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Analysis of Amino Acid Oxidation Catalyzed by Pyrroloquinoline Quinone, A Quantum Chemical Study

(ピロロキノリンキノンによる
アミノ酸の酸化反応に関する
量子化学計算を用いた研究)

Waka Uchida

打田 和香

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Chapter 1.

Introduction

There are various organic functional molecules. I focus on an oxidation-reduction catalyst, especially for pyrroloquinoline quinone (PQQ) in my doctor thesis. PQQ is one of quinone compounds of biological system. Many researchers have been studied about this PQQ using by biological, physiological, biochemical, chemical, physicochemical methods, and so on. It has not yet been settled whether PQQ is a vitamin or not. Here, at first, I describe the detail of vitamins and vitamin analogous compounds, at second, I describe representative examples of quinone of biological system (e.g. vitamin K and ubiquinone) among vitamins and vitamin analogous compounds. At last, I describe the detail of PQQ which is quinone of biological system and vitamin analogous compound.

Vitamin and vitamin analogous compound

History and overview of vitamin

The discovery of vitamin has been contributed by a lot of researchers for many years. The first discovery of vitamin is thiamine (vitamin B₁) in 1913 by Dr. Umetaro Suzuki. He found this complex as antiberiberi factor in rice bran at the research of beriberi. He named it aberic acid, and later changed its name into orizantin. In 1911, Dr. Casimir Funk extracted the same factor from rice bran. He named it vita-amine (vitamin) which meant vital amine for living system. At later, this name was changed to vitamin because of the discovery of vitamin which was not amine. This was origin of vitamin. In 1926, B.C.P.Jansen extracted pure crystal of thiamine because the antiberiberi factor which was discovered by Dr. Umetaro Suzuki and Dr. Casimir Funk included impurities. In 1936, R.R.Williams and A.Windaus determined a chemical structure of thiamine.

The name of vitamin A, B and C was named by J.C.Drummond in 1920. In 1913, E.V.MoCollum reported there were unknown and vital nutrient factors for development of mouse which were fat-soluble and water-soluble complexes, and in 1915 he named them fat-soluble A and water-soluble B, respectively. And also, in 1907 A.Holst et al. succeed in causing guinea pigs to develop scurvy (scurbutus). In 1919, J.C.Drummond named this antiscorbutic factor water-soluble C. And then, he named fat-soluble A vitamin A, water-soluble B vitamin B, and water-soluble vitamin C. And also, there were different natures in vitamin B, which were weak and strong against heat. He named them vitamin B₁ and vitamin B₂, respectively.

The vitamin is a vital organic compound for organism which is required in

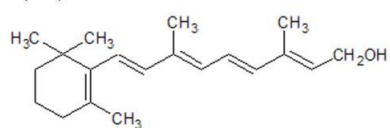
limited amounts, but cannot be synthesized in biological system of human and needs to be taken in from outside of body. There are 13 species of vitamin which have been reported until now, and shown in Table 1 and Figure 1. The families of vitamin B and vitamin C are water-soluble vitamin, and while, those of vitamin A, vitamin D, vitamin E, and vitamin K are fat-soluble vitamin.

Table 1. The list of fat-soluble vitamin (a) and water-soluble vitamin (b).

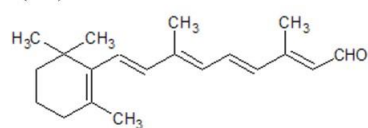
vitamin	name
Fat-soluble	
Vitamin A	retinol retinal retinoic acid 3-dehydroretinol 3-dehydroretinal 3-dehydroretinoic-acid
Vitamin D	ergocalciferol (D ₂) cholecalciferol (D ₃)
Vitamin E	α -tocopherol β -tocopherol γ -tocopherol tocotrienol
Vitamin K	phylloquinone (K ₁) menaquinone (K ₂) menadione (K ₃)

Vitamin	name
water-soluble	
Vitamin B ₁	thiamine
Vitamin B ₂	riboflavin
Vitamin B ₆	pyridoxine pyridoxal pyridoxamine
Vitamin B ₁₂	cobalamin
folate	Folate
niacin	nicotinic acid nicotinic-acid amino
pantothenic acid	pantothenic acid
biotin	biotin
Vitamin C	ascorbic acid

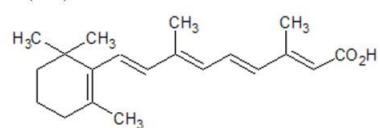
(a1)



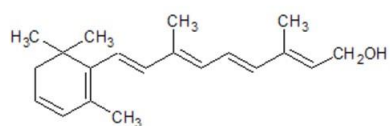
(a2)



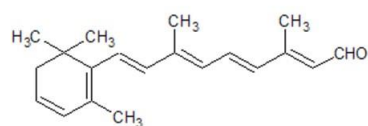
(a3)



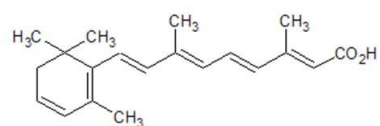
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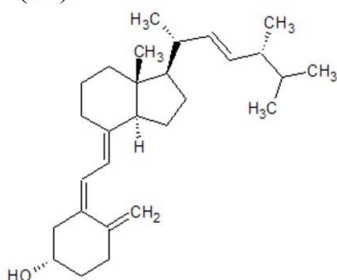
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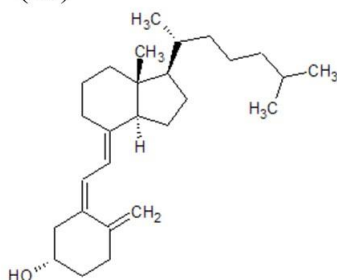
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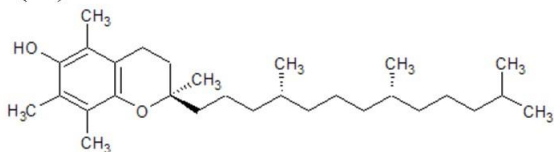
(b1)



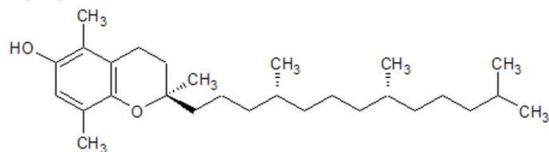
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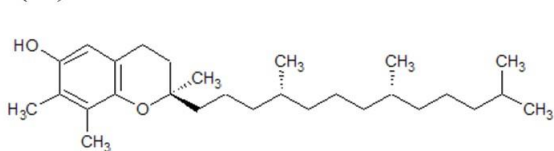
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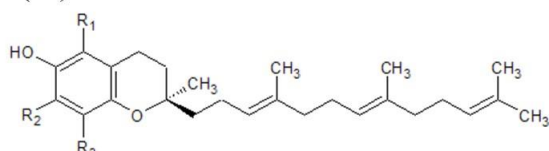
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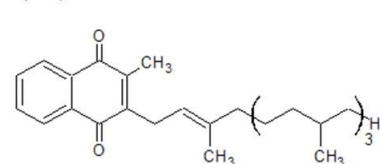
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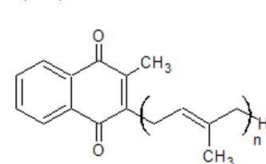
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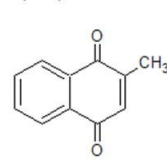
(d1)



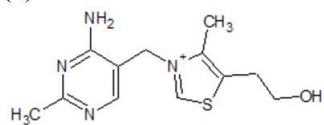
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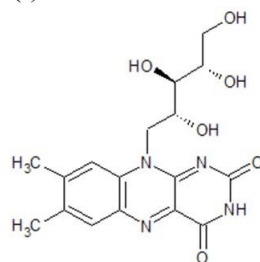
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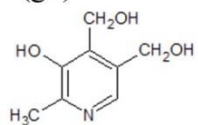
(e)



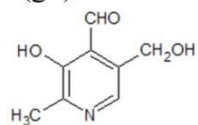
(f)



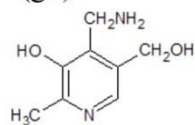
(g1)



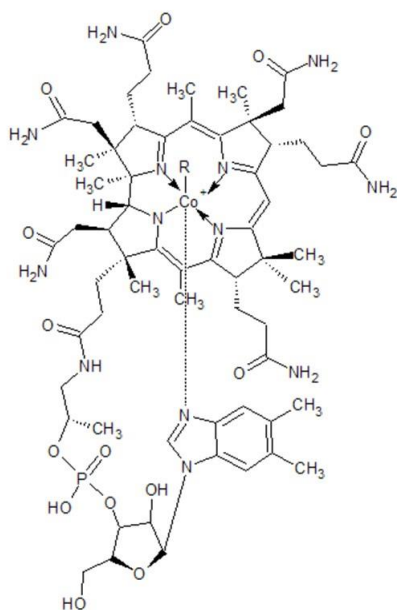
(g2)



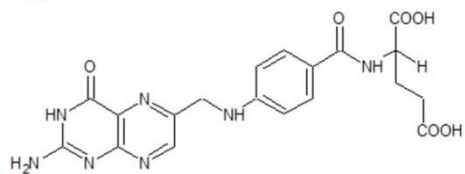
(g3)



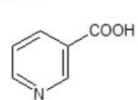
(h)



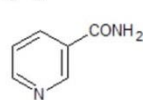
(i)



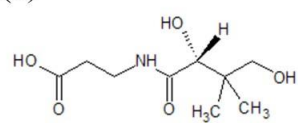
(j1)



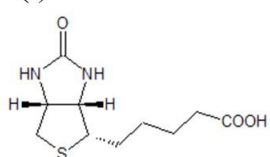
(j2)



(k)



(l)



(m)

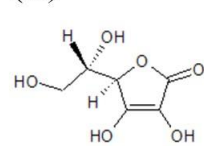


Figure 1. The structures of fat-soluble vitamin, (a1)retinol, (a2)retinal, (a3) retinoic acid, (a4) 3-dehydroretinol, (a5) 3-dehydroretinal, (a6) 3-dehydroretinoic-acid, (b1) ergocalciferol (D₂), (b2) cholecalciferol (D₃), (c1) α -tocopherol, (c2) β -tocopherol, (c3) γ -tocopherol, (c4) tocotrienol, (d1) phyloquinone (K₁), (d2) menaquinone (K₂), and (d3) menadione (K₃). And also, the structures of water-soluble vitamin, (e) thiamine, (f) riboflavin, (g1) pyridoxine, (g2) pyridoxal, (g3) pyridoxamine, (h) cobalamin, (i) folate, (j1) nicotinic acid, (j2) nicotinic-acid amino, (k) pantothenic acid, (l) biotin, and (m) ascorbic acid.

There are three main approaches on the discovery of vitamin. One is the case that researchers studied about a disease of unknown cause, expected a factor which has possibility of prevention and cure existed in foods and then they succeeded to extract them. Second is the case that researchers suspected other unknown nutrient was required to development because animals didn't grow up well even they used feeds which were consisted of known nutrients during animal culture experiments, and then they demonstrated its factor was vital for human. And last is the case that researchers discovered growth factor of microorganism and then they also found this factor was vital for human.

The other complex which is also required limited amounts for human and except for vitamin is hormone. The difference between hormone and vitamin is that the former is synthesized in body, and the latter is not synthesized in body and has to take in from outside of the body. However there are some exceptions for vitamin. For example, some vitamin is synthesized in body, but this production didn't meet the vital amount, that is why we need to take in from outside of the body. That is why we call

them vitamins.

There is another complex which has physiological function in relatively minute amounts. That is mineral. The difference between mineral and vitamin is that the former is inorganic compound and the latter is organic compound.

As described above, vitamins are not basically synthesized in human body. Human utilizes complex which are synthesized by microorganisms and plants as vitamins. Animals include many vitamins, but the origin of supply of vitamin in animal body is the accumulated vitamins which were taken in from microorganisms and plants. Animal themselves synthesized a few vitamins in their bodies. Vitamins are usually broken down in our body and excreted to outside the body.

Vitamin has another roll except for nutrient. Many researchers have focused on possibilities of sufficient doses of vitamins as prevention and cure of diseases in these days.

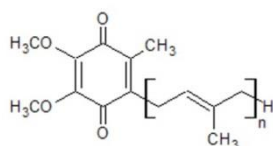
Discovery of vitamin analogous compounds

There are many compounds except for above-mentioned compounds (vitamins) which have specific biological function. They are called vitamin analogous compound. The name and structure are shown in Table 2 and Figure 2.

