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Oxygen radical-mediated oxidation reactions of an alanine peptide motif – density functional theory and transition state theory study

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Abstract

Background

Oxygen-base (O-base) oxidation in protein backbone is important in the protein backbone fragmentation due to the attack from reactive oxygen species (ROS). In this study, an alanine peptide was used model system to investigate this O-base oxidation by employing density functional theory (DFT) calculations combining with continuum solvent model. Detailed reaction steps were analyzed along with their reaction rate constants.

Results

Most of the O-base oxidation reactions for this alanine peptide are exothermic except for the bond-breakage of the C_α-N bond to form hydroperoxy alanine radical. Among the reactions investigated in this study, the activated energy of OH α-H abstraction is the lowest one, while the generation of alkylperoxy peptide radical must overcome the highest energy barrier. The aqueous situation facilitates the oxidation reactions to generate hydroxyl alanine peptide derivatives except for the fragmentations of alkoxy alanine peptide radical. The C_α-C_β bond of the alkoxy alanine peptide radical is more labile than the peptide bond.

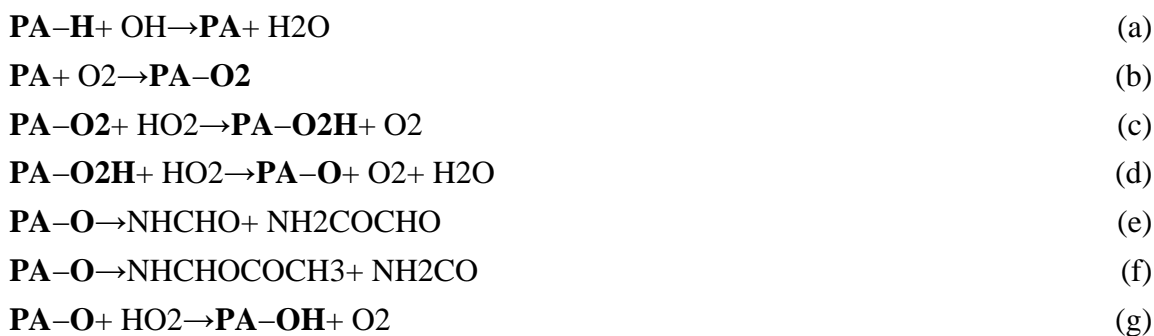
Conclusion

the rate-determining step of oxidation in protein backbone is the generation of hydroperoxy peptide radical via the reaction of alkylperoxy peptide radical with HO₂. The stabilities of alkylperoxy peptide radical and complex of alkylperoxy peptide radical with HO₂ are crucial in this O-base oxidation reaction.

Introduction

During the past decade, there was a rapidly increasing interest in oxidative damage to proteins, and its relevance to aging and pathological disorders [1-12]. The oxidation mechanism was constructed by identifying the trace of possible reaction intermediates and it is surmised that the mechanism is composed of a series reactions with HO₂ radical, as suggested by Stadtman et al. [2-4]. However, the details of reactions are still undetermined. These reaction mechanisms can provide scientists with some clues to aid in the design of medicines or nutrients to slow down the aging process or to decrease the probability of the age-related diseases. Obviously, an understanding of the processes of the protein oxidation is important, particularly as life expectancy increases. To the best of our knowledge, there are only few articles using ab initio or density functional theory (DFT) methods to study the oxidative damage of proteins through structural factors [13-20]. The main goals of the previous studies were to estimate the stability of carbon-center radicals and the strength of the C-H bond via bond dissociation energy calculations. In our previous study, it was found that the α-H located on a β-sheet is more difficult to abstract than one located on an α-helix [21]. However, this finding contradicts the results reported by Rauk et al. [18] and Owen et al. [22,23]. There were several articles about the OH H-abstraction from several amino acids [24-28]. Huang and Rauk [29] performed a series of theoretical calculations on reactions theoretically starting from an alkylperoxyl radical. Wood et al. [30] studied the C-C backbone fission of four small alkoxy radicals. To our best knowledge, there are no studies regarding the kinetic and thermodynamic aspects of the overall oxidation processes in proteins and therefore it becomes the most crucial issue in understanding the protein oxidative damage.

To facilitate the calculation, acetyl(Ala)NH₂, HC(O)NHCH(CH₃)C(O)NH₂ denoted as **PA-H**, was chosen to model a simple alanine peptide, as used in the previous studies [13,29], to investigate the oxidation reactions mediated by reactive oxygen species (ROS). In this study, a simple peptide represents a peptide before any oxidation reactions take place. The following reaction scheme is the oxidation reaction process adopted from those suggested by Stadtman, [1-4] but without the metal catalysis in the reactions.



Where **PA-** represents (CHO)NHC(CH₃)CO(NH₂), an α -C center radical of this alanine peptide. Base on the previous theoretical study[31] for CH₃ reacting with HO₂, an association channel of **PA** reacting with HO₂ was also considered in this study.



the rate constants of the above reactions at 298.15 K was calculated using transition state theory to understand their reaction kinetics. However, the effect of tunneling was not considered in this study. The solvent effect was also simulated by using continuum model in order to investigate the influence of an aqueous solution environment on the protein oxidation reactions. Through this study, we hope to shed some light on the process of protein oxidation.

Computational method

The geometry optimizations and frequency calculations of all species in this study, such as reactants, intermediates, transition states (**TS**) and products, were carried out at B3LYP/6-31 G(d) level. The final energy values were calculated at BHandHLYP/6-31 + G(d,p) level with ZPE correction. This BHandHLYP calculated level is rather computationally inexpensive and sufficient to obtain the relative energies as described in the previous studies [24] about OH α -H abstraction from alanine and glycine, in spite of its small basis set. The conductor-like polarizable continuum model (CPCM) using UAHF cavities at B3LYP/6-31 + G(d,p) level was suggested as a proper method to obtain the free energies of aqueous solvation [32]. Therefore, in this study CPCM was used to estimate the aqueous solvation free energy of all species. The rate constants were calculated as the following equation

$$k^{TST}(T) = \frac{k_b}{h} \frac{Q^\ddagger}{Q_A Q_B} \exp\left(\frac{-E_0}{k_b T}\right) \quad (1)$$

where Q is partition function, which can be obtained in Gaussian output file; E_0 is the energy with zero-point correction; k_b is Boltzmann constant; h is Planck constant and T is temperature. The transition states were verified by harmonic vibrational frequency analysis with only one negative frequency and checked their reaction pathways with intrinsic reaction coordinate (IRC) analysis by connecting the associated reactants and products. As to the ΔH_{rxn} value, we just used those reported in Gaussian output. We used the structures optimized in the gas phase to do single point calculation with CPCM at B3LYP/6-31 G(d,p) level without zero-point correction. There are several issues are worth noting for the proposed oxidation mechanism before entering the discussion section. In **Reaction b**, as mentioned in the introduction section, alkylperoxy peptide radical is an important intermediate in the

