folding, enzymatic reactions, *etc.*), the theoretical methods applied (e.g., hybrid methods such as QM/MM) must include a very large system in order to account for all important interactions. On the other hand, proteins are not designed to be oxidized. Oxidation is an undesirable process that can involve a residue, which can be 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 well known that oxidative attacks occur randomly and non-specifically because of the very high reactivity and low selectivity of the oxidant in many cases, *e.g.*, OH radical (the most common radical for protein damage in biological systems). In addition, because of the lack of unsaturations in protein backbones, the electronic effects cannot propagate further than two sigma bonds. Consequently, the rest of the protein has no important effect on the oxidation process, and a simplified model like the one used in this study is excellent to study protein damage and repair. Furthermore, this molecular model of proteins has also been tested experimentaly.[17] The measured rate constant for the reaction of the N-formyl-tryptophanamide radical with ascorbate is 1.4×10^8 $M^{-1}s^{-1}$, while the repair of the same lateral amino acid damaged residue (*i.e.*, tryptophanyl) in the proteins chymotrypsin, pepsin, lysozyme and β-lactoglobulin with ascorbate led to k values of 1.6×10^8 $M^{-1}s^{-1}$, 1.8×10^8 $M^{-1}s^{-1}$, 8.3×10^7 $M^{-1}s^{-1}$ and 2.2×10^7 $M^{-1}s^{-1}$, respectively. Of the four proteins studied, the greatest discrepancy in k for the amino acid repair between a protein and the model is found with β-lactoglobulin, relative to which the k of the model is only six times larger. However, the agreement in k with the other three proteins and the model is very good. Therefore, the validity of the simplified molecular model for proteins used in this paper has been demonstrated. Additional examples of publications where very similar models have been used are listed in Ref. 23.

The antioxidant used in this work is the anionic form of dihydrolipoic acid (dihydrolipoate, DHL), shown in Figure 3, the reduced form of lipoic acid which contains two thiol groups. Dihydrolipoic acid has shown to have antioxidant properties through hydrogen-transfer reactions involving the thiol groups.[24] Hence, the radical-repair reactions studied are H-transfer reactions from each of the two –SH sites in DHL (labelled S(1) and S(2), see Figure 3) to each of the carbon-radicals that can be formed from previous attacks to the lateral chain of the protein model used. Hydrogen-transfer reactions have also been the preferred repair mechanism for the amino acid regeneration of guanosine carbon-centred radicals by glutathione.[25]

The differences found in the experimental rate constants for the repair of the same residue in the four proteins previously discussed[17] could be an effect of the microenvironment (hydrophobic or hydrophilic) of the damaged amino acid within each protein. This idea will be tested in this paper by studying the repair reactions with DHL both in water and in a non-polar solvent.

2. Computational Details

All electronic calculations were performed with the Gaussian 09 software.[26] Geometry optimizations and frequency calculations were carried out using the M06-2X[27] functional and the 6-31++G(d,p) basis set, using ultrafine grid, in conjunction with the SMD continuum model[28] using water as solvent to mimic the most usual biochemical environment. Additional calculations in a non-polar media using pentyl ethanoate as solvent were also performed.

Spin contamination was checked for all the radical species studied and deviations from the correct value ($\langle S^2 \rangle$ = 0.75) were lower than 1.25%. Table S1 displays the $\langle S^2 \rangle$ values for the transition states (TSs) in both solvents. Since it has been previously established that for differences within 10% error the results obtained can be trusted,[29] spin contamination in the present work is negligible and the calculated energies are reliable.

The M05-2X and M06-2X functionals have been recommended and tested by its developers for kinetic calculations.[27] M05-2X has been successfully used by independent authors[30-35] and M06-2X was developed as an improvement over its predecessor and it performs better for radical-molecule reactions.[36] 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.[28] It is important to mention that, according to the developers, SMD can be successfully used for optimization and frequency calculations in solution[37] while other continuum solvation models such as COSMO have been disqualified for thermodynamic corrections in solution.[38]

Thermodynamic corrections at 298.15 K were included in the calculation of the relative energies. In addition, the solvent cage effects have been included according to the correction proposed by Okuno,[39] which takes into account the free volume theory.[40]

Rate constants (k) were calculated applying conventional transition state theory (TST) using eq 1. In this expression, $\Delta G^{\neq}$ is the standard Gibbs energy of activation; k_B and h are the Boltzmann and Planck constants, respectively; σ represents the reaction path degeneracy, which accounts for the number of equivalent reaction paths, and κ is the tunneling correction, which cannot be ignored in reactions where hydrogen atoms are transferred.