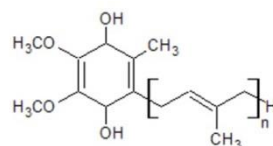
Table 2. The list of vitamin analogous compound.

vitamin analogous compound
carotenoid
flavonoid
Vitamin F
choline
inositol
<i>p</i> -aminobenzoic acid
carnitine
Vitamin P
lipoic acid
orotic acid
Vitamin U
pangamic acid
biopterin
Ubiquinone (CoQ)
pyrroloquinoline quinone

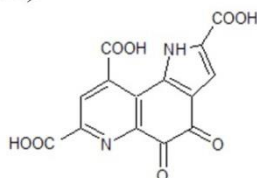
(a1)



(a2)



(b1)



(b2)

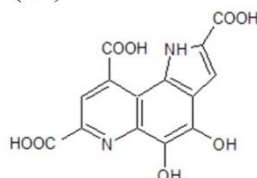


Figure 2. The structures of some vitamin analogous compound, (a1) oxidized ubiquinone (a2) reduced ubiquinone, (b1) oxidized PQQ and (b2) reduced PQQ.

(Further information can be seen in here [1].)

Quinone of biological system (vitamin K and ubiquinone)

In my doctor thesis, I focus on PQQ which is quinone of biological system. Among above-mentioned vitamins and vitamin analogous compounds, there are some quinone of biological system, that is vitamin K and ubiquinone. I describe the details of vitamin K and ubiquinone at this section.

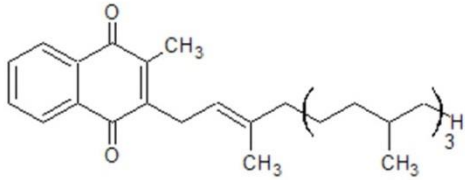
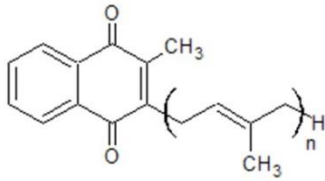
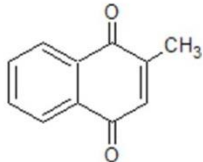
Vitamin K (fat-soluble vitamin)

History

In 1929, Dr. Henrik Dam observed subcutaneous bleeding and anemia when he gave fat-free meals to chickens on the study of sterol. After the detailed researches, he found that antihemorrhagic substance existed in liver and several plants extract and was fat-soluble. In 1935, Dr. Henrik Dam named this substance vitamin K. It was named after blood coagulation (Koagulation in german). Until 1939, two native form vitamins were isolated. Vitamin K₁ and vitamin K₂ were isolated from alfalfa and rotten fish, respectively. These vitamins are different from melting point.

The compound which has vitamin K activity in Table 3 has in common 2-methyl-1,4-naphthoquinone ring. There are different sub chains at position 3 of this ring. In nature, vitamin K occurs as two forms, one is vitamin K₁ (phylloquinone) (Phylloquinone 2-methyl-3-phytyl-1,4-naphthoquinone) which is synthesized by plants, and another is vitamin K₂ (Menaquinone) (Menaquinone-n 2-methyl-3-multiprenyl-1,4-naphthoquinone) which is mainly synthesized by microorganism and forms a family from the length of sub chain at position 3 of 2-methyl-1,4-naphthoquinone ring (Table 3). And also, there is no sub chain at position 3 of 2-methyl-1,4-naphthoquinone ring, which is called vitamin K₃ (menadione) (Table 3). This is alkylated to MK-4 in tissue of birds and mammals, which is a MK-4 transformation intermediate or a synthetic.

Table 3. The list of vitamin K.

Vitamin K	Structure
VK ₁	
VK ₂ (MK-n)	
VK ₃	

It was reported that vitamin K was a coenzyme which was needed to translate from glutamic acid residue (Glu) of vitamin K-dependent protein to γ -carboxyglutamic acid residue (Gla), which meant vitamin K-dependent carboxylation. This detail of mechanism is shown in Figure 3.

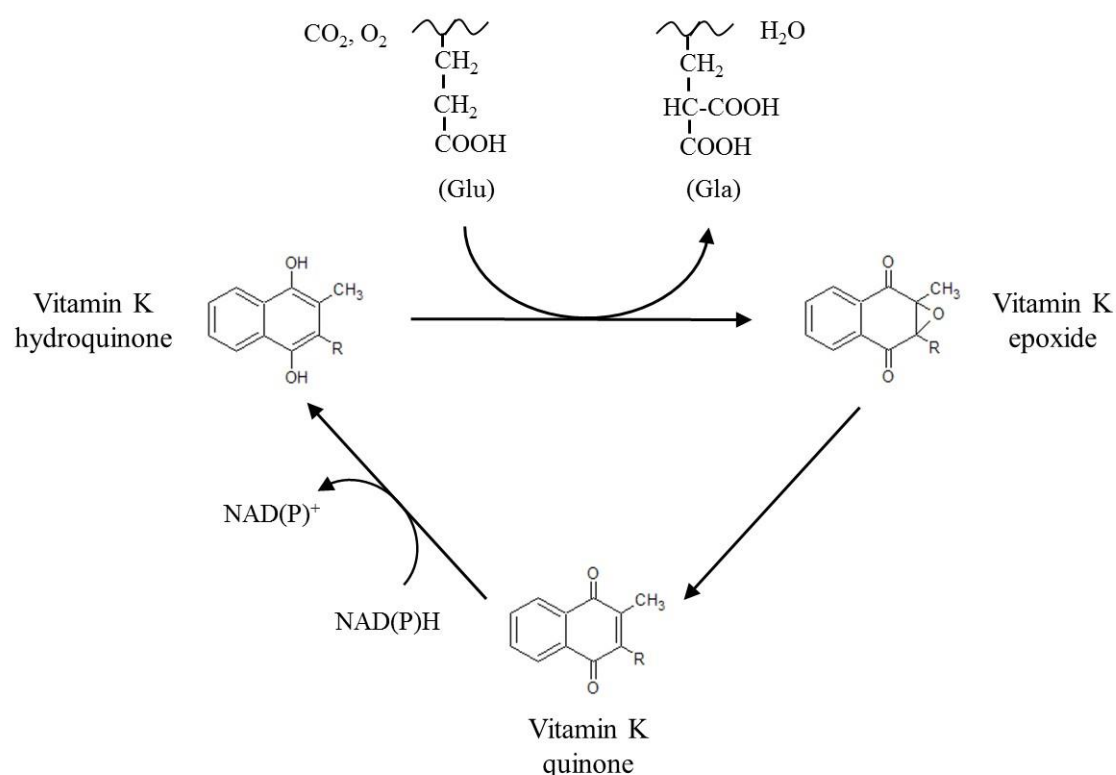


Figure 3. The vitamin K cycle [2].

Vitamin K-containing substance

Among native form vitamin K, phylloquinone (vitamin K₁) which is synthesized by chloroplast of plants is included in green and yellow vegetables and beans, and while Menaquinone (MK-n : n=4~14; vitamin K₂) synthesized by bacteria and animals is included in meats, chicken eggs, dairy products, fermented food, and so on.

Behavior and physiological effect of vitamin K within a living organism

Vitamin K is absorbed from small intestine, taken into chylomicron and carried to liver via lymphatic (Figure 4). After that, vitamin K is moving in the blood and then

carried to peripheral tissue (Figure 4). Vitamin K has effects on blood coagulation, arteriosclerosis and bone metabolism (Figure 4).

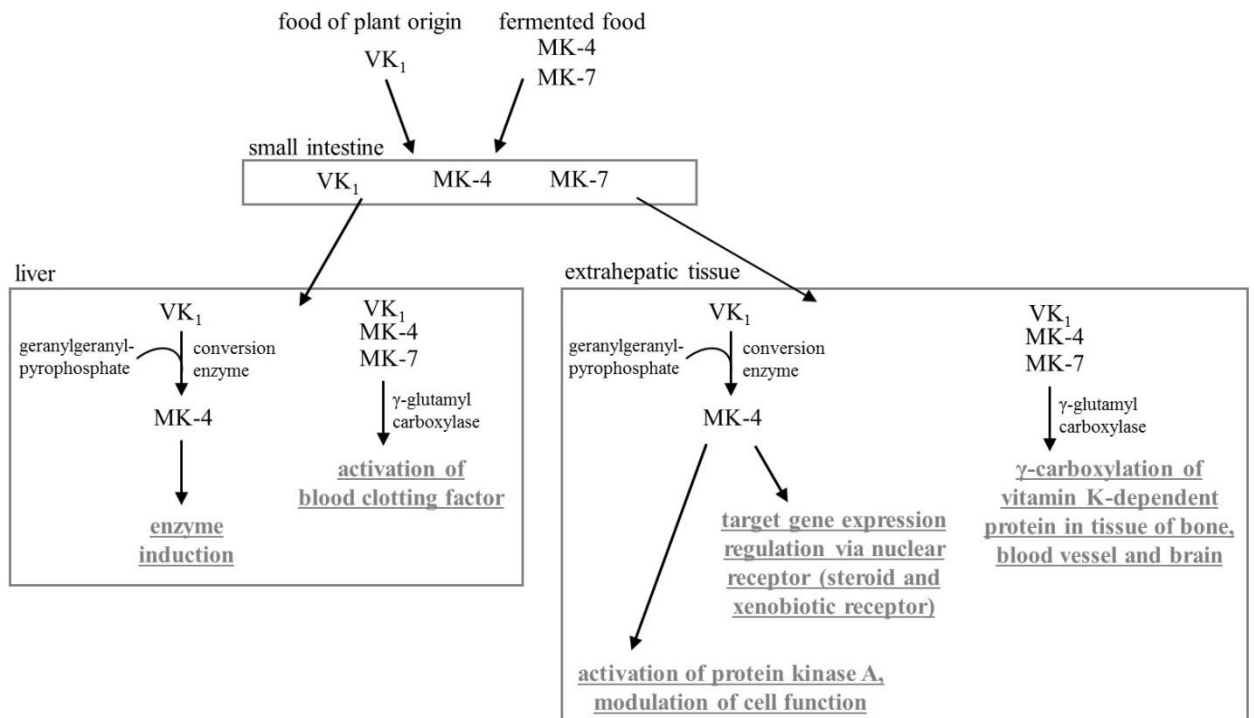


Figure 4. The behavior and physiological effect of vitamin K within a living organism [2].

Blood coagulation

There are two pathways of blood coagulation by vitamin K, which are extrinsic and intrinsic ones in Figure 5. Damage of tissue is a trigger, the pathway of blood coagulation follows like the process shown in Figure 5, and eventually blood becomes to be stopped by a chunk of cross-linked fibrin. In this case, vitamin K works as a cofactor on the system of blood coagulation. Vitamin K is involved with blood coagulation factor (prothrombin (factor II), factor VII, factor IX, factor X).

On the other hand, thrombin which works on the process of blood coagulation in Figure 5 has an anti-blood coagulation activity. This is involved in blood coagulation control factor (protein C, protein S) and then inhibits blood coagulation. Protein C and protein S, incidentally, are vitamin K-dependent proteins. Therefore, vitamin K is vital vitamin for acceleratory effect and inhibitory effect on the mechanism of blood coagulation.

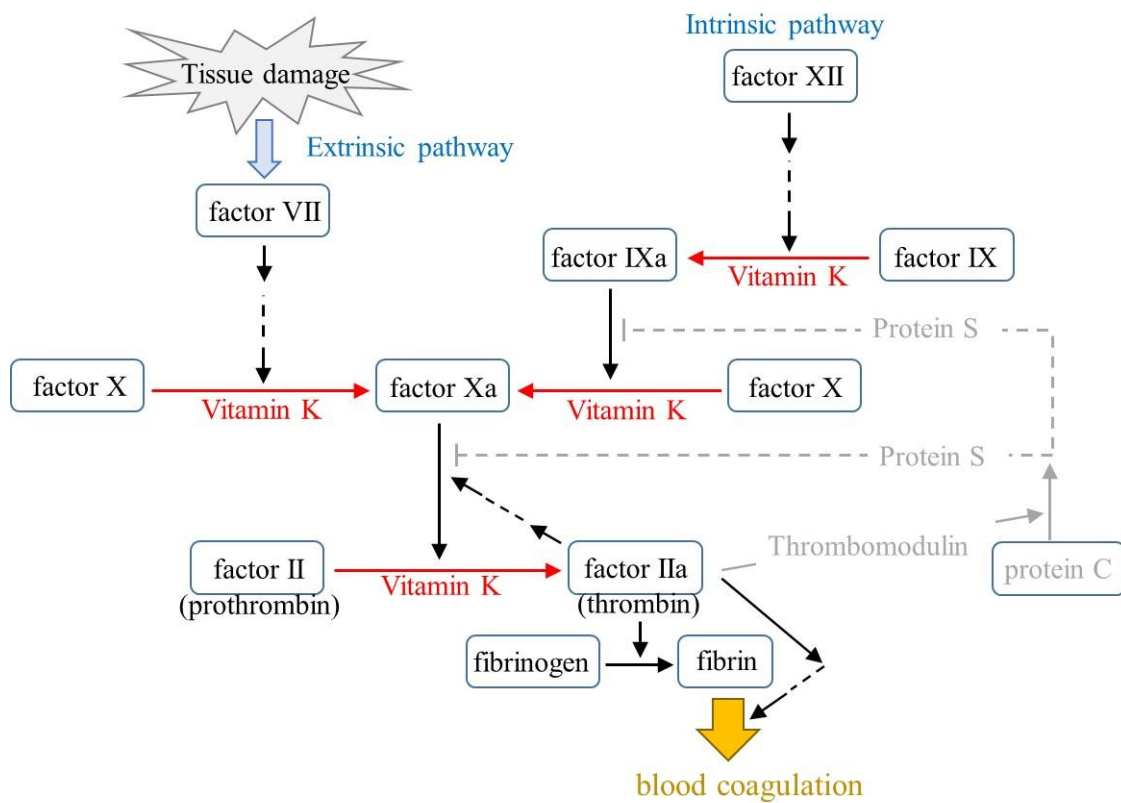


Figure 5. The role of vitamin K on the mechanism of blood coagulation [2].

