Journal of Structural Chemistry. Vol. 52, No. 5, pp. 870-875, 2011

A QUANTUM CHEMICAL STUDY OF THE FORMATION OF 2-HYDROPEROXY-COELENTERAZINE IN THE Ca^{2+}-REGULATED PHOTOPROTEIN OBELIN

L. Yu. Antipina,[1,3] F. N. Tomilin,[1] E. S. Vysotskii,[2] and S. G. Ovchinnikov[1,3]

UDC 577.332

The Ca^{2+}-regulated photoprotein obelin determines the luminescence of the marine hydroid *Obelia longissima*. Bioluminescence is initiated by calcium and appears as a result of the oxidative decarboxylation related to the coelenterazine substrate. The luciferase of the luminescent marine coral *Renilla muelleri* (RM) also uses coelenterazine as a substrate. However, three proteins are involved in the *in vivo* bioluminescence of these animals: luciferase, green fluorescent protein, and Ca^{2+}-regulated coelenterazine-binding protein (CBP). In fact, CBP that contains one strongly bound coelenterazine molecule is the RM luciferase substrate in the *in vivo* bioluminescent reaction. Coelenterazine becomes available for oxygen and the reaction with luciferase only after binding CBP with calcium ions. Unlike Ca^{2+}-regulated photoproteins, the coelenterazine molecule is not activated by oxygen in the CBP molecule. In this work, by means of quantum chemical methods the behavior of substrates in these proteins is analyzed. It is shown that coelenterazine can form different tautomers: CLZ(2H) and CLZ(7H). The formation of 2-hydroperoxy-coelenterazine is studied. According to the obtained data, these proteins use different forms of the substrates for the reaction. In obelin, the substrate is in the CLZ(2H) form that affords hydrogen peroxide. In RM, coelenterazine is in the CLZ(7H) form, and therefore, CBP is not activated by oxygen.

Keywords: coelenterazine, 2-hydroperoxy-coelenterazine, *Obelia longissima*, *Renilla muelleri*.

INTRODUCTION

Bioluminescence is the result of a biochemical reaction in which chemical energy converts into light energy. In the course of the reaction, the substrate is oxidized by an enzyme. All chemiluminescent reactions need molecular oxygen and proceed with the formation of intermediate complexes: organic peroxide compounds. In the decomposition of these complexes, the energy is released that excites the molecules of a compound responsible for the light emission.

One of the most studied bioluminescent systems is the photoprotein responsible for the luminescence of marine animals. To them belong the Ca^{2+}-regulated photoprotein obelin isolated from the hydropolyp *Obelia longissima* (OL) and the Ca^{2+}-regulated coelenterazine-binding protein (CBP) extracted from the luminescent marine coral *Renilla muelleri* (RM) [1, 2]. Unlike Ca^{2+}-regulated photoproteins, in the CBP molecule, the coelenterazine molecule is not activated by oxygen. From the single crystal X-ray diffraction (XRD) data it is seen that the structures of the substrates and the OL and RM environments noticeably differ (Fig. 1) [3-5].

[1]L. V. Kirensky Institute of Physics, Siberian Division, Russian Academy of Sciences, Krasnoyarsk; antipinalyu@gmail.com. [2]Institute of Biophysics, Siberian Division, Russian Academy of Sciences, Krasnoyarsk. [3]M. F. Reshetnev Siberian State Aerospace University, Krasnoyarsk. Translated from *Zhurnal Strukturnoi Khimii*, Vol. 52, No. 5, pp. 900-905, September-October, 2011. Original article submitted January 26, 2010; revised October 8, 2010.