$$k = \sigma\kappa\frac{k_B T}{h}e^{-\frac{\Delta G^{\neq}}{RT}} \quad (1)$$

Some of the k values calculated using eq 1 are in the diffusion-limit regimen, *i.e.*, they are greater than 10^8 $M^{-1}s^{-1}$. Accordingly, the apparent rate constant (k_{app}) cannot be directly obtained from TST calculations, and the Kimball-Collins[41] theory (see eq 2) is used instead.

$$k_{ap} = \frac{k_D k}{k_D + k} \quad (2)$$

In eq 2, k is the rate constant obtained from TST calculations using eq 1, and k_D is the steady-state Smoluchowski[42] rate constant for an irreversible bimolecular diffusion-controlled reaction, calculated using eq 3.

$$k_D = 4\pi R D_{AB} N_A \quad (3)$$

In eq 3, R denotes the reaction distance between the reactant species A (the protein radical) and B (DHL), N_A is Avogadro's number, and D_{AB} is the mutual diffusion coefficient of the reactants A and B, calculated using eq 4. D_A and D_B have been estimated applying the Stokes-Einstein[43] approach using eq 5.

$$D_{AB} = D_A + D_B \quad (4)$$

$$D = \frac{k_B T}{6\pi\eta a} \quad (5)$$

Referring to eq 5, η denotes the viscosity of the solvent (for water, $\eta = 8.91 \times 10^{-4}$ Pa s) and the value a is the radius of the solute assuming it is spherical.

The methodology used in this work has been previously proven to accurately reproduce experimental rate constants in solution.[44]

3. Results and Discussion

The protein model used, N-formyl-leucinamide, has two peptide bonds (shown in red in Figure 1). Of the two most common tertiary conformations proteins can adopt (alpha-helix and beta-sheet), only the alpha-helix is considered in this study and dihedral angles have been oriented accordingly.

A leucine side-chain is attached to the C_α atom of the central amino acid. We have studied the repair of the four types of C-centred radicals (labelled α, β, γ and δ, see Figure 2) that are the product of the damage by free radicals, even though it has been shown that the alpha position is usually not damaged.[22,23b,45-47] Since N-centred radical have not been experimentally found in this type of studies, we have not considered them. The optimized structures of these species are shown in Figure S1 of the Supplementary Information (SI) section.

Thus, the hydrogen-transfer repair reactions studied are:

Radical α + DHL-S(1)H → Protein + DHL-S(1)· α(1)
Radical β + DHL-S(1)H → Protein + DHL-S(1)· β(1)
Radical γ + DHL-S(1)H → Protein + DHL-S(1)· γ(1)
Radical δ + DHL-S(1)H → Protein + DHL-S(1)· δ(1)

Radical α + DHL-S(2)H → Protein + DHL-S(2)· α(2)
Radical β + DHL-S(2)H → Protein + DHL-S(2)· β(2)
Radical γ + DHL-S(2)H → Protein + DHL-S(2)· γ(2)
Radical δ + DHL-S(2)H → Protein + DHL-S(2)· δ(2)

3.1. Thermodynamic study

Table 1 displays the calculated standard Gibbs free energies of reaction (ΔG°). All reactions are exergonic; hence, spontaneous at room temperature. Repair with S(1) leads to ΔG° values that are more negative (i.e., more spontaneous reactions) than for the equivalent repair reaction with S(2) in aqueous solution, but the opposite is observed in the non-polar solvent. This is a

consequence of the fact that the DHL-S(1) radical is 0.63 (1.54) kcal/mol more stable than DHL-S(2) in water (pentyl ethanoate), in agreement with the degree of substitution of the bonded carbon atoms.

According to the Bell-Evans-Polanyi principle, the less exergonic a reaction, the slower it should be. Hence, according to the values of $\Delta G°$ the expected rate constant trend for the repair reaction should be: $\delta > \beta > \gamma > \alpha$.

Table 1. Calculated Gibbs free energies (in kcal/mol) of reaction ($\Delta G°$) and activation ($\Delta G^{\neq}$), and apparent rate constant (k_{app}, $M^{-1}\,s^{-1}$) for the repair reactions of N–formyl–leucinamide in two solvents at 298.15 K.