oxidation chain reactions of many chemical and biological systems due to its stability [33-37]. An alkylperoxy peptide radical can be generated easily from an α -C peptide radical surrounded by oxygen molecules. There are rotational isomers for this radical, which can be located by rotating the oxygen molecule with respect to the α -C-C bond, followed by structural optimization. After locating the stable structures, their heat of formation values can be calculated. In **Reaction d**, although **PA-O₂Hs** are rather difficult to obtain via **Reaction c**, as stated in previous subsection, it can be generated without energy barrier by attaching the terminal O of HO₂ directly to the carbon radical site of **PA**, Reaction h. Therefore, it is important to study the reaction between **PA** and HO₂ in present study. Apart from **PA-O₂Hs**, the related isomers can be classified according to the rotation of the C _{α} -O or O-O bond. The H-migration reactions involved in **Reaction c and g** are crucial for the oxidation mechanism. Without them, the reactions involve higher energetic barriers. In **Reaction c**, the spin densities of hydroperoxyl radical (HO₂) [38,39] and alkylperoxy alanine peptide radical (**PA-O₂s**) are mostly located on the terminal oxygen. From literature [40,41], the most stable structure of HO₂ dimer is HOO-OOH. This implies that the first step of **Reaction c** is to generate **PA-O₂OOH** by attaching a HO₂ to the terminal O of **PA-O₂s**. After three consecutive H-migration reactions, the products of the **Reaction c** can be obtained. **Reaction g** is a reaction involving an HO₂ radical attacking an alkoxy alanine peptide radical (**PA-O**), generating a hydroxyl alanine peptide derivative, i.e., **PA-OH**, and O₂. Similarly, owing to the dominant spin density of **PA-O** located on O, the pre-reactive species **PA-O₂OOH** are formed first and then **Reaction g** occurs via three consecutive H-migrations. All calculations were performed with Gaussian03 package [42].

Result and discussion

The focus of the present study is the oxidation reactions taking place at protein backbone, and therefore, no other intermediates, such as those generated via the rotation of the bonds in backbone, were considered. The relative energies were shown under the molecular labels in Figures 1,2,3,4,5,6,7 and the values without parentheses were in gas phase, while those in parenthesis were taken the free energies of aqueous solvation into consideration. The following discussion was organized according to the reaction types of the previously mentioned reaction scheme.

Figure 1 The mechanism and structures of the OH α -H abstraction reaction from **PA-H**, with the relative energy in kJ mol⁻¹ at 0 K

Figure 2 The conversion mechanism between the **PA-O₂** isomers and their corresponding structures with the relative energy in kJ mol⁻¹ at 0 K

Figure 3 The most probable mechanism of **Reaction c**, **PA-O₂ + O₂H** \rightarrow **PA-O₂H + O₂**, with the lowest reaction barriers and the relative energy in kJ mol⁻¹ at 0 K

Figure 4 The conversion mechanism between the **PA-O₂H** isomers and their corresponding structures with the relative energy in kJ mol⁻¹ at 0 K

Figure 5 The most probable mechanism of **Reaction d**, **PA-O₂H + O₂H** \rightarrow **PA-O + H₂O + O₂**, with the lowest reaction barriers and the relative energy in kJ mol⁻¹ at 0 K

Figure 6 The mechanism of Reaction *e* and Reaction *f* and the related fragmentations of PA-O, with the relative energy in kJ mol⁻¹ at 0 K

Figure 7 The most probable mechanism of Reaction *g*, PA-O + O₂H → PA-OH + O₂, with the lowest reaction barriers and the relative energy in kJ mol⁻¹ at 0 K

(a) Reaction *a*: the generation of an α-C center peptide radical by OH α-H abstraction

The mechanism of the α-H abstraction reaction by OH radical from alanine peptide –PA-H was presented in Figure 1. A pre-reactive complex **IN1-a-PA** is formed first, followed by H₂O elimination to generate an α-C center radical peptide intermediate, **PA**. The energy barrier of the OH α-H abstraction from **PA-H**, is 22.9 kJ mol⁻¹ in gas phase compared with 24.6 kJ mol⁻¹ in an aqueous environment. Their corresponding rate coefficients of these two reactions, without tunnel effect consideration are 4.38 × 10⁷ M⁻¹ s⁻¹ and 2.24 × 10⁷ M⁻¹ s⁻¹, respectively. The rate constant we found is one order lower than that for oligopeptides [8,43] (*k* ~ 10⁸) and two order lower than that for cyclic peptides [8,44] (*k* ~ 10⁹) in aqueous solutions. However, they are close to the rate constant [9] for free alanine, 7.7 × 10⁷ M⁻¹ s⁻¹, at pH ca. 7.

(b) Reaction *b*: Alkylperoxy peptide radical generation through molecular oxygen molecular addition to α-C center peptide radical

Three different conformers of alkylperoxy alanine peptide radical can be found, i.e., **PA-O₂-a**, **PA-O₂-b** and **PA-O₂-c**. There is no energy barrier for this oxygen addition reaction. Interestingly, the rate constants of cyclic peptides with oxygen were measured, ca. 10⁹ M⁻¹ s⁻¹ [44], which indicates that it could be barrierless and controlled by diffusion. In the gas phase, the most stable conformer is **PA-O₂-b**, consistent with the previous study [29], followed by **PA-O₂-a** and **PA-O₂-c** is the least stable one among these three. The optimized structures and the interconversion mechanisms among these three isomers were shown in Figure 2. However, including solvent effect, **PA-O₂-c** becomes more stable than **PA-O₂-a**. **PA-O₂-b** is a stable intermediate since its heat formation is not very large, 55.7 kJ mol⁻¹ in gas phase and 64.7 kJ mol⁻¹ in aqueous phase. All the energy barriers of the interconversion among **PA-O₂s**, by rotating the C_α-O bond, are pretty small and lower than the energy required for dissociating the oxygen molecule directly.

(c) The generation of hydroperoxy alanine peptide intermediate (PA-O₂H) via Reactions *c*

The products of the **Reaction c** require three consecutive H-migration reactions as shown in Equation (2).