 0022-4766/11/5205-0870 © 2011

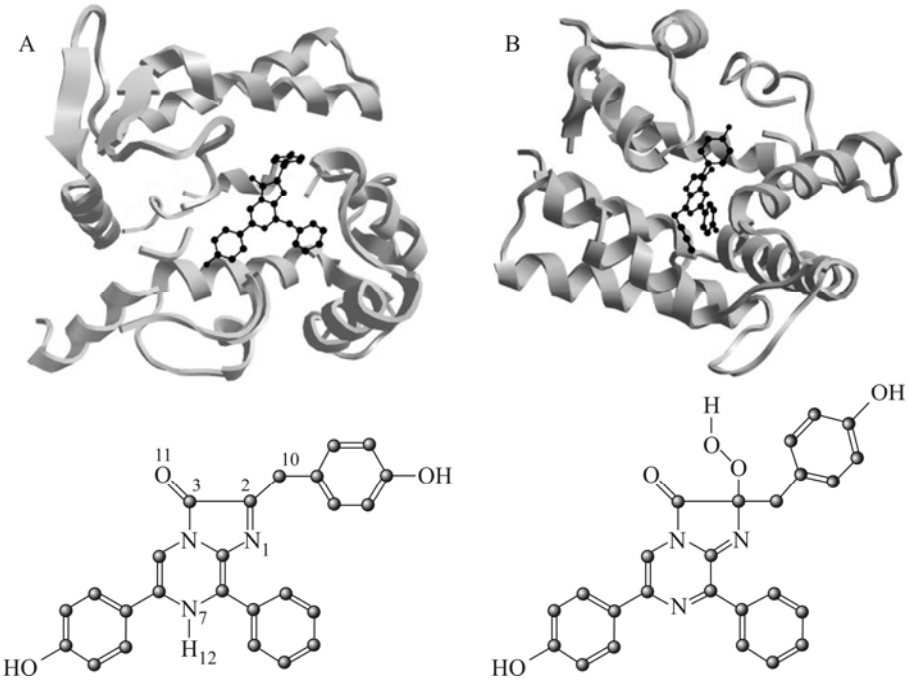

Fig. 1. Structure of proteins and their substrates. A is the protein extracted from *Renilla muelleri* and its substrate (coelenterazine); B is the protein extracted from *Obelia longissima* and its substrate (2-hydroperoxy-coelenterazine).

The question why the coelenterazine molecule is activated by oxygen in OL and not activated in RM still remains unclear. In this work, we analyzed the oxygen activation of coelenterazine using quantum chemical methods. They have proved themselves to be effective in studying processes occurring in biological systems [6-8]. In order to analyze the atomic structure and electronic charges of CLZ(7H) and CLZ(2H), the density functional theory (DFT) and Hartree–Fock (HF) methods were applied with the B3LYP hybrid potential and the 6-311** basis set [9-11]. The calculations were performed using the GAMESS program [12]. In order to take into account van der Waals forces and hydrogen bonds between the molecules in the calculation of reaction mechanisms in the presence of water and/or amino acid molecules, we employed semiempirical PM3 and PM6 methods [13-16]. The semiempirical calculations were performed using the MOPAC2007 program. Transition states were calculated by the quadratic synchronous transition (QST) option implemented in the HyperChem7 program. For the investigation we took the structures of two proteins (OL and RM), the data for which were obtained from the single crystal XRD study.

RESULTS AND DISCUSSION

A few mechanisms of the formation of CLZ hydrogen peroxide are proposed in the literature [17, 18]. In all cases, it is suggested that the process proceeds through the formation of an anion (Fig. 2).

In the *in vitro* studies of coelenterazine behavior in the basic medium, a rapid decarboxylation reaction is observed, which is accompanied by luminescence with the formation of coelenteramide. This fact is supported by our calculations. An approximate pattern of coelenterazine behavior in the basic medium is shown in Fig. 2B. The calculation was carried out in the presence of hystidine and water molecules by semiempirical PM3 and PM6 methods. Because of the presence of molecular oxygen in the system, the first two reaction steps were calculated in the triplet state, and the latter two were calculated in the singlet state. Bond changes in the reaction course from structure 1 to structure 4 are presented in Table 1.

The energy barrier for the anion formation reaction ($CLZ(7H) + OH^- \rightarrow CLZ(7-) + H_2O$) is sufficiently low (56 kJ/mol), therefore, the reaction must proceed quite readily. This agrees with the experiment, according to which in the