	Solvent: water			Solvent: pentyl ethanoate		
Radical	$\Delta G°$	$\Delta G^{\neq}$	k_{app}	$\Delta G°$	$\Delta G^{\neq}$	k_{app}
Repaired with S(1)						
α(1)	-6.36	11.29	7.6×10^4	-8.60	12.51	2.4×10^4
β(1)	-16.14	7.99	1.7×10^7	-16.87	6.90	1.1×10^8
γ(1)	-11.27	7.65	1.5×10^7	-11.54	9.45	1.5×10^6
δ(1)	-16.97	6.00	9.2×10^8	-17.71	7.65	3.1×10^7
Repaired with S(2)						
α(2)	-5.73	9.96	3.1×10^5	-7.06	9.78	8.4×10^5
β(2)	-15.51	3.09	2.3×10^9	-15.33	9.13	2.5×10^6
γ(2)	-10.65	4.67	1.2×10^9	-10.01	7.43	4.4×10^7
δ(2)	-16.35	5.89	1.0×10^9	-16.17	5.55	1.1×10^9

3.2. Kinetic study

The optimized structures of the calculated TSs in water are shown in Figures S2 and S3 of the SI section. The calculated standard Gibbs free energies of activation ($\Delta G^{\neq}$) are displayed in Table 1, together with the apparent rate constants of all repair reactions in both solvents considered. Even though the DHL radical resulting from the hydrogen transfer from S(1) is more stable, the $\Delta G^{\neq}$ values for the repair with S(2) are smaller than for those with S(1) in most cases. The non-corrected thermal (k) and diffusion-controlled (k_D) rate constants are listed in Table S2. The following paragraphs will first refer to the calculations in aqueous solution.

Since the difference in reactivity of the two thiol groups of DHL is not significant, we could attribute the $\Delta G^{\neq}$ differences to long-distance dynamic factors such as hydrogen-bonding interactions between the carboxylate group of DHL and NH groups in the backbone of the protein model used (see Figure S3 B) or to other intermolecular interactions, which seems to be the case of the TSs of radicals γ and δ (see Figures S3 C and D).

Large rate constants are calculated, except for the repair in the alpha position, as expected.[45] For the other three repair sites, most rate constants (k_{app}) are near or within the diffusion limit and the repair sequence is the following:

Repaired with S1: $\delta > \beta \approx \gamma$

Repaired with S2: $\beta > \gamma \approx \delta$

The rate constants for the repair with DHL-S(1)H show that the different sites are repaired according to the reactivity of radicals in the leucine moiety, *i.e.*, the radical of leucine damaged in δ, which is the most reactive because it is centred on a primary carbon, is repaired almost 100 times faster than the radicals in β and γ. This trend changes for the repair with DHL-S(2)H since the calculated k_{app} value for the repair reaction of the β radical is twice that of the repair reaction in the γ and δ radicals. This change could be related to the hydrogen bond that exists between the carboxylate group of DHL and an NH group that is part of the backbone of the protein model used (see Figure S3 B).

In all cases, the deviations of the expected reactivity trend can be explained by the strength of the intermolecular interactions with the carboxylate moiety, with the exception of the TSs for the δ position in which a hydrogen-bond-like interaction was found with the other S-H group.

However, if we analyze the overall rate constants ($k_{overall}$, shown in Table 2) calculated according to eq 6, we find that DHL can effectively repair all positions (except alpha) with very similar rate constants that are within the diffusion limit. Hence, the repairing sequence becomes: $\beta > \delta > \gamma > \alpha$. This "breakdown" of the Bell-Evans-Polanyi principle has been previously found for related systems such as the reaction of dihydrolipoic acid with free radicals[24] and those of glutathione with free radicals and damaged guanosine.[48,49]

$$k_{overall} = k_{repaired\ with\ S(1)} + k_{repaired\ with\ S(2)} \quad (6)$$

Table 2. Overall calculated rate constants ($k_{overall}$, $M^{-1}\ s^{-1}$) for the repair of N–formyl–leucinamide in two solvents at 298.15 K.

Reaction	Water	Pentyl ethanoate
Radical α + DHL-SH	3.9×10^5	8.6×10^5
Radical β + DHL-SH	2.3×10^9	1.1×10^8
Radical γ + DHL-SH	1.2×10^9	4.6×10^7
Radical δ + DHL-SH	1.9×10^9	1.1×10^9

If we define the standard Gibbs free energy of dissociation as the ΔG° of the process in which a hydrogen atom is removed from N–formyl–leucinamide to form each of the possible carbon-centered radicals, the calculated values for the formation of the alpha, beta, gamma and delta radicals are 58.3, 66.6, 61.3 and 67.4 kcal/mol, respectively. These values express the thermodynamic stability order of these radicals: $\alpha > \gamma > \beta > \delta$, which is the reverse reactivity order to repair them if we ignore the "breakdown" of the Bell-Evans-Polanyi principle previously discussed. The M06-2X stability order of these radicals is in agreement with the expected order taking into account that the delta radical is primary, beta radical is secondary, and the gamma and the alpha ones are tertiary.