According to the Equation (2), the possible conformations of **PA-OOOOH** were searched based on the orientation between **PA-O₂s** and HO₂ and the corresponding transition states and intermediate (**PA-OOO(H)O**) of the two consecutive H-migration reactions were found. Figure 3 showed the least energy pathway of **Reaction c** found in our calculation. The adduct (**IN1-c-PA**) of HO₂ with **PA-O₂-b** is only stable in gas phase (10.5 kJ mol⁻¹) but is unstable

in aqueous environment. The first H-migration, via **TS1-c-PA**, is the most difficult step in **Reaction c** and its energy barrier in kJ mol^{-1} are 195.3 in gas phase, and 165.0 in an aqueous phase. Their corresponding rate constants were 5.38×10^{-22} and $1.09 \times 10^{-16} \text{ s}^{-1} \text{ M}^{-1}$, respectively. Unstable as the intermediate **IN2-c-PA** is, it easily undergoes the second H-migration via **TS2-c-PA** and subsequently forms the product by losing O_2 . Therefore, to estimate the energy barrier and rate constant of **Reaction c** in the present study, only the first H-migration reaction was taken into consideration. The energy barriers were found in gas and in aqueous phases as 195.3 and 165.0 kJ mol^{-1} , respectively. Their corresponding rate constants in $\text{sec}^{-1} \text{ M}^{-1}$ were 5.38×10^{-22} and 1.09×10^{-16} , respectively. **Reaction c** can also take place via a direct abstraction channel, as shown in following equation



This is similar to the reaction of HO_2 with CH_3 and RO_2 as described in previous theoretical works [31,45,46]. However, we did not find any intermediate **PA-OO...HOO** that existed at singlet state, which is consistent with the previous study. Due to its high energy, triplet state **PA-OO...HOO** was not considered in the present study [45].

(d) The generation of hydroperoxy alanine peptide by a HO_2 addition to PA

The relative energy of **TS-r-O₂H-bbb**, which is the activated complex of the conversion reaction between **PA-O₂H-b** and **PA-O₂H-bb**, is lower than that of **PA-O₂H-bb** in gas phase. In aqueous phase, the strong solvation of **TS-r-O₂H-bbb** makes its relative energy even lower than those of both reactant and product for the interconversion reaction. These results indicate that the rotation of the O-O bond has a small or no energy barrier, therefore, the conformers formed by rotating the O-O bond were ignored and only the conformers formed by rotation of the $\text{C}_\alpha\text{-O}$ bond were considered in this study. Two conformers were found, **PA-O₂H-a** and **PA-O₂H-c**. The related optimized structures and conversion mechanism were presented in Figure 4.

In general, the conversion by rotation across $\text{C}_\alpha\text{-N}$ bond is difficult in gas phase but is rather easy in aqueous phase due to the strong solvation in its **TS**, **TS-r-O₂H-bbc**. Moreover, the conversions among **PA-O₂Hs** are even harder than the conversions among **PA-O₂s**. The most stable conformer of **PA-O₂Hs** in gas phase is **PA-O₂H-c** because the H in HO_2 can serve as a hydrogen bond (HB) donor [38-41,47,48], interacting with carbonyl O, which is consistent with the previous study [29,30]. It also can explain why **PA-O₂H-b** is more stable than **PA-O₂H-bb** in gas phase. However, **PA-O₂H-bb** becomes the most stable conformer among **PA-O₂Hs** when the aqueous solvation is taken into consideration. The dominant species of **PA-O₂Hs** in gas and in aqueous phases are **PA-O₂H-c** and **PA-O₂H-bb**, respectively. Their corresponding formation heats are -210.7 and $-175.6 \text{ kJ mol}^{-1}$, highly exothermic.

(e) Reaction d : $\text{PA-O}_2\text{H} + \text{HO}_2 \rightarrow \text{PA-O} + \text{H}_2\text{O} + \text{O}_2$

The alkoxy radical peptide intermediate (**PA-O**) can be generated by the hydroperoxy alanine peptide intermediate (**PA-O₂H**) first reacted with HO_2 and then followed by the loss of H_2O and O_2 . The reaction mechanism of **Reaction d** can be proposed as the following:



There exists a pre-reactive species, **PA-(O₂H)₂**, before the generation of products, i.e., **PA-O**, H₂O and O₂. Therefore, all probable conformations of **PA-(O₂H)₂**s were searched by taking into account all conformers of **PA-O₂Hs** interacting with HO₂, as found in the previous subsection. Through the above process, the generated **PA-(O₂H)₂**s were selected as the pre-reactive of **Reaction d** and their corresponding **TSs** were found. Meanwhile, the possible **TSs** of **Reaction d** were also searched by considering all conformers of hydroperoxy peptide intermediate (**PA-O₂Hs**) attacked by HO₂ directly from all probable directions. The found **TSs** were verified by tracing along the reaction pathway to find the related pre-reactive species. Finally, the lowest energy pathway among those we found were listed in Figure 5.

The structure of **IN-d-PA**, also a pre-reactive specie of **Reaction d**, has the terminal O of HO₂ connected to the amino H and the H of HO₂ close to the terminal O of the other HO₂ bonded to C_α. Consistent with previous studies [38-41,47,48] about the reaction with HO₂, its terminal O can serve as a HB acceptor and the H as a HB donor. These HB interactions stabilize the complex about 40.8 kJ mol⁻¹ in gas phase. Via **TS-d-PA**, **IN-d-PA** can generate **PA-O**, O₂ and H₂O, with the energy barrier in gas phase and in aqueous phase of 150.8 and 121.4 kJ mol⁻¹, respectively. Their corresponding rate constants are 1.56×10⁻¹³ and 2.20×10⁻⁸ s⁻¹ M⁻¹, respectively.

(f) The fragmentations of alkoxy radical peptide intermediate (**PA-O**): Reaction e and f

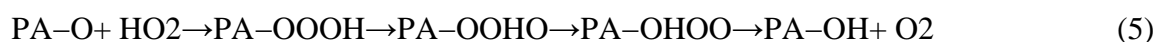
With a rather long C-C_α bond (1.607 Å), **PA-O** can break the C-C_α bond rather easily. Our calculated results also support this statement with the energy barrier, the reaction energies and corresponding rate constant in gas phase are 0.48 kJ mol⁻¹, 33.0 kJ mol⁻¹ and 1.05 × 10⁷ s⁻¹ M⁻¹, respectively. Their counterparts in aqueous phase are 43.6 kJ mol⁻¹, 34.8 kJ mol⁻¹ and 5.06 × 10⁶ s⁻¹ M⁻¹, respectively. These imply the probability for an alkoxy peptide radical to yield peptide fragmentation. The breakage of C_α-N bond in **PA-O** is more difficult than that of the C_α-C bond. As to the C_α-N bond breakage, the energy barrier, reaction energy and the corresponding rate constant in gas phase are 127.6 kJ mol⁻¹, 63.9 kJ mol⁻¹ and 7.50 × 10⁻¹⁰ s⁻¹ M⁻¹, respectively. Their corresponding values in aqueous phase are 131.4 kJ mol⁻¹, 47.0 kJ mol⁻¹ and 1.62 × 10⁻¹⁰ s⁻¹ M⁻¹, respectively. This data were also listed in Table 1 for comparison. The trend is in agreement with the previous theoretical study[29]. The optimized structures of their transition states and fragments for both C_α-N and C_α-C bond-breakage were presented in Figure 6. Because the backbone breaking reaction of the peptide was the main issue in this study, our products were all TS-like species but not the lowest-energy products. Therefore, the energy released in the studied reactions is lower than that found in the previous studies [29,30]. Interestingly, the backbone of the above transition states, **TS-e-PA** and **TS-f-PA**, are almost folded, and therefore, it will be rather difficult for these reactions to take place in proteins with restriction from the whole structure, as observed in this alanine peptide motif.