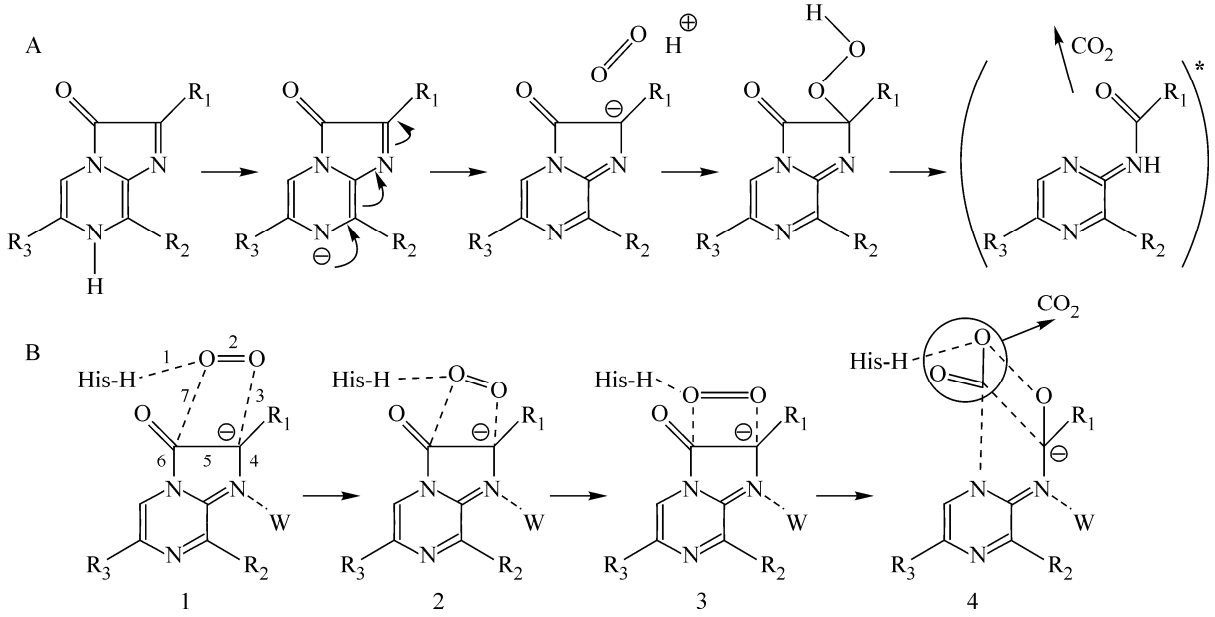

Fig. 2. Approximate mechanism of the coelenteramide formation. A is the mechanism proposed in the literature [14, 15]. B is the mechanism obtained from our calculations.

TABLE 1. Bond Length Change in the Calculation of the Coelenteramide Formation Reaction. Bond Numbers Correspond to Fig. 2B

Bond number	Bond length, Å	
	Structure 1	Structure 4
1	1.5	1.7
2	1.2	1.6
3	1.7	1.4
4	1.4	1.5
5	1.4	1.6
6	1.4	1.7
7	2.0	1.5

basic medium, the coelenteramide formation reaction proceeds almost instantly. The study of the kinetics of the processes occurring in the OL protein shows that the coelenterazine capture by the protein takes less than 3 s. Further transformation of coelenterazine into 2-hydroperoxy-coelenterazine involving oxygen proceeds in 2-3 h.

Due to the difficulties in the XRD measurements of photoproteins it is difficult to state experimentally what takes place in the system during the substrate capture by the apo protein, especially because the reaction proceeds very rapidly. There are only some crystal structures of photoproteins at different intermediate stages [3, 4]. In our case, these are OL containing 2-hydroperoxy-coelenterazine and RM containing inactivated coelenterazine (Fig. 1).

In the geometry optimization of the coelenterazine structure obtained by the XRD analysis of CBP in the amino acid environment of obelin, the coelenterazine structure decomposes because one of the coelenterazine substituents (R_3) crosses with the amino acid environment (Fig. 3A). Hence, we failed to find the optimal position of coelenterazine in the protein structure. Therefore, it was supposed that in OL and RM, coelenterazine was in different isomeric forms: CLZ(7H) in RM and CLZ(2H) in OL (Fig. 3B). This is likely to occur due to the different geometry of the cavity containing coelenterazine.

Then, in the presence of the oxygen molecule, the CLZ(2H) form reacts with the formation of a stable 2-hydroperoxy-coelenterazine structure. In Fig. 4, we present the scheme and energy diagram of this process.