The calculated rate constants in a non-polar media using pentyl ethanoate as solvent, as expected, are considerably smaller. The values obtained for the overall rate constants are 8.6×10^5, 1.1×10^8, 4.6×10^7 and 4.6×10^7 for the alpha, beta, gamma and delta repair reactions, respectively, *i.e.*, only the repair of the delta radical remains close to diffusion control, while the rate constant for the gamma repair is two orders smaller. The new results make our model more complete. Moreover, these results allow us to provide a plausible explanation for the differences found between the experimental rate constants for the repair of the same amino acid residue in four proteins.[17] The larger values could indicate that the residue is in a hydrophilic environment, while the smaller rate constants could indicate that it is in a hydrophobic pocket.

Conclusions

We can conclude that dihydrolipoic acid (DHLA) repairs with diffusion-controlled rate constants all but the α radicals in damaged leucine via H-atom transfer. In the absence of secondary stabilizing interactions, the thiol groups are capable of repairing all the other positions at relatively fast rates. Any additional stabilizing interaction involving the other thiol group (*e.g.*, see Figures S2 D and S3 D), or the carboxylate or amino moieties (e.g., see Figure 3 and Figure S3 B) will additionally stabilize the corresponding TS, accelerating the reaction. These reactions are fast enough to be considered a possible repair process for an initially damaged protein with a leucine lateral chain. These results could be extrapolated to glutathione because its structure is similar to that of DHLA.

We cannot claim that the TSs reported here lead to the lowest possible $\Delta G^{\neq}$ values because of the size and complexity of these systems. Nonetheless, if TSs with lower barriers could be located, the reactions would still be diffusion-controlled and the conclusions derived here would still apply. The same applies to cases in which additional parts of the protein (not considered in our simplified model) could lead to secondary interactions that additionally stabilize the TSs. The protein model used in the present work can be used to study the repair of protein moieties which are exposed to the biochemical environment, hence more prone to damage. To the best of our knowledge, this is the first theoretical kinetic study of the repair of damaged amino acids in proteins making use of electronic structure methods.

Acknowledgements

The authors gratefully acknowledge the Dirección General de Servicios de Cómputo Académico (DGTIC) at Universidad Nacional Autónoma de México. This work was partially supported by a grant from the DGAPA UNAM PAPIIT- IN220215, and the Project SEP-CONACyT 167430. R. C.-A. acknowledges CONACyT and the Natural Sciences and Engineering Research Council of Canada (NSERC) for financial support.

Supplementary Information

Supplementary information data associated with this article (Tables S1 to S2 and Figures S1 to S4; 21 pages) can be found in its online version. $\langle S^2 \rangle$ values for the calculated transition states in the two solvents studied (Table S1); Calculated rate constants (k and k_D in $M^{-1}\,s^{-1}$) for the repair reactions of N–formyl–leucinamide in two solvents at 298.15 K (Table S2); Optimized structures of the N-formyl-leucinamide radicals, the TSs (repaired radicals with the two sites of DHL), and the reaction products (Figures S1 to S4); M06-2X-SMD/6-31++G(d,p) Cartesian coordinates of the optimized geometries of the different reactants, products and TSs calculated in this study.

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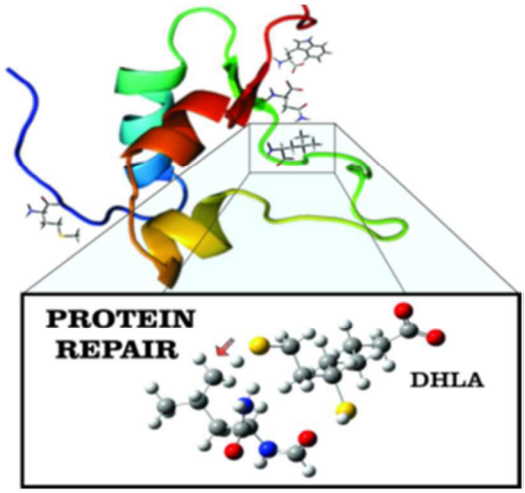
TOC



Dihydrolipoic acid repairs carbon-centred radicals at diffusion-controlled rates via HAT mechanism