Table 1 The energy barriers, reaction energies and rate constants of the two fragmentation reactions in Reaction f as investigated in the current study, with energy in kJ mol⁻¹ and rate constant in sec⁻¹ mol⁻¹

Bond to be fragmented	Gas phase			Aqueous phase		
	<i>E_a</i>	ΔH_{rxn}	<i>k</i>	<i>E_a</i>	ΔH_{rxn}	<i>k</i>
C-C _α	0.48	33.0	1.05 × 10 ⁷	43.6	34.8	5.06 × 10 ⁷
C _α -N	127.6	63.9	7.5 × 10 ⁻¹⁰	131.4	47.0	1.62 × 10 ⁻¹⁰

(g) Reaction g: it is a generation of a hydroxyl derivative through an alkoxy radical with HO₂

In order to generate the products, **Reaction g** needs to undergo three consecutive H-migrations as indicated in Equation (4).



Similar to **Reaction c**, we searched all possible **PA-OOHs**, then tried to find the corresponding **PA-OO(H)O** and the **TSs** for the two corresponding H-migration based on Equation (5). Figure 7 lists the lowest energy pathway of **Reaction g** that we have found. The association energy of **IN1-g-PA** to form the isolated **PA-O** and HO₂, is 46.7 kJ mol⁻¹ in gas phase. The terminal H of HO₂ interacts with carbonyl O of backbone. Similarly, the first H-migration via **TS1-g-PA** is the rate determining step and the second H-migration is almost barrierless since the relative energy of **TS2-g-PA** is lower than that of **IN2-g-PA**. Therefore, only the first H-migration from **IN1-g-PA** to **IN2-g-PA** via **TS1-g-PA** was considered to represent **Reaction g**. And, the energy barriers were found to be 127.6 kJ mol⁻¹ in gas phase and 131.4 kJ mol⁻¹ in aqueous phase, respectively. Their corresponding rate constants in were 7.50×10⁻¹⁰ and 1.62×10⁻¹⁰ s⁻¹ M⁻¹, respectively.

(h) The overview from reaction a to reaction g

Although, all the oxidation reactions for the alanine peptide considered in the present study are exothermic, except for the breakage of C_α-N bond in **PA-O**. Table 2 lists the energies and rate constants of all the mentioned reactions with barriers in both gas and aqueous phases. It indicates that OH α-H abstraction is the easiest step and the generation of hydroperoxy alanine peptide radical **PA-O2H** via **Reaction c** is the most difficult one. Except for those reactions involved the fragmentations of **PA-O**, the oxidation reactions are facilitated by the aqueous solvation, especially for the two most difficult steps, **Reaction c** and **d**. To generate the hydroperoxy peptide intermediate via **Reaction c** is rather difficult and should go through the HO₂ addition reaction to form α-C center radical peptide intermediate. With the participation of HO₂, in **Reaction c**, **d** and **g**, pre-reactive peptide intermediates, **IN1-c-PA**, **IN1-d-PA** and **IN1-g-PA**, were formed first, respectively. Their dissociation energies to separate into reactants were 10.5, 40.8 and 46.7 kJ mol⁻¹ in gas phase, respectively. However they all become unstable in aqueous environment. Because **IN1-c-PA** is the most unstable one, the effects that increase its stability will be the key factors to enhance the oxidation processes tha involving it. The generation of alkoxy alanine peptide radical is a critical step since it can break the C_α-C_β bond easily to yield the peptide backbone fragmentation.

Table 2 The energy barriers, reaction energies and rate constants of the oxidation reactions for the simple alanine peptide as investigated in the current study, with energy in kJ mol⁻¹ and rate constant in sec⁻¹ mol⁻¹

Reactions	Gas phase			Aqueous phase		
	<i>E_a</i>	ΔH_{rxn}	<i>k</i>	<i>E_a</i>	ΔH_{rxn}	<i>k</i>
(a)	22.9	-113.6	4.38×10 ⁷	24.6	-103.3	2.24×10 ⁷
(c)	195.3	-148.4	5.38×10 ⁻²²	165.0	-96.4	1.09×10 ⁻¹⁶
(d)	150.8	-129.3	1.56×10 ⁻¹³	121.4	-125.9	2.20×10 ⁻⁸

(e)	33.0	-0.84	1.05×10^7	34.8	-43.6	5.06×10^6
(f)	127.6	63.9	7.50×10^{-10}	131.4	47.0	1.62×10^{-10}
(g)	132.1	-231.7	1.46×10^{-10}	116.7	-167.0	8.19×10^{-8}

Conclusion

Theoretical O-base oxidation in protein backbone was performed, using an alanine peptide as a model, and focused on the peptide backbone. The solvent participating in oxidation procedure was not considered in this study, however, continuum model was used to estimate the influence of the aqueous phase. Several important features were found as shown in the following. Most of the oxidation reactions in this alanine peptide are exothermic, except for the breakage of the C_α-N bond from hydroperoxy alanine peptide radical. The OH α-H abstraction is the easiest step and the generation of alkylperoxy peptide radical is the most difficult one. The aqueous environment facilitates the oxidation processes, except for the fragmentations of alkoxy alanine peptide radical. The C_α-C_β bond of the alkoxy alanine peptide radical is more labile than the peptide bond. Generating a hydroxyl alanine peptide derivative from the alkoxy alanine peptide radical is feasible. The C_α-C_β fragmentation takes place easily and causes large structural deformation by yielding alkoxy peptide radical. Therefore, the rate determining step of oxidation in protein backbones is the generation of hydroperoxy peptide radical via the HO₂ addition reaction to form the alkylperoxy peptide radical. The stabilities of alkylperoxy peptide radical and the alkylperoxy peptide radical and HO₂ complex are important factors that influence the reaction rate.

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Authors' contributions

HYC and JYC performed all the calculations. SJ and TRJ analyzed the data. HFL and FYL initiated and designed the study and finalized the manuscript. All authors have read and approved the final version.

Competing interests

The authors declare that they have no competing interests.