Arteriosclerosis

Vitamin K-dependent protein MGP (matrix gla protein) was reported to inhibit strongly media sclerosis in blood vessel [3]. Therefore, many researchers have focused

on the relation between vitamin K and arteriosclerosis. It was considered that vitamin K was involved in arteriosclerosis as shown in Figure 6 [4-6], but the detail of mechanism has not yet been revealed.

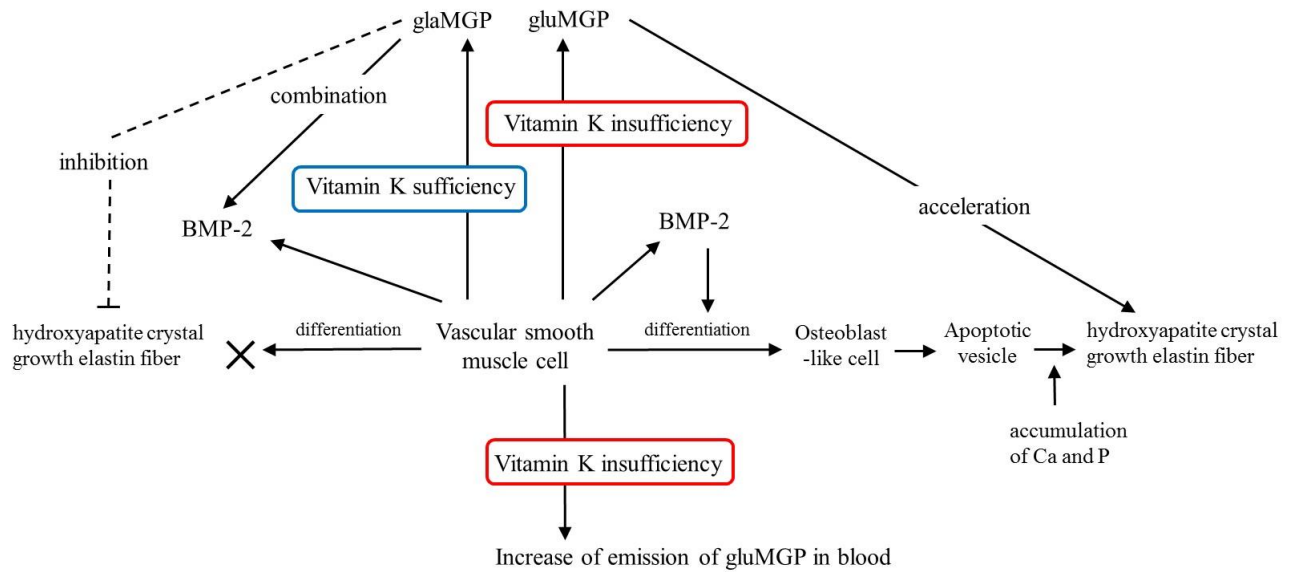


Figure 6. The inhibition of arteriosclerosis by vitamin K-dependent protein MGP (matrix gla protein) [2].

Bone metabolism

The bone is always remodeled by cooperative relations between osteoblast which works on a bone formation and osteoclasts which plays a role in bone resorption. In this osteoblast, vitamin K is considered to work as a roll shown in Figure 7.

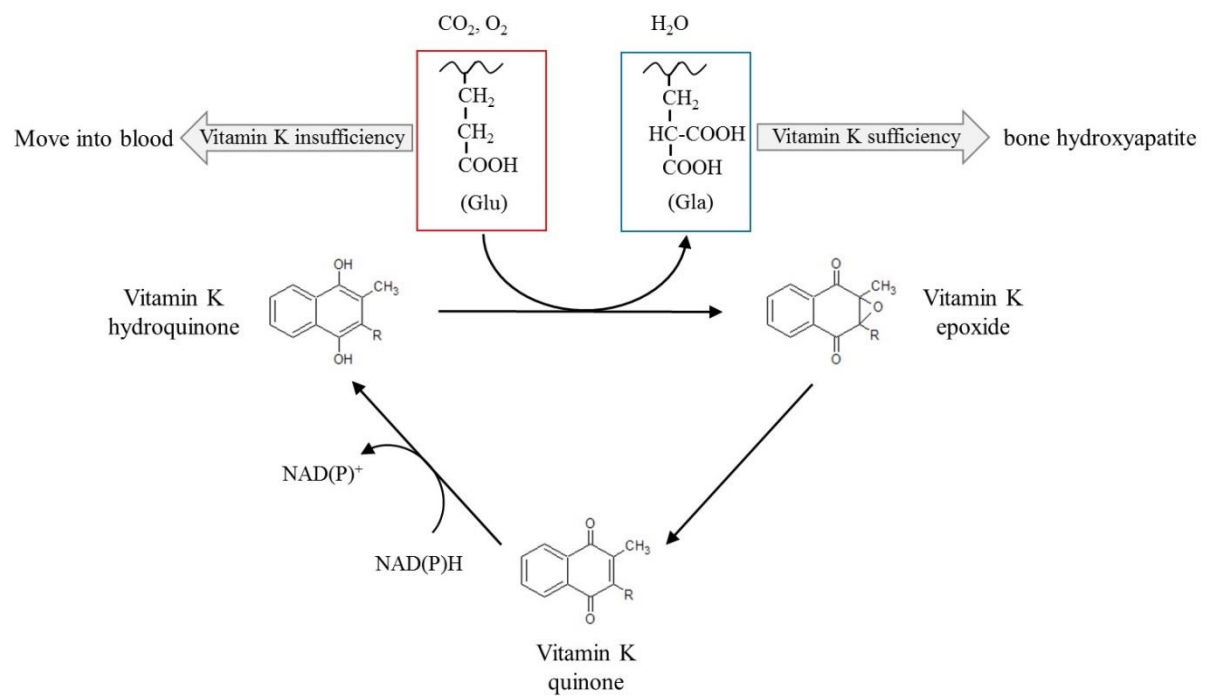


Figure 7. The activation of osteocalcin coupled to the vitamin K cycle [2].

The place of existence in vivo

CoQ is widely distributed in biological membrane [11].

Biochemical and physiological effects

Electron transport

The CoQ is an essential factor to the electron transport chain (Figure 9). The electron transport chain exists on mitochondrial inner membrane in eukaryote, and while, that exists on cytoplasmic membrane in prokaryote.

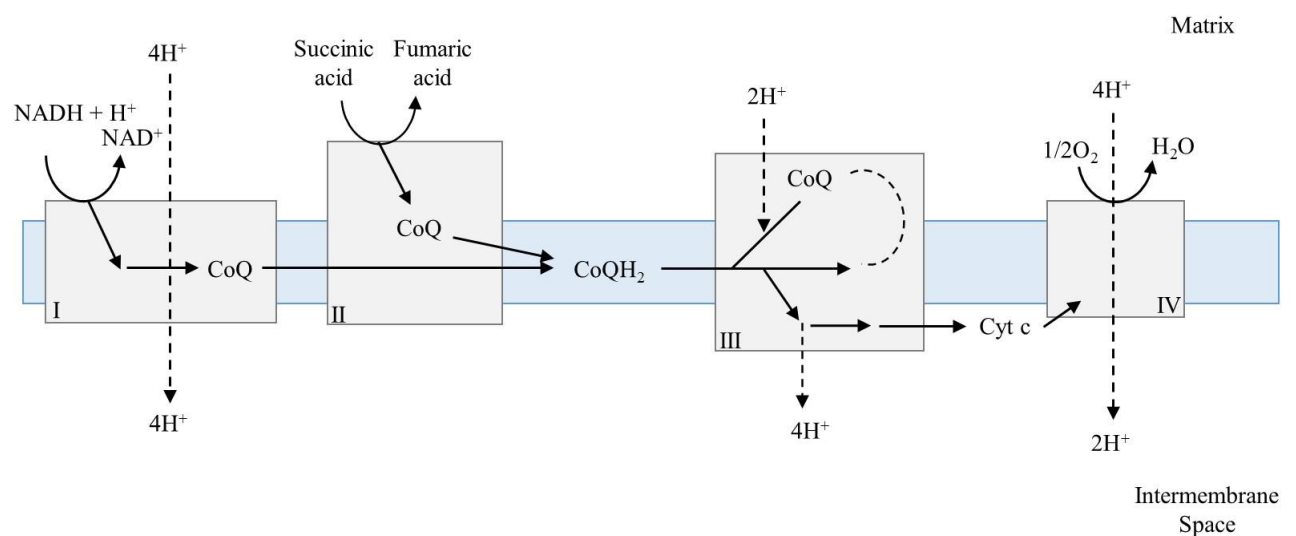


Figure 9. The outline of electron transport [1].

The main components of electron transport chain consist of four kinds of enzyme complexes and two kinds of electron carriers. The four kinds of enzyme complexes are NADH-CoQ oxidoreductase (Complex I), succinate-coenzyme Q reductase (Complex II), CoQH₂-cytochrome-c oxide reductase (Complex III, *bc₁* complex), and cytochrome c oxidase (Complex IV). The two kinds of electron carriers

are CoQ and cytochrome c (cyt c). On the electron transport chain, CoQ is involved in electron transfers and proton (H^+) transport via the membrane at Complex I and between Complex II and Complex III. The overall reaction on each complex are “ $NADH + H^+ + CoQ + H^+_{in} \rightleftharpoons NAD^+ + CoQH_2 + 4H^+_{out}$ ” on Complex I, “ $succinic\ acid + CoQ \rightleftharpoons fumaric\ acid + CoQH_2$ ” on Complex II, “ $CoQH_2 + 2cyt\ c_{ox} + 2H^+_{in} \rightleftharpoons CoQ + 2cyt\ c_{red} + 4H^+_{out}$ ” on Complex III, and “ $4cyt\ c_{red} + O_2 + 8H^+_{in} \rightleftharpoons 4cyt\ c_{ox} + 2H_2O + 4H^+_{out}$ ” on Complex IV (Figure 9).

The CoQ in lipid membrane exists almost ten times the amount of other electron carriers (molar ratio) existing in mitochondrial inner membrane. A part of CoQ exists in binding to proteins. The CoQ acts like “ $CoQ\ (ubiquinone) \rightleftharpoons SQ\ (ubisemiquinone) \rightleftharpoons CoQH_2\ (ubiquinol)$ ” which is shown in Figure 10. The SQ occurs not in the free state which is reactive but in binding to protein. Complex I, Complex II and Complex III have the binding site of CoQ or pocket.

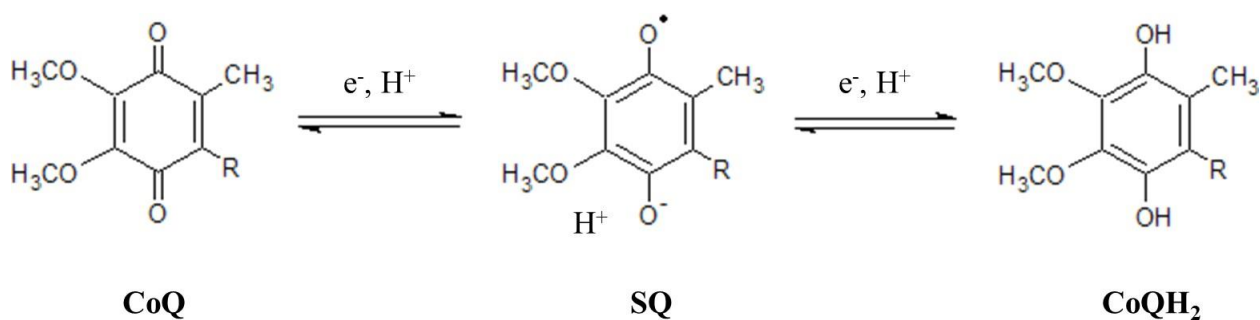


Figure 10. The change of form of CoQ by redox reaction [1].

Antioxidant effect

In 1966, for the first time, Dr. Mellors and Dr. Tappel reported an antioxidant effect of CoQ_6H_2 (reduced CoQ) [12]. There are homologues which have different

length of sub chain. It was reported that the length of sub chain was generally shorter, the strength of antioxidant effect was larger [13]. The two mechanisms of antioxidant by CoQH₂ were proposed, which were direct action and indirect action [14] (Figure 11).