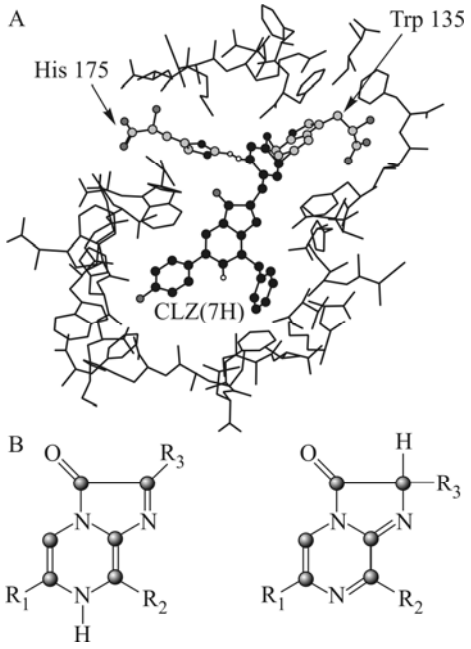

Fig. 3. A is the coelenterazine position in the CLZ(7H) form in protein obeline. B is the isomeric transition between 2-(4-hydroxy-benzyl)-6-(4-hydroxyphenyl)-8-phenylimidazo-[1,2- α]pyrazine-3(7H)-one (CLZ(7H)) and 2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl)-8-phenylimidazo-[1,2-α]pyrazine-3(2H)-one (CLZ(2H)).

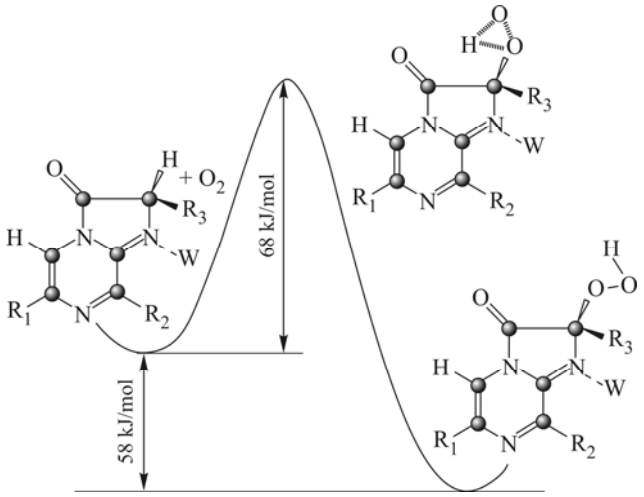

Fig. 4. Mechanism of the hydrogen peroxide formation.

Chemical reactions were modeled as follows. The CLZ molecule in the presence of water and the oxygen molecule in the ground triplet state were calculated as the initial point. The reaction product was the 2-hydroperoxy-coelenterazine structure and the water molecule. Then using the QST method we calculated the reaction paths by finding the maximum energy along the reaction coordinate on the potential energy surface [19]. In order to find the first order saddle point (i.e. the transition state), the maximum should be found in one (and only one) direction, while the minimum should be found in all directions. The transition state is characterized by the point in which all first energy derivatives are zero with regard to the geometric parameters (as for the geometry optimization), and the second derivative of the matrix (Hessian) has one and only one negative eigenvalue. The synchronous transition (ST) method enables the calculation of the transition state of the system and meshes with quasi-Newton methods for the transition state calculation. The transition state is calculated by the ST method in two ways. The linear synchronous transition (LST) method searches for the energy maximum along the linear path between the reagents and products. As a result of this method, it is possible to obtain a structure with two and more negative eigenvalues. The QST method is the improved LST method that makes it possible to find the energy maximum along the parabola relating the reagents and products and the energy minimum for all directions perpendicular to the parabola [19].

Since the oxygen molecule is in the ground triplet state, the addition reaction of oxygen in the direction from reactants to the transition state was calculated in the triplet state, i.e. in the calculations, a barrier for the forward reaction was in fact obtained. Considering that the reaction product has the singlet state, we suppose that at the moment of addition of the oxygen molecule (probably, in the transition state) the spin state changes from triplet to singlet, and then the reaction proceeds in the singlet state to reaction products. We assume this to occur under the effect of polar molecules when bonds in the oxygen molecule insignificantly change and become polarized.

The formation of 2-hydroperoxy-coelenterazine can be described in one stage with an intermediate state when the C2 proton passes to oxygen with the subsequent formation of 2-hydroperoxy-coelenterazine. According to the calculations, the activation barrier of this reaction is 68 kJ/mol.