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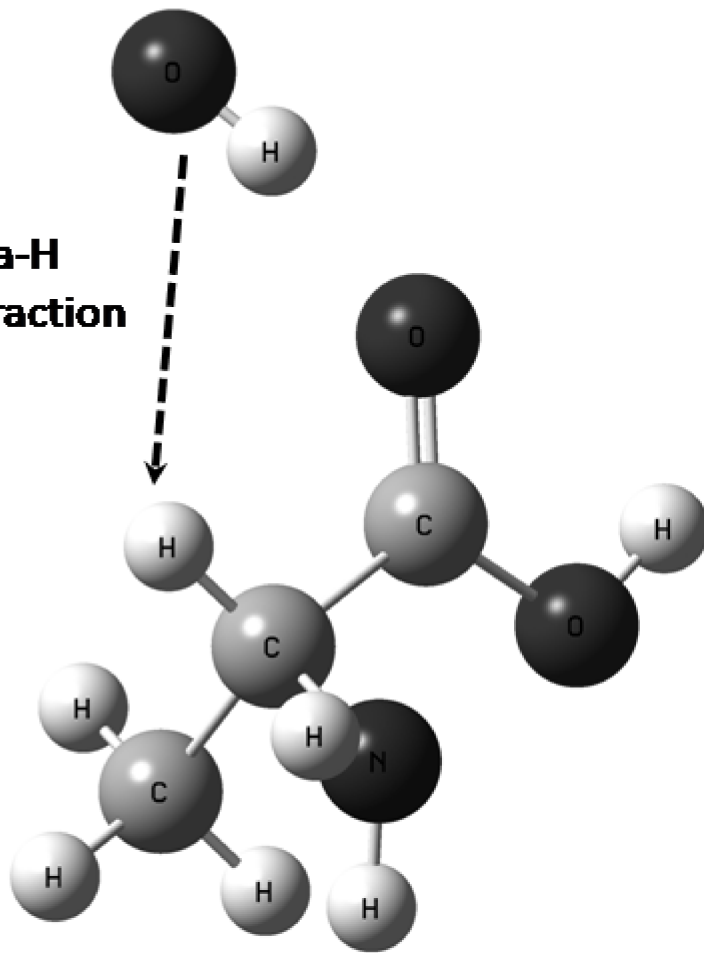
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**alpha-H
abstraction**