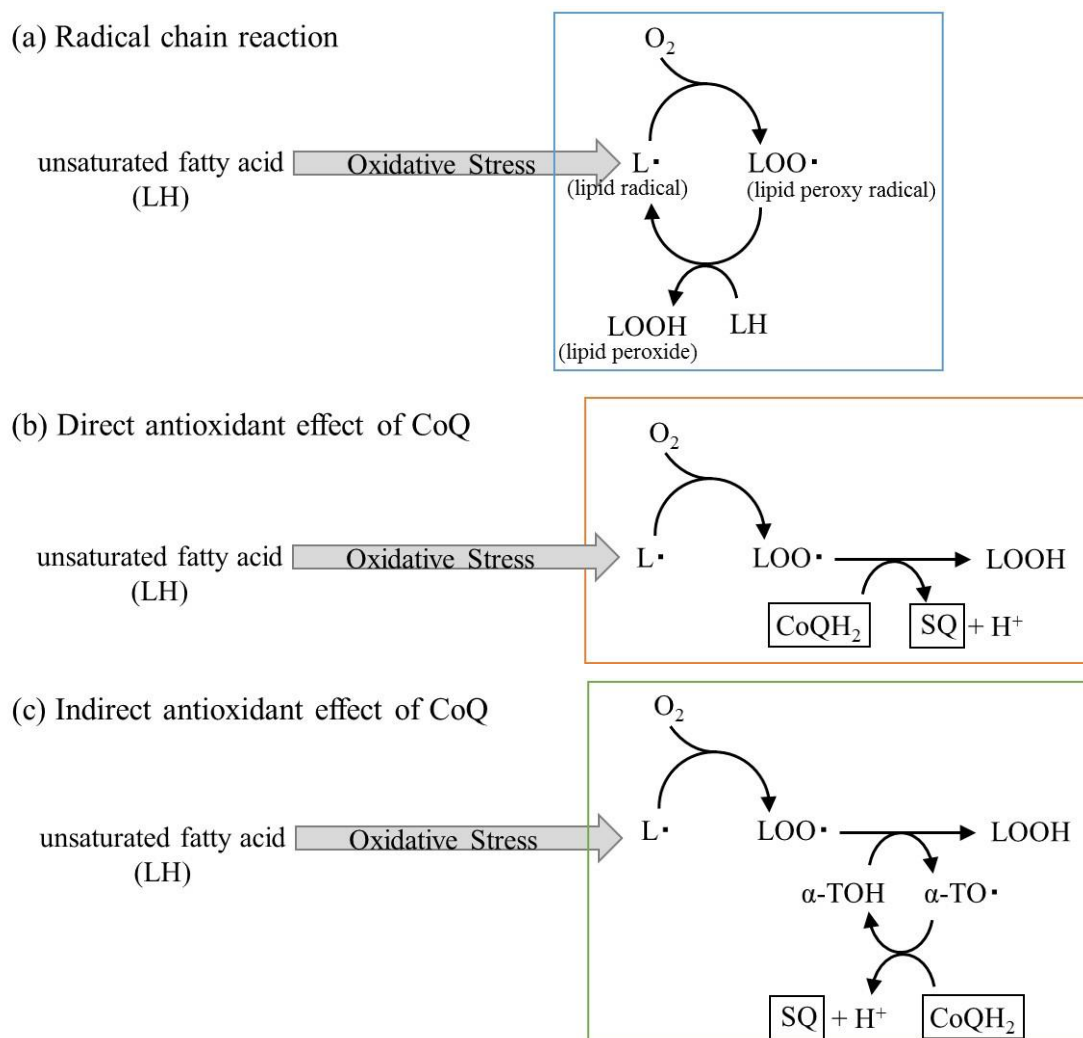


Figure 11. The mechanisms of antioxidant by CoQH₂ [1]. (a) Radical chain reaction, (b) Direct antioxidant effect of CoQ, and (c) Indirect antioxidant effect of CoQ.

CoQ needs to exist as CoQH₂ (reduced form) in order to have antioxidant effect, but CoQH₂ is very unstable substance in air, therefore, CoQH₂ is immediately

oxidized to CoQ (oxidized form). However, it was reported that many part of CoQ in human body and animal body (e.g. rat, and so on) (in the plasma and in the liver) exist as CoQH₂ [11,15]. Furthermore, on the experiments of injecting CoQ into human or animals, CoQH₂ was detected in the blood and within a tissue. Consequently, it is suggested the mechanism of two-electron reduction of CoQ and keeping CoQ CoQH₂ within the cell. In fact, until now, enzymes which have a possibility of two-electron reduction of CoQ₁₀ within the body were reported, which were NAD(P)H, lipoamide dehydrogenase, thioredoxine reductase 1, glutathione reductase, and NADPH-dependent CoQ reductase [16-19]. It is considered that these enzymes are involved in oxidation-reduction cycle of CoQ and may protect the body from reactive oxygen species.

PQQ (quinone of biological system)

History

J.G. Hauge discovered Pyrroloquinoline quinone (PQQ) as a bacterial redox cofactor in 1964 [20]. And then, Anthony et al., Salisbury et al. and Duine reported it as methoxatin [21-26]. Salisbury et al. and Duine et al. reported the structure of PQQ in 1979 [27,28]. PQQ is the third redox cofactor following $\text{NAD}^+/\text{NADP}^+$ and FAD/FMN . PQQ is identified as a bacterial redox cofactor, but it has not yet been revealed that mammal has PQQ. In 2003, Dr. Takaoki Kasahara and Dr. Tadafumi Kato reported that PQQ is a cofactor which is involved in metabolic pathways of lysine in mouse [29], but there are no reports of proving directly. Until now, 13 kinds of vitamins were found and many researchers have been considered PQQ is a 14th vitamin, but it is not settled whether PQQ is vitamin or not. PQQ is mainly made from gram-negative bacteria, works as a redox cofactor in bacteria. In plants, in plants of the family Liliaceae, it was reported that PQQ prompts pollen tube growth and the growth of vegetables (e.g. tomato and cucumber).

Chemical reactivity of PQQ

PQQ is an o-quinone and has a pyridine ring and a pyrrole ring. A pyridine ring and a pyrrole ring have the opposite electronic properties. Therefore, PQQ is a special structure. At the position of o-quinone of PQQ, a redox reaction occurs. And also, PQQ has three carboxyl groups (Figure 12), therefore, it is very freely soluble in water and heat stable. The color of PQQ presents pink. PQQ are reported to react to various substances. The overall of chemical reactivity are shown in Figure 12.

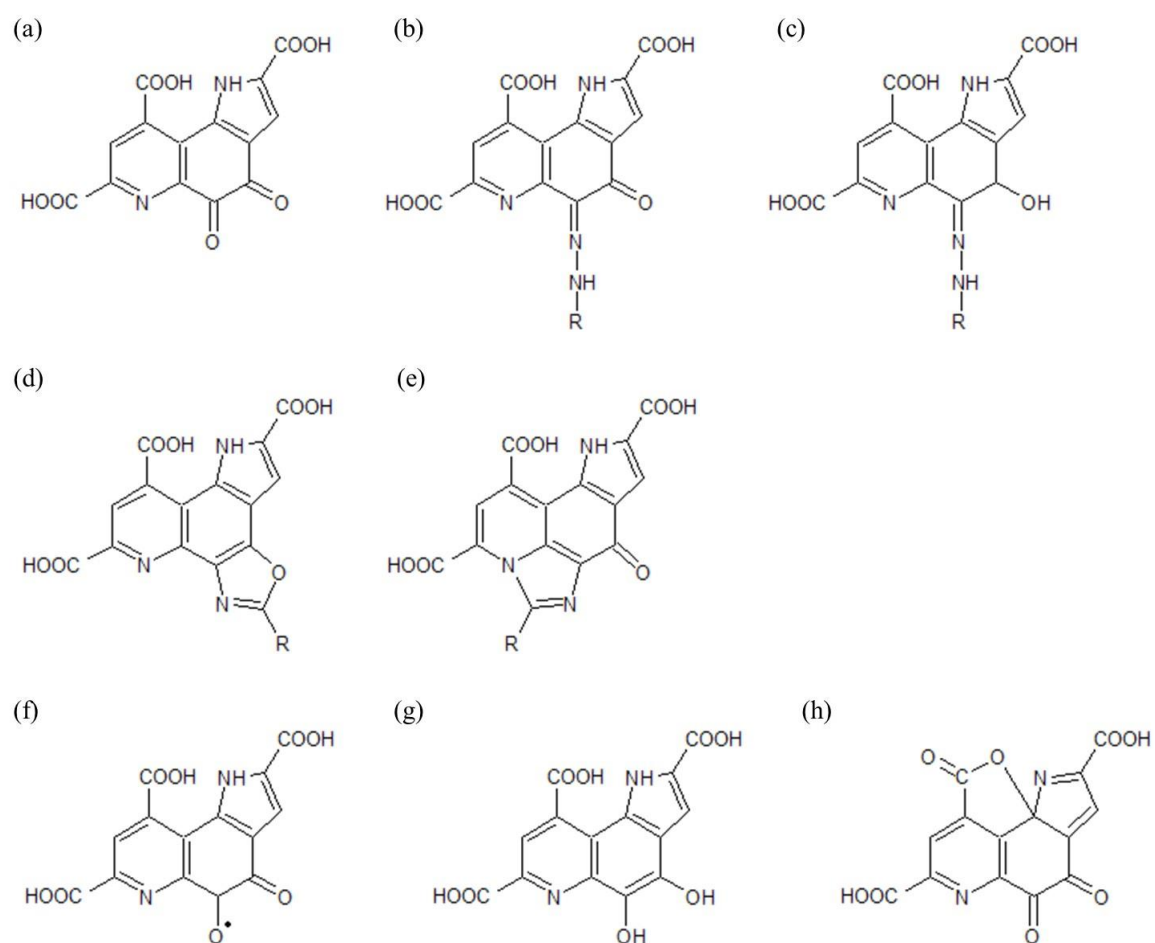
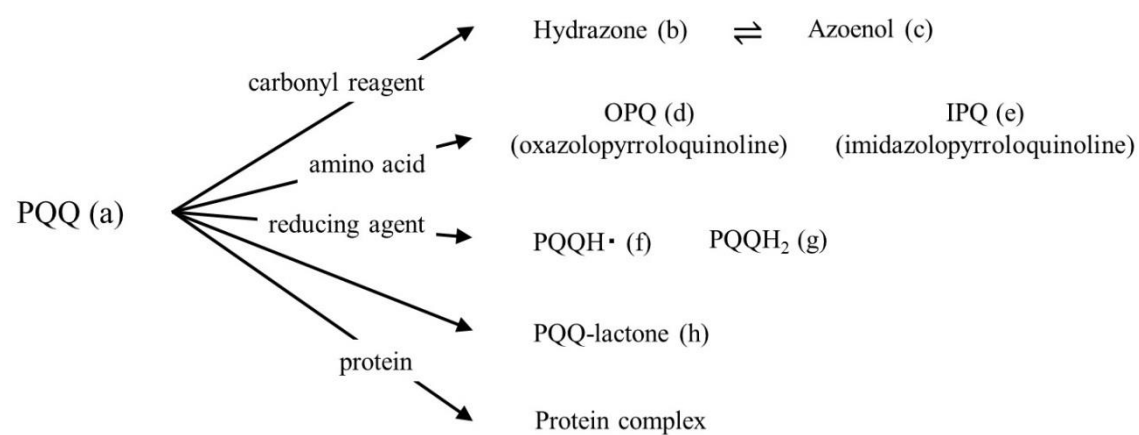


Figure 12. The overall of chemical reactivity of PQQ [1].

Determination method [30,31]

Until now, six approaches were reported on the determination method.

- (1) The method of measuring of remodeling activity of enzymes which need PQQ as coenzyme [32].
- (2) The method of high performance liquid chromatography (HPLC).
- (3) The method of nitroblue tetrazolium (NBT) (NBT/Glycinate assay), which is the assay of redox cycle using glycinate [33-39].

On this method, quinone oxidizes glycine and then changes to hydroquinone. At that time, glycine changes to dehydrogenated glycine. Hydroquinone reacts with oxygen and consequently superoxide anion is formed. And then NBT reacts with this and changes to formazan. The reduction of NBT to formazan is measured spectrophotometrically at 530 nm.

This method is used in the experiments which are referred at chapter 2 and 3.

- (4) The method of GC/MS, which is the method that PQQ is handled by using phenyltrimethylammonium (PTMA) and then is analyzed as a volatile derivative [40].
- (5) The enzyme-linked immunosorbent assay (ELISA) which uses antibody of PQQ that is controlled as hapten.
- (6) The method of LC/MS, which is the method of detecting PQQ by using mass spectrometry after the complex is separated or eluted by the HPLC method [41].

PQQ-containing substance

PQQ is widely distributed in foods of plants (Table 4).