TABLE 2. Charge Distribution on the Atoms Calculated at the DFT and HF Level in the 6-311** Basis Set. Atomic Numbers Correspond to Fig. 1

Atom	CLZ(7H)		CLZ(2H)	
	HF/6-311**	DFT/6-311**	HF/6-311**	DFT/6-311**
N(1)	–0.41	–0.55	–0.61	–0.54
C(2)	0.01	0.21	–0.04	–0.04
C(3)	0.59	0.55	0.76	0.57
N(7)	–0.62	–0.67	–0.59	–0.53
C(10)	–0.15	–0.30	–0.23	–0.24
O(11)	–0.51	–0.57	–0.57	–0.48
H(12)	0.24	0.26	0.19	0.15

In OL, the amino acid environment seems to force coelenterazine to pass to the CLZ(2H) form. When the coelenterazine molecule is captured from the *in vitro* solution, the amino acid environment bends the R_3 substituent at an angle of 125° with respect to the pyrazine base of the molecule. In the CLZ(7H) structure, this substituent is at an angle of 180°. Here the bonds are rearranged as a whole, and hydrogen passes from N(7) nitrogen to C(2) carbon. At the same time, the electron density on the atoms also changes. On C(2) carbon a charge of –0.04 appears (Fig. 1, Table 2). On hydrogen bonded to C(2) a charge of 0.15 appears. All this results in that the oxygen molecule is attracted to C(2).

CONCLUSIONS

In our opinion, in *Obelia longissima* and *Renilla muelleri*, coelenterazine is in two different isomeric forms: CLZ(2H) and CLZ(7H) respectively. This is caused by the different amino acid environment of proteins, which sets the geometry of coelenterazine.

Oxygen activation of coelenterazine with the formation of 2-hydroperoxy-coelenterazine is possible only for the CLZ(2H) structure, and therefore coelenteramide does not form in *Renilla muelleri*. For the CLZ(7H) structure the interaction reaction with the oxygen molecule proceeds without the formation of the intermediate hydrogen peroxide molecule until the formation of coelenteramide and is accompanied by the light emission.

The authors are grateful to the researchers of the Siberian Federal University P. V. Avramov and A. A. Kuzubov for their assistance in the work.

The work was supported by RFBR (07-04-00930-a), the "Molecular and Cell Biology" Program of the Presidium of the Russian Academy of Sciences, and the Program of the Siberian Division of the Russian Academy of Sciences (project No. 2) within the implementation of the Federal Targeted Program "Scientific and Scientific Pedagogical Personnel of Innovative Russia, 2010" (P333 and P213).

REFERENCES

1. E. S. Vysotskii, C. V. Markova, and L. A. Frank, *Mol. Biol*., **40**, No. 3, 404 (2006).
2. O. Shimomura, *Bioluminescence: Chemical Principles and Methods*, World Scientific Publishing Co. Pte. Ltd. (2006).
3. G. A. Stepanyuk, Z-J. Liu, S. S. Markova, et al., *Photochem. Photobiol. Sci*., **7**, 442 (2008).
4. Z-J. Liu, E. S. Vysotski, C-J. Chen, et al., *Protein Science*, **9**, 2085 (2000).
5. E. S. Vysotski and J. Lee, *Acc. Chem. Res*., **37**, 405 (2004).
6. F. N. Tomilin, L. Yu. Antipina, and E. S. Vysotski, *Dokl. Biochem. Biophys*., **422**, 279 (2008).
7. F. N. Tomilin, L. Yu. Antipina, and E. S. Vysotskii, *Biofiz.*, **54**, No. 4, 630 (2009).
8. S. Nakai, M. Yasui, M. Nakazato, et al., *Bull. Chem. Soc. Jpn*., **76**, 2361 (2003).

9. P. Hohenberg and W. Kohn, *Phys. Rev. Lett*., **136**, B864 (1964).
10. W. Kohn and L. Sham, *Phys. Rev. Lett*., **140**, A1133 (1965).
11. A. D. Becke, *J. Chem. Phys*., **98**, 5648 (1993).
12. M. W. Schmidt, K. K. Baldridge, J. A. Boatz, et al., *J. Comp. Chem*., **14**, 1347 (1993).
13. J. J. P. Stewart, *J. Comp. Chem.,* **10**, 209 (1989).
14. J. J. P. Stewart, *J. Mol. Model*., **10**, 155 (2004).
15. J. J. P. Stewart, *Computational Chemistry*, Colorado Springs, USA, CO (2007).
16. J. J. P. Stewart, *J. Mol. Model*., **13**, 1173 (2007).
17. F. G. Prendergast, *Nature*, **405**, 291 (2000).
18. O. Shimomura, *J. Microscopy*, **217**, No. 1, 3 (2005).
19. C. Peng and H. B. Schlegel, *J. Chem*., **33**, 449 (1993).