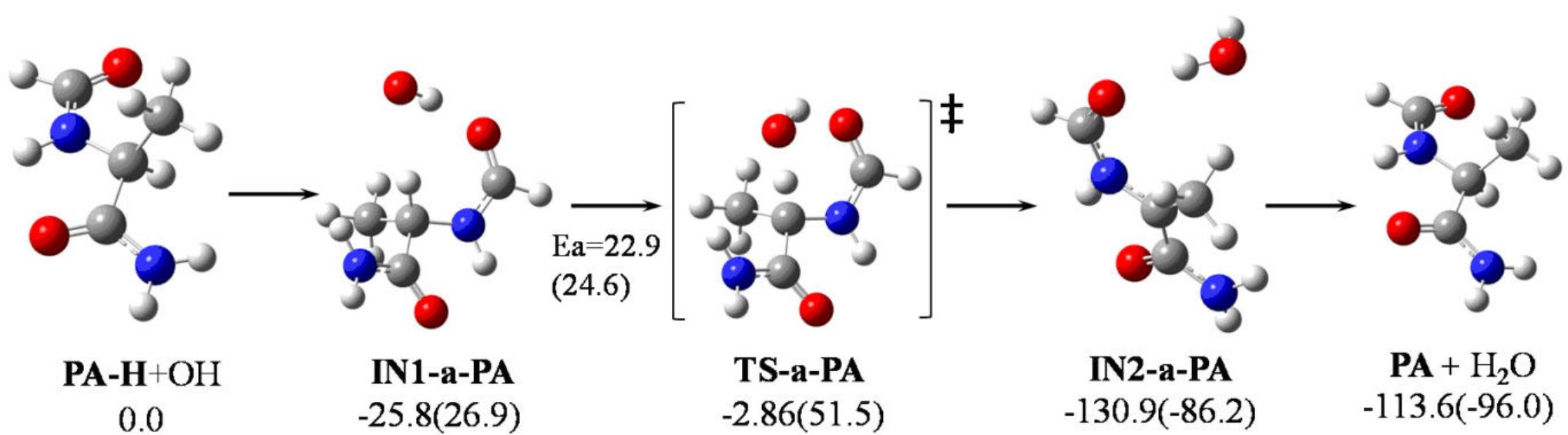


Figure 1

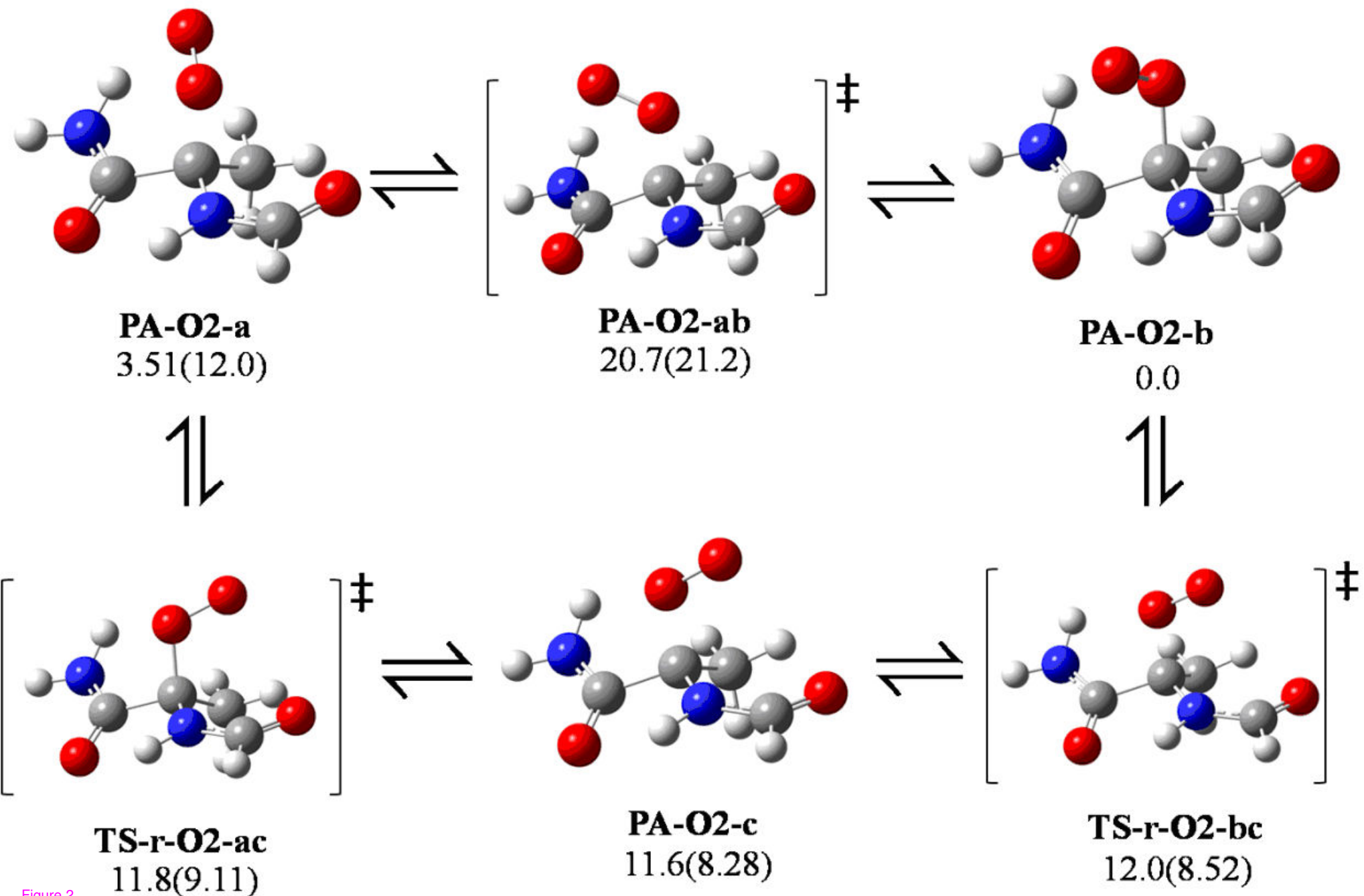


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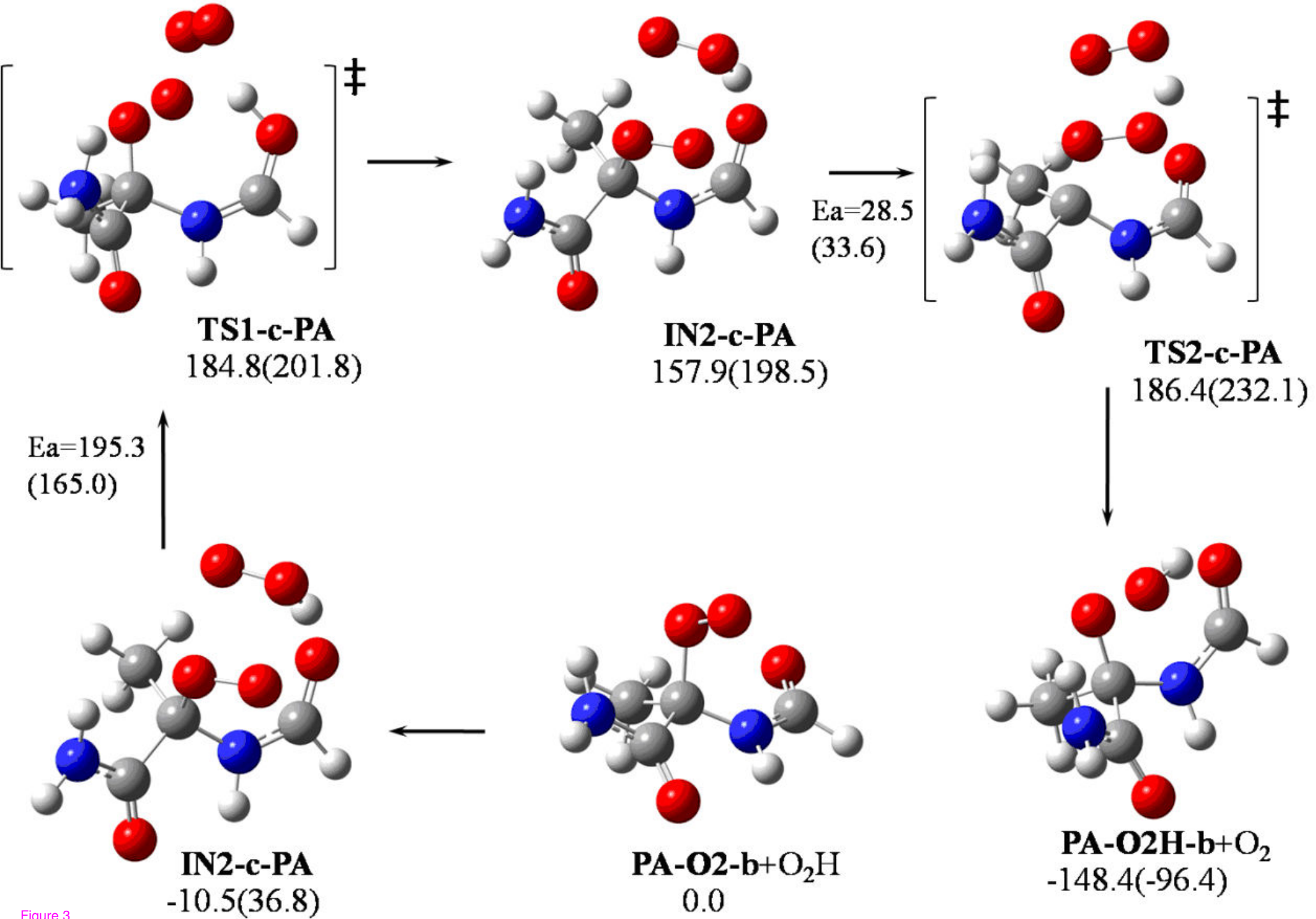