Table 4. The list of PQQ-containing plants [30].

plants	PQQ (ng/g)	plants	PQQ (ng/g)
broad bean	17.8 ± 6.8	apple	6.1 ± 1.4
green soybean	9.3 ± 3.8	banana	12.6 ± 3.8
Irish potato	16.6 ± 7.3	kiwifruit	27.4 ± 2.6
sweet potato	13.3 ± 3.7	orange	6.8 ± 2.2
parsley	34.2 ± 11.6	papaya	26.7 ± 8.6
cabbage	16.3 ± 4.0		
carrot	16.8 ± 2.8		
celery	6.3 ± 2.4		
bell pepper	28.2 ± 13.7		
spinach	21.9 ± 6.2		
tomato	9.2 ± 1.8		

PQQ enzyme [1,22,42-44]

PQQ is the third oxidation-reduction coenzyme following NAD • NADP and FMN • FAD, as mentioned above. PQQ-dependent enzymes (PQQ enzymes) which have been revealed the function until now were found in alpha-proteobacteria, beta-proteobacteria, and gamma-proteobacteria. PQQ enzymes exist in periplasm of bacteria which is a region between cell wall and cytosolic membrane, and serve as an electron carrier to the electron transport chain in cytosolic membrane. There two kinds of PQQ enzyme distinguished from the difference of property toward periplasm, which are called “soluble enzyme” and “membrane-bound enzyme”, respectively. The former is that PQQ enzymes exist as being soluble in periplasm, and the latter is that PQQ enzymes bind strongly to the periplasm side of cell membrane. And also, there are PQQ enzymes which have not only PQQ but also heme c as an prosthetic group. These enzymes are called quinohemoprotein. And while, PQQ enzymes which have only PQQ

are called quinoprotein. PQQ enzymes need to bind a calcium ion for the redox activity [1]. This placement is shown in Figure 13. A calcium ion coordinate with oxygen atom at position 5 of carbonyl group of PQQ, nitrogen atom next to oxygen atom at position 5 of carbonyl group, and oxygen atom of carboxyl group next to the nitrogen.

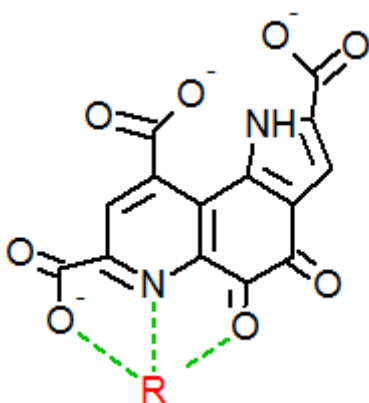


Figure 13. The placement of calcium ion (R) on PQQ.

Distinguished by substrate of PQQ enzymes, there are alcohol dehydrogenase that PQQ enzymes use alcohol as substrate and that is the large amount of PQQ enzyme among various PQQ enzymes, and glucose dehydrogenase that PQQ enzymes use glucose as substrate. And the other substrate is sugar alcohol. In the next paragraph, I describe alcohol dehydrogenase and glucose dehydrogenase of PQQ enzymes.

PQQ alcohol dehydrogenase (PQQ-ADH) [23,43,45,46]

PQQ enzymes which exist most widely among these enzymes are alcohol dehydrogenases (ADH). For example, there are methanol dehydrogenase (MDH), ethanol dehydrogenase, 1-butanol dehydrogenase, and so on. The quinoprotein which has been studied most for a long time among these ADH is MDH, which exists in

methanol assimilating bacteria that utilizes methanol as a sole source of carbon (Figure 14).

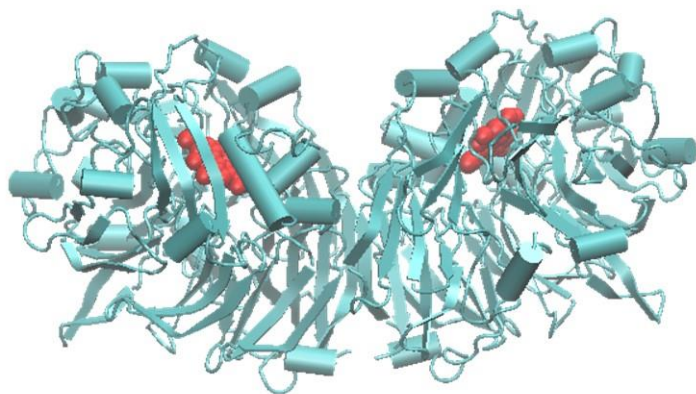


Figure 14. One example of the X-ray structure of MDH [47]. PQQ molecules are represented by red color and van der Waals view, and atoms except for PQQ are represented by gray color and cartoon view.

PQQ-ADH other than MDH which use alcohol as substrate are divided into quinoprotein and quinoxemoprotein, which are called “Type-I ADH (qADH)” and “Type-II ADH (qhADH)”, respectively. ADH of acetic acid bacteria are membrane-bound enzymes which have three subunit containing quinoxemoprotein and cytochrome c, and called “Type-III ADH”. The following are typical examples of qADH and qhADH.

A typical example of Type-I ADH (qADH) is an ethanol dehydrogenase (qEDH) that is produced when oxidative bacteria (e.g. *Pseudomonas aeruginosa*, and so on) produce ethanol (Figure 15). This structure of enzyme is similar to that of MDH, however, this enzyme does not react much with methanol, but react much with primary alcohol having short carbon chain (e.g. ethanol).

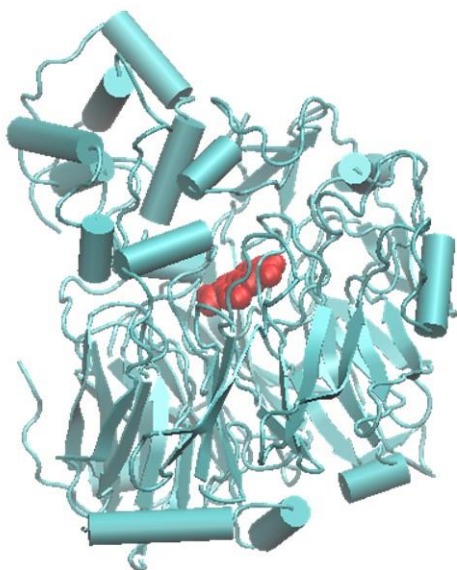


Figure 15. One example of the X-ray structure of qADH [48]. PQQ molecules are represented by red color and van der Waals view, and atoms except for PQQ are represented by gray color and cartoon view.

Typical example of Type-II ADH (qhADH) are alcohol dehydrogenases, ADH-IIB and ADH-IIG (Figure 16(a) and 16(b)). Those enzymes are produced by *Pseudomonas putida* HK5 that assimilates ethanol dehydrogenase (qhEDH) and main-chain alcohol of *Comamonas testosteroni*. *Pseudomonas putida* HK5 produces three different kinds of ADH by utilizing different alcohols as a source of carbon. Among three ADHs, ADH-IIB and ADH-IIG are Type-II ADH, which are produced by the product of primary alcohol of medium chain (e.g. 1-butanol) and by the product of 1,2-propanediol and glycerol, respectively.

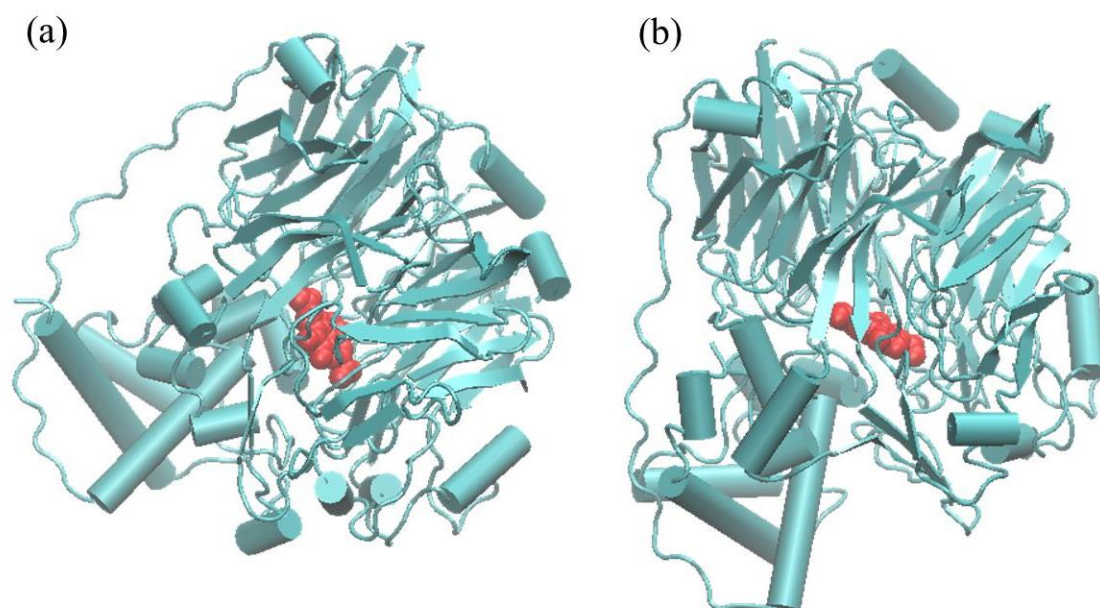


Figure 16. One example of the X-ray structure of (a) ADH-IIB [49] and (b)ADH-IIG [50]. PQQ molecules are represented by red color and van der Waals view, and atoms except for PQQ are represented by gray color and cartoon view.

PQQ glucose dehydrogenase (PQQ-GDH) [44,45]

PQQ enzymes which exist second most widely after PQQ-ADH are glucose dehydrogenases (GDH). There are two kinds of GDH, which are soluble enzyme (sGDH) that exists in *Acinetobacter* (Figure 17) and membrane-bound enzyme (mGDH) that distributes widely in acetic acid bacteria, *Pseudomonas*, intestinal bacteria, and so on in *Proteobacteria*. These two enzymes are different from structures and functions. The structure of sGDH is greatly different from other PQQ enzymes. The place binding to PQQ is an inversion one. The mGDH of *Escherichia coli* (*E. coli*) is produced as an apoenzyme in order not to have a PQQ biosynthesis system, and oxidizes glucose to gluconic acid only when PQQ is taken in from outside.

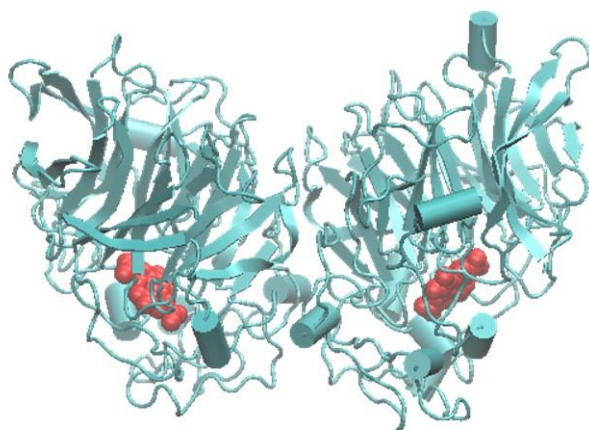


Figure 17. One example of the X-ray structure of sGDH [51]. PQQ molecules are represented by red color and van der Waals view, and atoms except for PQQ are represented by gray color and cartoon view.

Physiological function of PQQ [1,52]

Physiological role of PQQ in animal has not yet been revealed, but PQQ is considered to play important roles within a living organism, therefore, various studies are reported.

Antioxidant potential

PQQ is reported to work as a radical scavenger (antioxidant) and remove efficiently reactive oxygen species (ROS) (e.g. $\cdot\text{O}_2^-$, $\cdot\text{HO}$, and so on). It is supposed that PQQ work in organism as an antioxidant like Glutathione, vitamin C, vitamin E, and uric acid.

And also, PQQ is considered to involve in important pathway which is important to cell signaling [53-57]. The following function (cell proliferation, mitochondriogenesis and oxidative metabolism, and apoptosis-cell death) shown in

Figure 18 are considered. I describe the detail of these functions in the following paragraph.

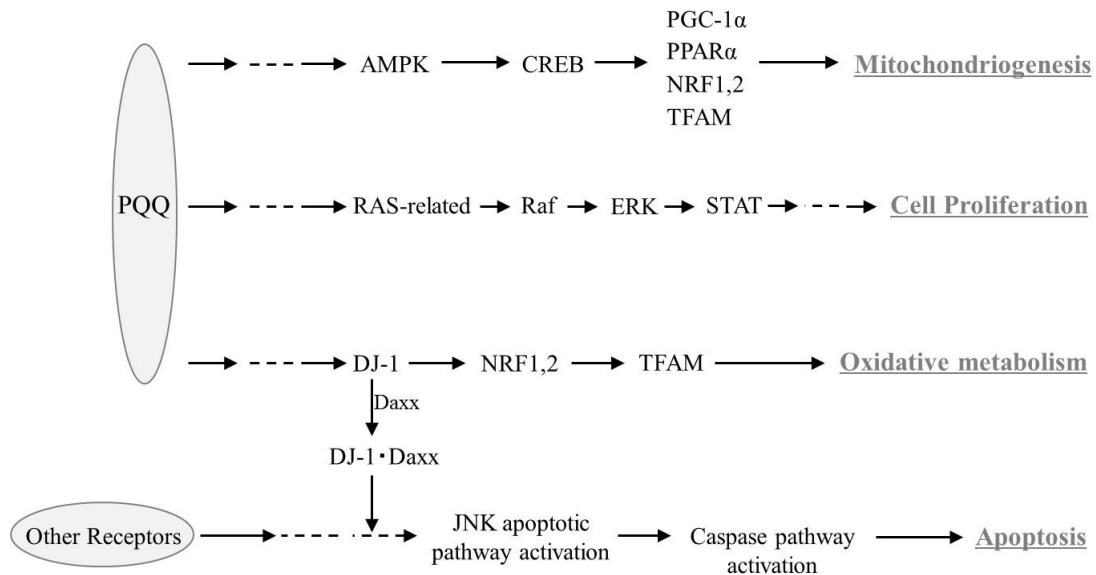


Figure 18. The physiological function of PQQ [52].