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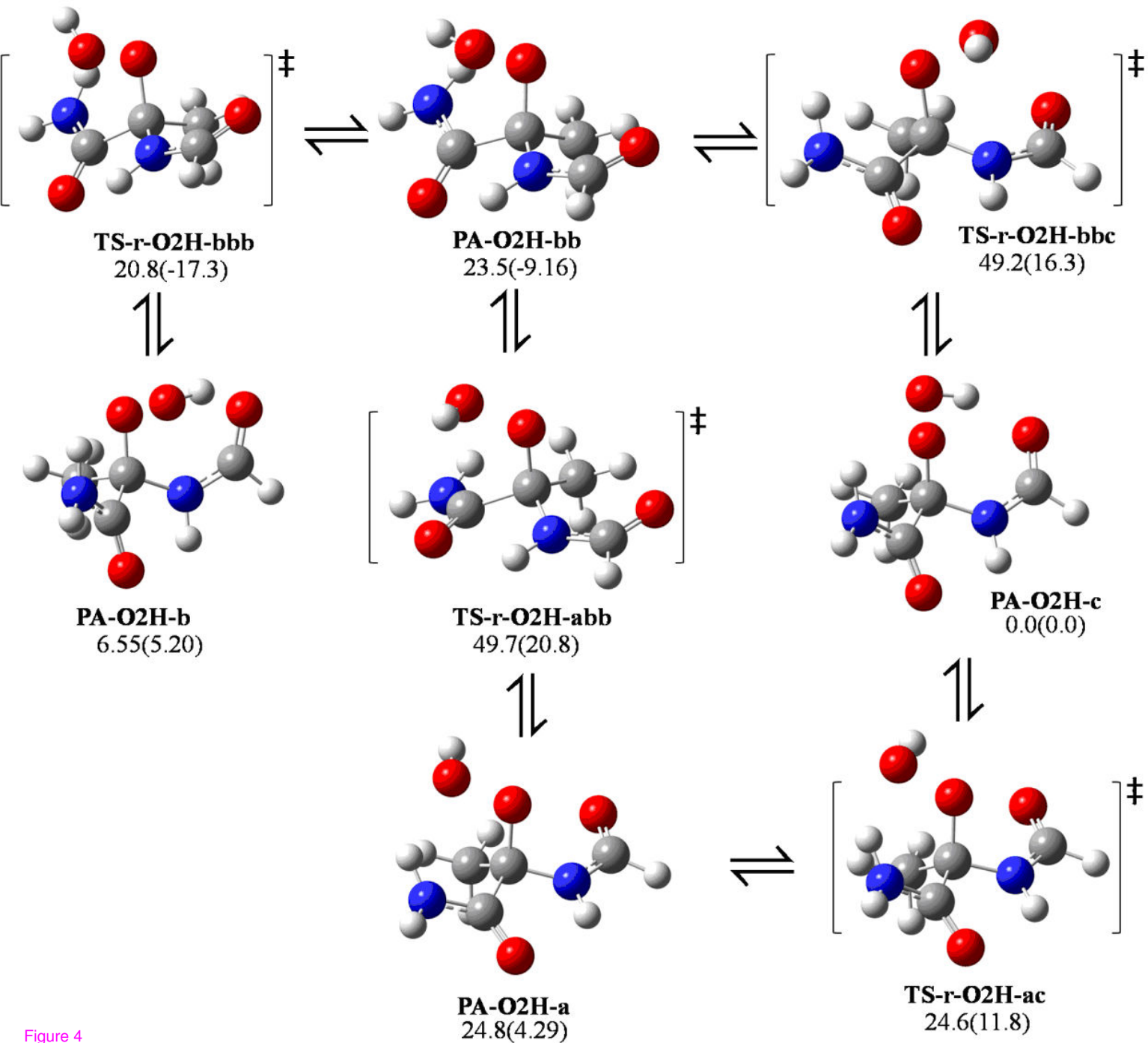


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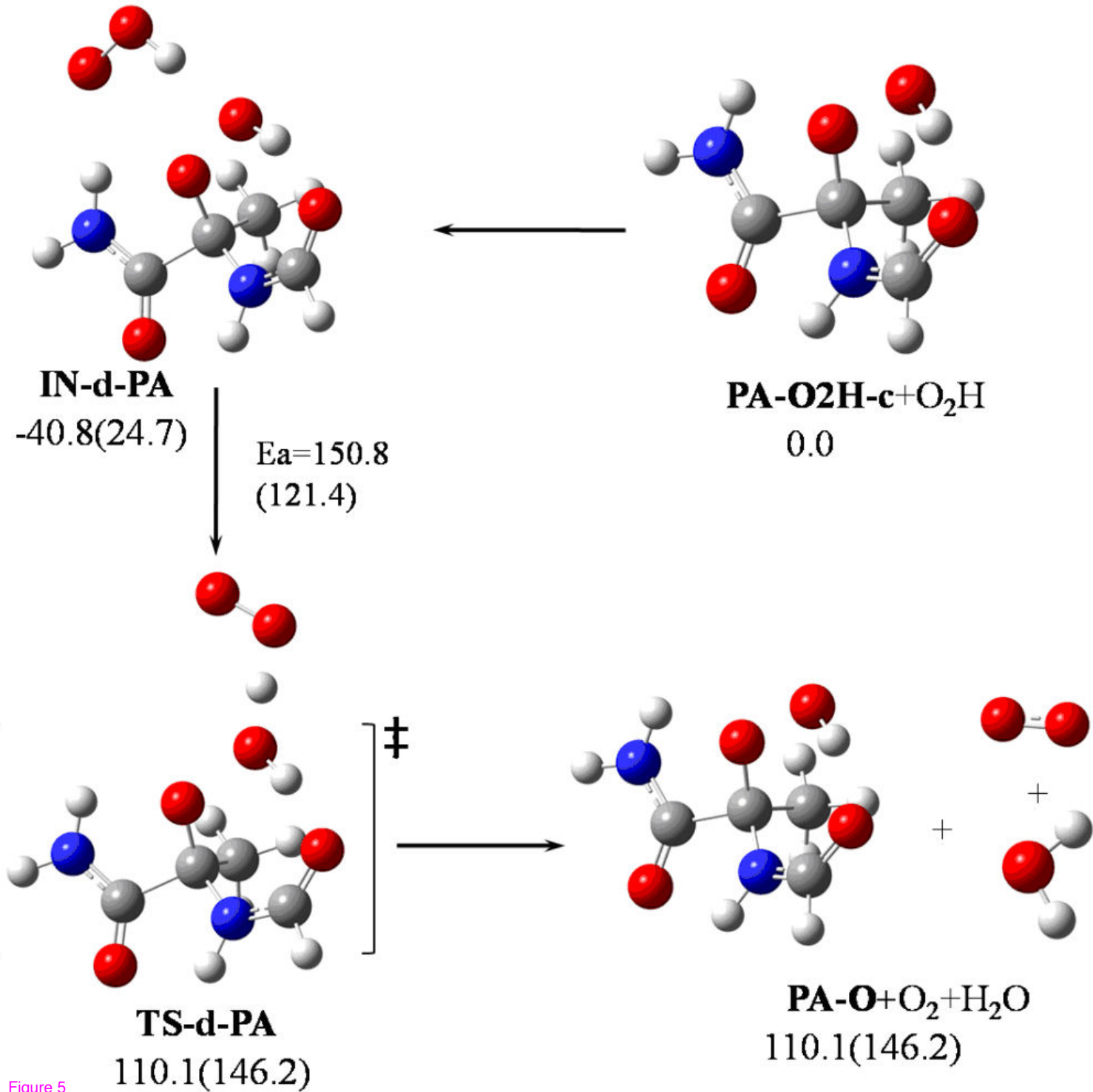