Mitochondriogenesis

Mitochondrial respiratory control is important because it is involved in energy utilization, protection from reactive oxygen species, and so on which is important for our health. Mitochondrial DNA depletion and mutations are associated with diseases (e.g. cardiomyopathy, developmental delays, and so on) [58], therefore, optimal mitochondrial function for health are required. PQQ is proposed to involve in this mitochondrial-related events (Figure 18). These many mitochondrial-related events are controlled by peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1 α) and nuclear respiratory factors. And the interactions between this PGC-1 α and PQQ have been reported [58-60].

PGC-1 α mentioned above is a transcriptional coactivator. This regulates genes which are involved in energy metabolism [59,60]. There is a direct link between an external physiological stimulus and the regulation of mitochondriogenesis by an interaction between PGC-1 α and the protein and an association between PGC-1 α and multiple transcription factors. PGC-1 α is also associated with a reduction in reactive oxygen species [59].

On PGC-1 α -related cell signaling [33,57,61-63], activation begins with signals. This signal is from 5'AMP-activated protein kinase (AMPK) or one of the many mitogen-activated protein (MAP) kinases which are linked to various cell surface receptors [58,59]. After that, like pathway shown in Figure 18, cAMP response element binding protein (CREB; a transcription factor which binds to some DNA sequences that are called cAMP response elements) is activating in combination with nuclear respiratory factors 1 and 2 (NRF), and mitochondrial transcription factors (e.g., Tfam), and eventually, it is considered to lead to the increased mitochondriogenesis [61-63].

Cell proliferation

PQQ is also considered to influence the activity of ras [56]. Ras is an oncogene which is important to signal transduction processes that is involved in growth and development. As a pathway shown in Figure 18, by the affection of PQQ, the signals of ras, Raf, ERK(a protein kinase that functions in the ras-signaling pathway to activate other components of transcription which is activators, co-activators, and transcription factors), and signal transducers and activators of transcription (STAT; essential to control of cell growth, survival, and differentiation) are activated in turn [64], and eventually, this signal of pathway is considered to connect to cell proliferation.

Oxidative metabolism and Apoptosis-cell death

PQQ is also reported to affect the activity of DJ-1 [55]. DJ-1 has a relation with cellular oxidative stress responses. If autosomal-recessive mutations in DJ-1 happen, Parkinson's disease is caused. It is suggested that an interaction between DJ-1 and PQQ facilitates and fine-tunes overall cellular regulation [55]. As a pathway shown in Figure 18, DJ-1 is considered to involve in oxidative metabolism. And while, DJ-1 has a relation with Daxx (a multifunctional protein which is associated with apoptotic events). DJ-1 • Daxx is capable of modulating apoptosis by inactivating the Janus kinase (JNK) cell-signaling pathway. And eventually, as a pathway shown in Figure 18, apoptosis-cell death is considered to occur after JNK apoptotic pathway activation and caspase pathway activation.

Summarized the proposed physiological function of PQQ (Figure 18), the crosstalk between the signals (i.e. interaction among ras, DJ-1 and numerous kinases) leads to control cell proliferation, apoptosis, and mitochondriogenesis.

(Further information can be seen in here [1].)

Summary and the purpose of my doctor thesis

Vitamin and vitamin analogous compound are considered to have various functions. However, PQQ has not yet been revealed much. And furthermore, the structure of PQQ is special, that is why PQQ is considered to have a possibility of special function. Therefore, I think the study of PQQ proceeds further development of the study. In my doctor thesis, I focus on the antioxidant function of PQQ. This function is widely known and therefore gained considerable attention from many researchers. The understanding of its redox mechanism is a key to proceed further development in the fields of medicine, engineering, sitology, pharmacy, and chemistry. For example, PQQ was used as a model for the dehydrogenation reaction of an amine to design a bio-inspired catalyst. Therefore, understanding the function of coenzyme PQQ is an indispensable basis for the development of designing a superior catalyst-molecule.

The purpose of my doctor thesis is to understand the redox mechanism of PQQ. At first, I investigated the mechanism of glycine oxidation catalyzed by PQQ in aqueous solution. And then, I investigated the comparison of three amino acids (glycine, alanine and valine) oxidation catalyzed by PQQ in aqueous solution. Moreover, the role of cation was investigated. I report the detail of results following the chapter.

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Chapter 2.

Mechanism of glycine oxidation catalyzed by pyrroloquinoline quinone in aqueous solution

(Waka Uchida, M. Wakabayashi, K. Ikemoto, M. Nakano, H. Ohtani, S. Nakamura, Chem. Phys. Lett. 620 (2015) 13.)

Abstract

The mechanism of glycine oxidation reaction by PQQ in aqueous solution was investigated. A new crystal structure of PQQ under alkaline conditions was referred to calculate on a quantum chemical basis. Two mechanisms are investigated by this calculation: a “stepwise” mechanism, namely, a nucleophilic attack on C5 or C4 by the nitrogen atom of glycine, and proton and electron transfer to PQQ. The second mechanism is a “concerted” mechanism, namely, simultaneous reaction, which does not include the nucleophilic attack and include proton and electron transfer to PQQ.

Introduction

In 1964, J.G. Hauge discovered pyrroloquinoline quinone (PQQ) as a bacterial redox cofactor [1]. After that discovery, Anthony[2,3], Salisbury[4], and Duine[5] et al. reported it as methoxatin. The structure of PQQ has a redox site with a quinone moiety that can be hydrogenated to a diphenol. PQQ has two forms: “normal” (oxidized) and “reduced”. The active site of bacterial glucose dehydrogenase has PQQ as a co-enzyme, which is used in blood-sugar-level sensors used by diabetics to determine when to inject insulin [6,7]. These sensors require PQQs that have a high redox cycling potential. Known as a functional material for both animals and humans, PQQ is contained in highly fermented food and breast milk [8]. The reported functions include protection of neurons, improvement of growth, a drop in blood-sugar level, and mitochondrial activation. It has also been reported that reduced PQQ, which can be formed by reduction of PQQ by using NADH, an alcohol, and an amine, is related to most of these functions [9-11].

The antioxidant function of PQQ, for which the reduced form is much more active than the oxidized form, is also widely known [12]. PQQ has therefore gained considerable attention from many researchers. The understanding of its redox mechanism is a key to developing it further in the fields of medicine, engineering, sitology, pharmacy, and chemistry. Recently, a bio-inspired catalyst was designed. PQQ was used as a model for the dehydrogenation reaction of an amine [13]. To design a superior small molecule catalyst, understanding the function of coenzyme PQQ is therefore an indispensable basis for the development of catalyst-molecule.

Enzyme proteins containing PQQ are called quinoproteins, and the two main ones are reported, methanol dehydrogenase (MDH) [4,14-17] and glucose

dehydrogenase (GDH) [18-20], catalyze the oxidation of methanol and glucose to formaldehyde and glucolactone, respectively. The X-ray structures of these proteins have been reported and have been used for theoretical studies on the mechanism of methanol or glucose oxidation [21-27]. Among these theoretical studies and other studies (which did not use the x-ray structure), two mechanisms were reported: addition-elimination and/or hydride transfer [21-30]. In the protein environment, hydride transfer can be considered. In solution environment, however, hydride transfer is somewhat difficult to understand from the viewpoint of a hydride-producing source. We have been motivated to study the mechanism of the dehydrogenation reaction by PQQ, focusing on a simple system in solution from the viewpoint of quantum chemistry.

An amino acid may mostly coexists with PQQ as an amine and function as a reducing agent [31]. Amino acids can be found in food, medium, serum, and cytoplasm. PQQ acts as an effective catalyst for mediating the redox cycle, not only inside those proteins but also in the absence of proteins. PQQ is soluble in water, stable under heat, and has the ability to catalyze repeated redox reactions in aqueous solution [32]. In an appropriate environment, the number of repeated redox reactions by PQQ is larger than that by many bioactive quinones or enediols [8,32,33]. Under these appropriate conditions, the reaction of PQQ with glycine occurs better than that with other amino acids [34]. The present study therefore focuses on the mechanism of the PQQ-catalyzed glycine oxidation.

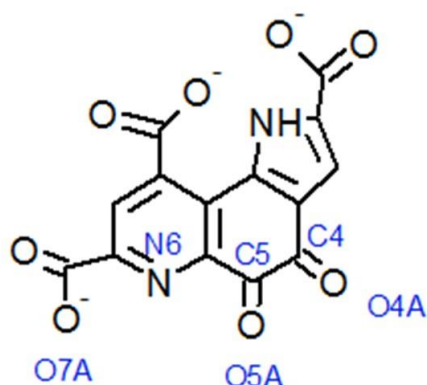
The crystal structures of PQQ have been reported as an acetone adduct, a metal complex, and a disodium salt [4,35,36]. Glycine oxidation (which is the subject of the current study) is conducted in alkaline solution; however, the state of PQQ under

alkaline conditions is not known. In this study, therefore, to determine the state of PQQ, a crystal of PQQ was produced under alkaline conditions [37]. Herein, in reference to the crystal structure thereby obtained, the mechanism of glycine oxidation catalyzed by PQQ in aqueous solution was investigated by using quantum chemical calculations. We should be careful for the comparison with enzymatic reaction, since our study is on a homogeneous catalyst in aqueous solution.

We present here a study on the reaction mechanisms reflecting the above-described PQQ crystal structure, molecular structures, electronic states, and relative Gibbs free energies of each point on the reaction pathways. The stepwise reaction mechanism (including nucleophilic attack by glycine) and the concerted mechanism were examined.

Computational details

Geometry optimizations and frequency analyses were performed by using the density functional theory [38] with the B3LYP hybrid functional [39-41]. The 6-31G(d,p) basis set [42,43] was used for all atoms. Gibbs free energies at 298.15 K were calculated from the frequency analyses of optimized structures at each equilibrium geometry or transition state (TS). The intrinsic reaction coordinate (IRC) searches [44] were carried out and confirmed that the TS was linked to reactants and products. The effect of water as a solvent was considered by using the polarizable continuum model (PCM) [45,46]. Moreover, additional explicit water molecules were included in the calculation if needed. Gaussian 09 [47] was used for all calculations.



The labels of the atoms in PQQ (C4, C5, O4A, O5A, N6, and O7A) are shown above. A calculation system was constructed from one PQQ molecule, one K^+ cation, two explicit water molecules, and one OH^- anion, as shown later and also in the supplementary information (SI) (see Figure S1). From the viewpoint of electrostatic interactions and coordination bonds, a K^+ cation interacts with O5A, N6, and O7A atoms, because the K^+ is included in the solution of experiments [33]. This position of K^+ in the initial structure of calculation system is supported by X-ray structures. A metal ion was located at the above-mentioned place in the recent crystal structure under

alkaline conditions [37], which was the common feature to the past X-ray structures. For example, a K^+ cation was located at the same position reported by the X-ray structure of PQQ containing K^+ [48], and moreover, a Na^+ cation was located at the same position reported by the X-ray structure of PQQ containing Na^+ [35]. Furthermore, in theoretical studies using the x-ray structures of MDH or GDH, the initial structures include Ca^{2+} at the same position mentioned above in their calculation system [21-24,30].

The experiments that we study currently were performed in aqueous solution at alkaline pH condition [8,33]. Although the solvent effect was considered by using a PCM method, two H_2O and one OH^- molecules were incorporated explicitly in the calculation systems. As for constructing the initial structures of calculation system, first, an oxygen atom of H_2O or OH^- was placed near one of the hydrogen atoms in the amino group of glycine so that it forms a hydrogen bond. This placement induces polarization and therefore induces a minus charge on the nitrogen atom of glycine. This placement enables a nucleophilic attack on C4 or C5 by the nitrogen atom at the first step of the reaction (Figure 1). In the next step, another H_2O or OH^- molecule was located near the former H_2O or OH^- so that it forms a hydrogen bond. In the last step, another H_2O or OH^- molecule was located near the second H_2O or OH^- to form a hydrogen bond. One of the H_2O or OH^- molecules forms a hydrogen bond with the O4A atom of PQQ (see Figure S1).

In regard to the search for possible reaction pathways, it was important to treat these H_2O and OH^- molecules explicitly (i.e., not simply by PCM). The H_2O or OH^- molecule nearest to one of the hydrogen atoms of the amino group of glycine was found to be very significant. A water molecule was located around the K^+ cation, and the

difference of energies was monitored (see Figure S2 and Table S1). Examples with more water molecules than described here are shown in the SI and suggest that the TS energy does not change much (less than 2 kcal/mol) with the additional water molecules (see Figure S3 and Table S2).

Results and Discussion

Mechanism of glycine oxidation by PQQ in aqueous solution.

Previously reported theoretical studies were mostly performed on the mechanism of methanol or glucose oxidation under a protein environment (not on a homogeneous catalyst in solution) [21-26]. Although the system of interest is in solution, reflecting the previous studies, the current study examined two mechanisms (Figure 1): first, a stepwise mechanism (including nucleophilic attack by glycine) and, second, a concerted mechanism.

Nucleophilic attack on the stepwise mechanism

As for the first reaction, nucleophilic attack by a nitrogen atom of glycine on C5 (Figure 1(a)-upper) or C4 (Figure 1(a)-lower) of PQQ, C5 is addressed first (because it is the most important one for conclusion of this study). It is natural to assume the nucleophilic attack because the charge around these carbon atoms is slightly positive due to the electro-negativity difference between oxygen and carbon. On the other hand, the nitrogen atom of glycine has a lone pair of electrons and is enough rich in electrons to approach C4 or C5.

Proton transfer following nucleophilic attack on the stepwise mechanism

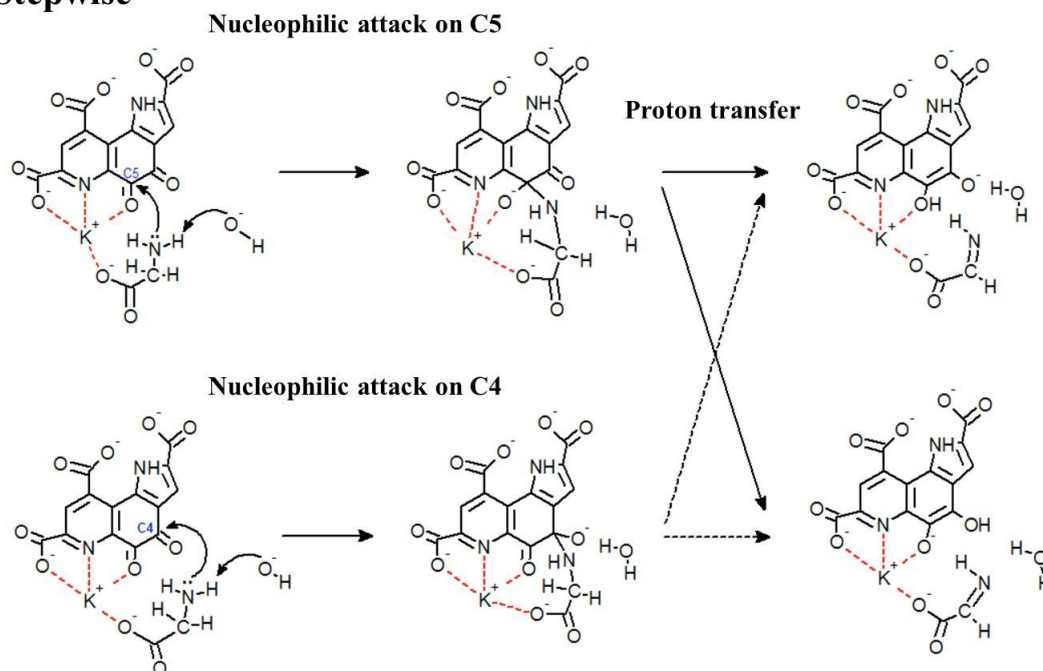
As for the second reaction, proton transfer [Figure 1(a)], a hydrogen atom on the α -carbon of glycine is extracted as a proton by the lone pair of electrons of O4A or O5A. At the same time, two electrons (which form the bond between the nitrogen atom of glycine and one of the carbon atoms) transfer to the PQQ molecule. As a result, the single bond between C4 and C5 becomes a double bond. The nitrogen atom and the α -carbon of glycine thereby form a double bond. Thus, the glycine oxidation is completed. Conventionally, the reduced form of PQQ is described so that both O4A and

O5A are hydrogenated. Once either O4A or O5A becomes OH, the PQQ can become the reduced form, since it easily receives one proton from one of the water molecules in the surroundings.

Concerted mechanism

The concerted mechanism includes three events that occur simultaneously [Figure 1(b)]; (i) transfer of one of the hydrogen atoms on α -carbon of glycine to O5A or O4A, (ii) transfer of one of the hydrogen atoms on the nitrogen atom of glycine to a neighboring H₂O or OH⁻ molecule, and (iii) transfer of a pair of electrons around the nitrogen atom of glycine to the PQQ molecule, which forms a double bond between C4 and C5 [Figure 1(b)].

(a) Stepwise



(b) Concerted

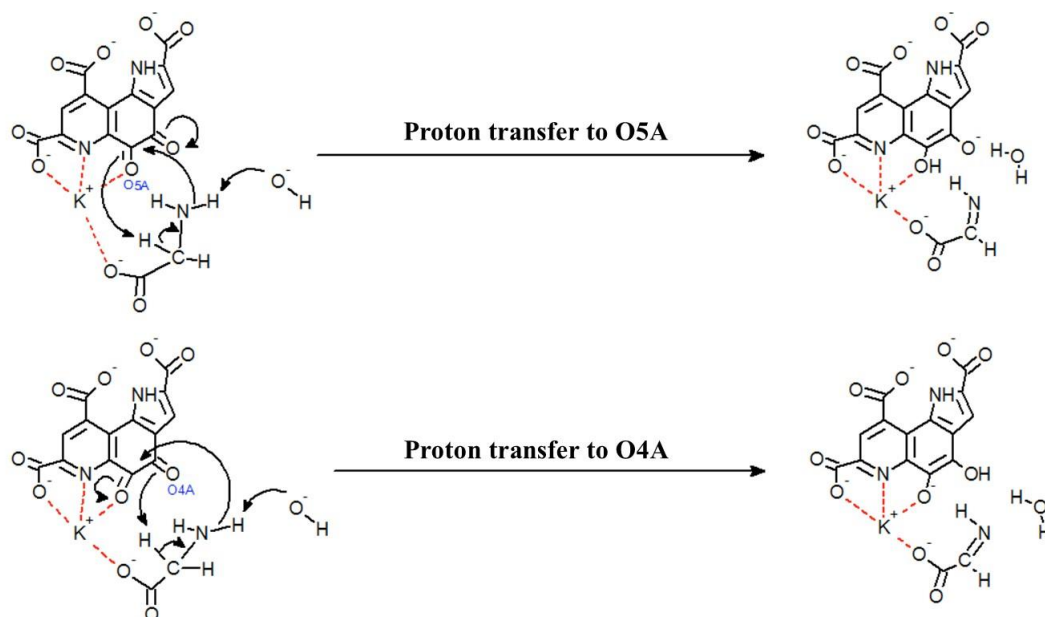


Figure 1. A stepwise mechanism (a) and a concerted mechanism (b). Stepwise mechanism (a) has two modes of nucleophilic attack on C5 or on C4. The first step is nucleophilic attack by the nitrogen atom of glycine and the second step is proton transfer and elimination reaction. A concerted mechanism (b) is a proton transfer to PQQ, a proton extraction by the surroundings and electron transfer to PQQ. It does not include nucleophilic attack.

Reactant structure

The geometry of the reactants with oxidized PQQ is optimized first. Here, this state is abbreviated to OX. The detail of OX is given in the SI (see Figure S1, and figures in the following discussions). The calculation system contains one PQQ molecule, one K^+ cation, two explicit water molecules, and one OH^- anion. The K^+ cation was located stably among O7A, N6, and O5A by coordination bonds. In addition, the oxygen atom of the carboxyl group of glycine forms a coordination bond with K^+ ,

which provides the foothold for glycine and makes the nucleophilic attack possible (Figure 1). Moreover, the nitrogen atom of the amino group of glycine is located very close to the C5 and C4 atoms, which allows the nitrogen atom to attack the C5 or C4 atoms as a nucleophilic addition reaction.

Nucleophilic attack on C5

The calculated results of the nucleophilic reaction that starts from a nucleophilic attack on C5 (Figure 1(a)-upper), the relative Gibbs free energies and optimized structures, are shown in Figures 2 and 3, respectively. When the nitrogen atom attacks C5, one of the hydrogen atoms of the amino group of glycine moves toward the nearest oxygen atom of the H₂O or OH⁻ molecules [Figures 3(a), 3(b), and 3(c)]. The TS energy is calculated to be 3.2 kcal/mol [Figure 2, TS1(C5)], relative to the reference energy; OX is the reference (0.0 kcal/mol). The mode with imaginary frequency at TS1(C5) corresponds well to the mode that describes the nucleophilic reaction (see Movie 1 in SI).

The nucleophilic attack reaction produces the intermediate (INT), of which the energy is 0.8 kcal/mol lower than that of the reactant OX (Figure 2). The electronic structure around C5 shifts from sp² to sp³ hybridization. The nitrogen atom of glycine forms a bond to C5 [C5-N=1.57Å; Figure 3(c)].

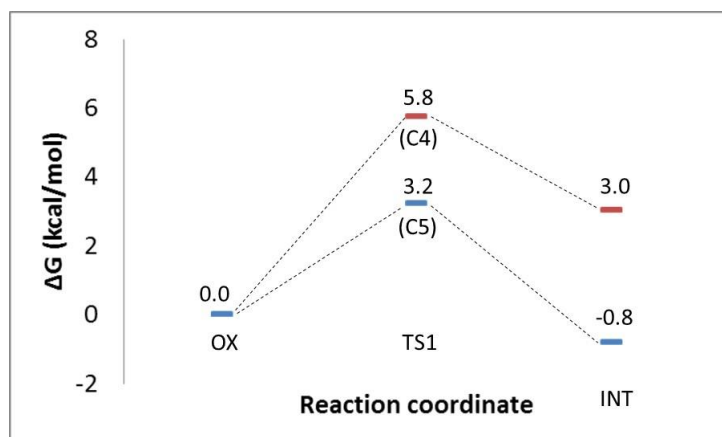


Figure 2. The Gibbs free energies of OX, transition state (TS) 1, and intermediate (INT).

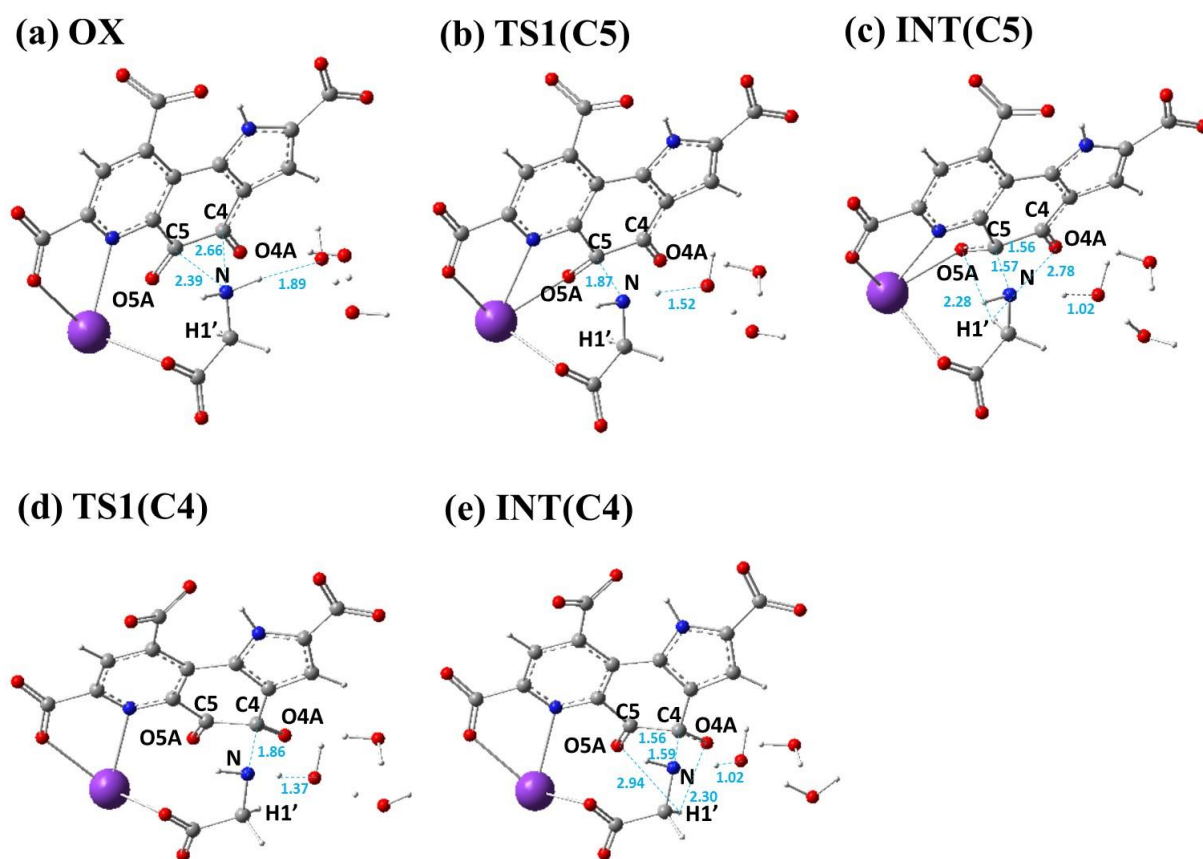


Figure 3. The optimized structures of (a) OX, (b) TS1(C5), (c) INT(C5), (d) TS1(C4), and (e) INT(C4). The distance is in Å.