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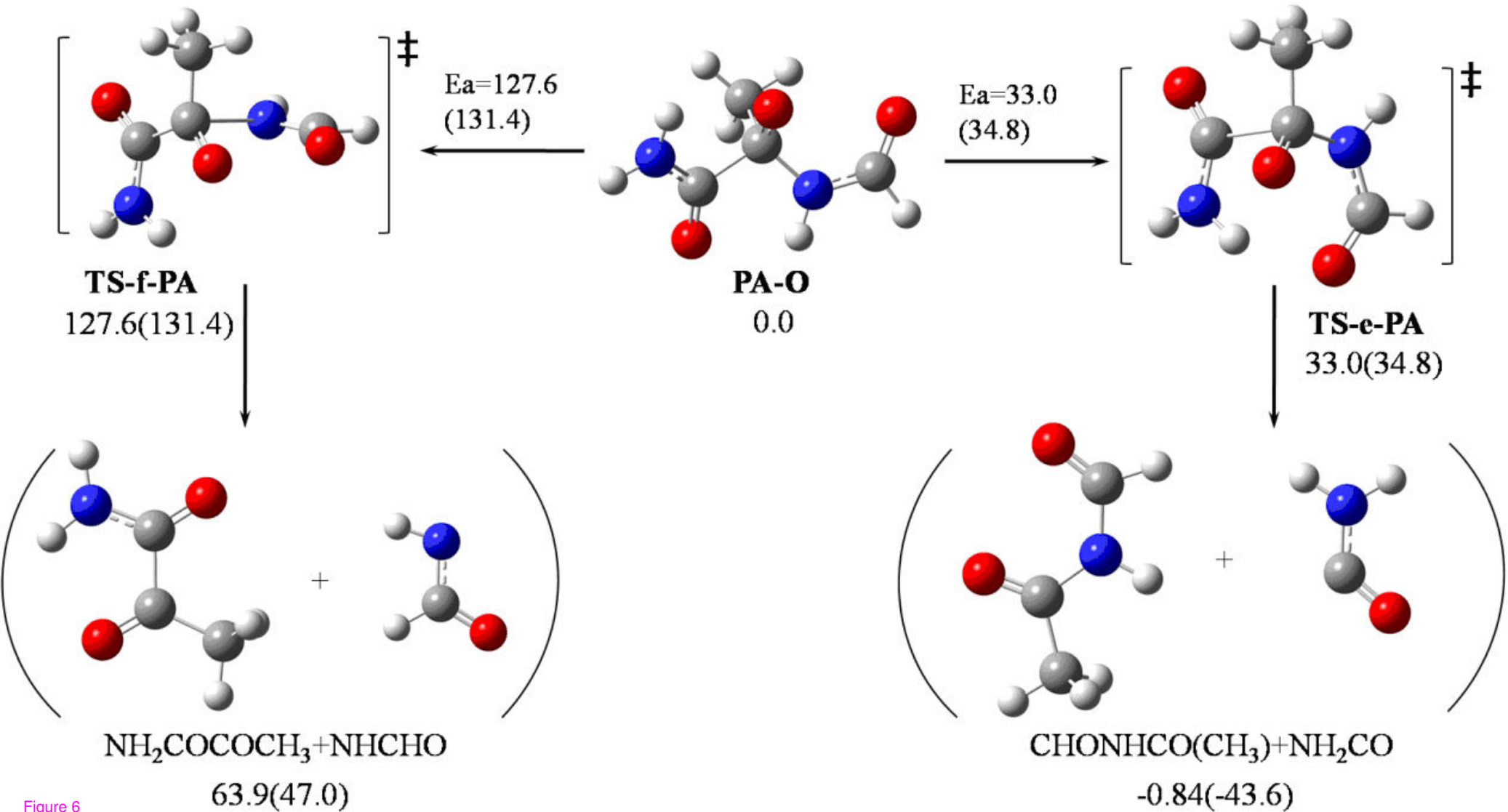
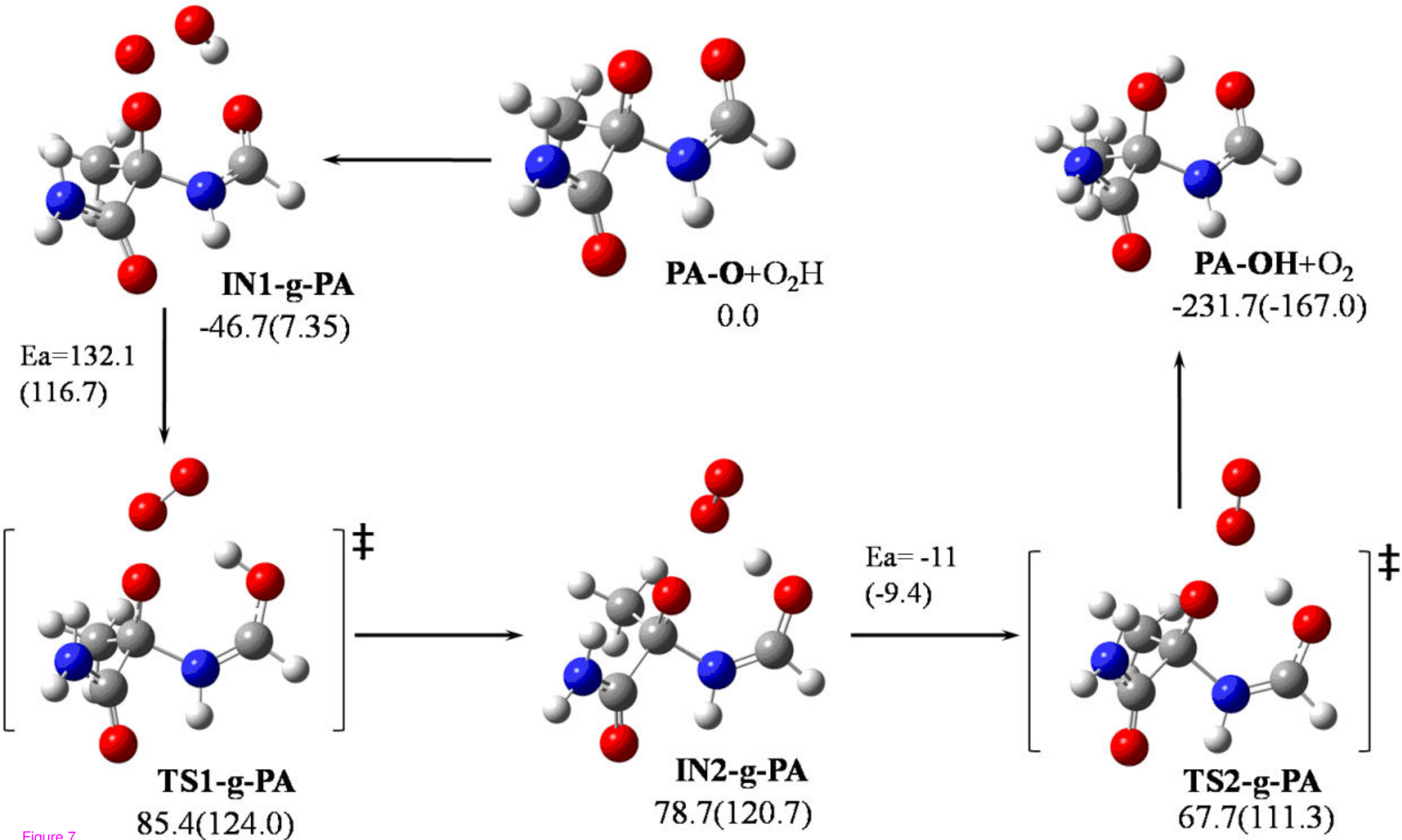


Figure 6



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