Nucleophilic attack on C4

The calculated results of the nucleophilic reaction that starts from a nucleophilic attack on C4 (Figure 1(a)-lower), the energies and optimized structures, are shown in Figures 2 and 3, respectively. When the nitrogen atom attacks C4, one of the hydrogen atoms of the amino group of glycine moves toward the nearest oxygen atom of the H₂O or OH⁻ molecules [Figures 3(a), 3(d) and 3(e)]. From OX to INT, the electronic structure around C4 changes from sp² to sp³ hybridization. The nitrogen atom of glycine forms a bond to C4 [C4-N=1.59Å; Figure 3(e)]. The TS energy of this reaction is 5.8 kcal/mol (Figure 2, TS1(C4)). The mode with imaginary frequency represents an attack on C4 by the nitrogen atom of glycine (see movie 2 in SI). The energy of TS1(C4) is higher than that of TS1(C5) by 2.6 kcal/mol (Figure 2). Furthermore, the energy of INT, which is the energy of the state after the nucleophilic attack, is 3.0 kcal/mol higher than that of OX (Figure 2). If the Boltzmann distribution is taken into account, the nucleophilic attack on C5 shown in Figure 1(a)-upper can be considered a major reaction. This consideration is supported by the calculated charge distribution of C5 or C4 of OX by Mulliken population analysis and the distance between the nitrogen atom of glycine and C5 or C4 (see Tables S3). The charge of the nitrogen atom is negative, so the nitrogen atom can easily attack a more-positively-charged atom. The charge of C5 is more positive than that of C4 and the distance between nitrogen and C5 is shorter than that between nitrogen and C4. The energy of TS1(C5) is lower. Considering further Mulliken population analysis for two systems; one is only one PQQ, the other is one PQQ and one K⁺, the calculated charge distributions indicate that C5 is more positive than C4 (see Figure S4 and Table S4).

The crystal structure of methanol adduct of the trimethyl ester of PQQ was

reported and the position of methanol addition was C5 [49]. This result is in line with the current result. Furthermore, theoretical studies on the scheme of addition-elimination under a protein environment focused on the addition of methanol or glucose to C5 of PQQ [21,24,25,30]. These standpoints are consistent with the result of current study, namely, the nucleophilic attack of nitrogen on C5 is a major reaction.

Proton transfer following a nucleophilic attack on C5

At the state after a nucleophilic attack on C5 (at INT(C5)), O5A, C5, the nitrogen atom of glycine, the α -carbon of glycine and one of the hydrogen on the α -carbon form a pentagon geometry. O4A, C4, C5, the nitrogen atom of glycine, the α -carbon of glycine and one of the hydrogen on the α -carbon form a hexagon geometry which is actually twisting in space (Figure 3(c)). This structure facilitates proton transfer from the α -carbon of glycine toward O5A or toward O4A (Figure 1(a)). The hydrogen labeled H1' in Figure 3(c) is the closest hydrogen on the α -carbon of glycine to PQQ.

TS energy of proton transfer to O5A is 9.7 kcal/mol, while that to O4A is 18.2 kcal/mol (Figure 4, TS2). In fact, the necessary energy from INT to TS2 in proton transfer to O5A is 10.5 kcal/mol, while that from INT to O4A is 19.0 kcal/mol. In other words, proton transfer to O4A is more difficult than that to O5A.

The mode with imaginary frequency at TS2 corresponds clearly to the reactions of proton transfer. Furthermore, the modes showed the characteristic profile of the reaction. When the distance between C5 and O5A or that between C4 and O4A increases, the distance between the nitrogen atom and the α -carbon of glycine decreases, and vice versa (see Movies 3, and 4 in SI). From INT to TS2, the proton transfers to

O5A, the bond between C5 and the nitrogen atom of glycine is lost (C5-N=1.57Å, Figure 3(c); C5-N=2.47Å, Figure 5(a)). Geometries shows that the electronic structure around the nitrogen atom shifts from sp³ to sp²-like, and around C5, it returns to sp²-like. In case of proton transfer to O4A, it is the same reaction as that for transfer to O5A (C5-N=1.57Å, Figure 3(c); C5-N=2.75Å, Figure 5(c)).

The addition-elimination mechanism for considering methanol or glucose oxidation under the protein environment has been reported. In particular, proton transfer from methanol or glucose to O4A and that from surrounding amino acids to O5A was reported [21,24,25,30]. By contrast, glycine oxidation in our calculation system is conducted in aqueous solution. Two possible proton transfer mechanisms to PQQ (O4A or O5A) were thus studied.

As a result of the proton transfer, INT becomes the product of the reduced PQQ form (RED) [Figures 3(c), 5(b), and 5(d)]. The energy of RED after proton transfer to O5A is -19.2 kcal/mol, and that corresponding to O4A is -13.8 kcal/mol (Figure 4). The α -carbon of glycine changed from sp³ to sp², and the bond between the nitrogen and α -carbon of glycine became a double bond [Figures 5(b) and 5(d)]. Either O4A or O5A here is a negative oxygen ion (O⁻) [Figures 5(b) and 5(d)]. It can be readily hydrogenated by protonation from one of the water molecules in the surroundings. With that hydrogenation, the formation of RED is complete.

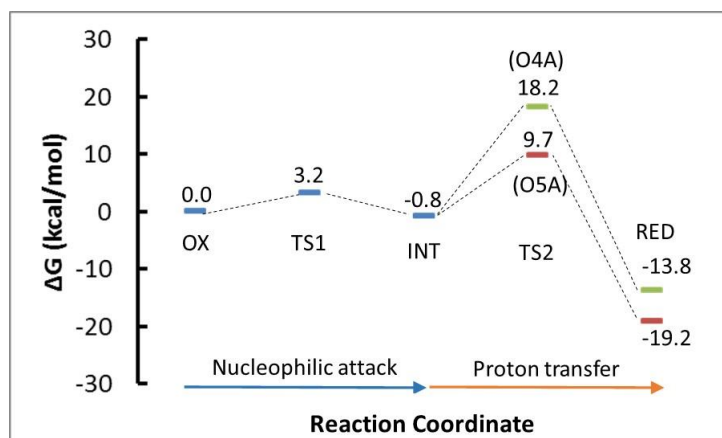


Figure 4. The Gibbs free energies of OX, transition state (TS) 1, intermediate (INT), TS2 and RED. Those of OX, TS1, and INT are the same in Figure 2 (blue).

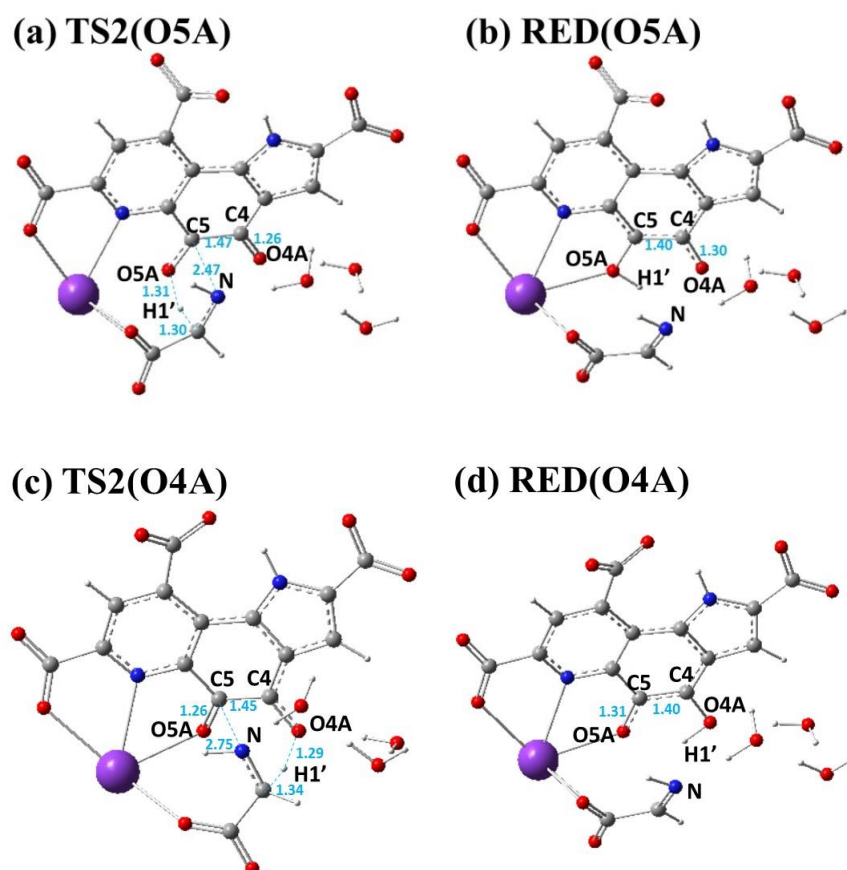


Figure 5. The optimized structures of (a) TS2(O5A), (b) RED(O5A), (c) TS2(O4A), and (d) RED(O4A). The distance is in Å. Those of OX, TS1 and INT of Figure 4 are shown

in Figures 3(a), 3(b) and 3(c).

Concerted reaction

The possibility of an alternative mechanism to stepwise mechanism, that is, a concerted mechanism [Figure 1(b)], was investigated as described in the following. The energies and optimized structures are shown in Figures 6 and 7, respectively. From OX to RED via only one TS, the concerted proton and electron transfer along the reaction paths were observed [Figures 7(a) - (e)].

This concerted reaction is facilitated by the OX structure. K^+ cation of OX provides the foothold for glycine, the nitrogen atom of glycine is very close to the C4 or C5 atom, and consequently one of the hydrogen atoms of the α -carbon in glycine is very close to O4A or O5A (see Figure S1 and Figure 7(a)).

The nearest hydrogen atom of the α -carbon in glycine is labeled H1' in Figure 7(a). The TS energy of proton transfer to O5A is 16.3 kcal/mol, and that to O4A is 23.2 kcal/mol (Figure 6). Same as the case of stepwise reaction, the proton transfer to O4A is more difficult than that to O5A. The TS(O5A) was obtained by loose convergence criteria. (Notice that it was obtained by tight convergence criteria when we add one more explicit water molecule (see Figure S5).)

TS of proton transfer to O5A or to O4A are shown in Figures 7(b) and 7(d), respectively. The mode with imaginary frequency represents the H1' transfer to O5A or O4A, respectively (see movies 5 and 6 in SI). The structures differ from those of TS2 of proton transfer to O5A or O4A in Figure 1(a), respectively (Figures 5(a), 5(c), 7(b), and 7(d), see movies 3, 4, 5, and 6 in SI). This concerted reaction does not include nucleophilic attack, instead, the electron and proton transfer occur in a concerted

manner with a single transition state.

In the reaction, from OX to TS of proton transfer to O5A or O4A, the H1' moves to O5A or O4A, the electronic structure around the nitrogen atom of glycine changes from sp³ to sp²-like, and one of the hydrogen atoms of the amino group in glycine moves to the nearest oxygen atom of the H₂O or OH⁻ molecules [Figures 7(a), 7(b), 7(d), and 7(e)]. The electronic structure around the α -carbon of glycine changes from sp³ to sp²-like, and the bond between the nitrogen and α -carbon atoms in glycine becomes a double bond. If the Boltzmann distribution is taken into account, both concreted reactions can be considered difficult ones.

As the proton transfers, OX changes to RED (C4-C5=1.54Å in Figure 7(a); C4-C5=1.47Å in Figure 7(b); C4-C5=1.40Å in Figure 7(c); C4-C5=1.46Å in Figure 7(d); C4-C5=1.40Å in Figure 7(e)). The energy of RED after proton transfer to O4A is -13.7 kcal/mol, and that to O5A is -19.3 kcal/mol (Figure 6). Either O4A or O5A here is a negative oxygen ion (O⁻) [Figures 7(c) and 7(e)]. It can be readily hydrogenated by protonation from one of the water molecules in the surroundings. With that hydrogenation, the formation of RED is complete.

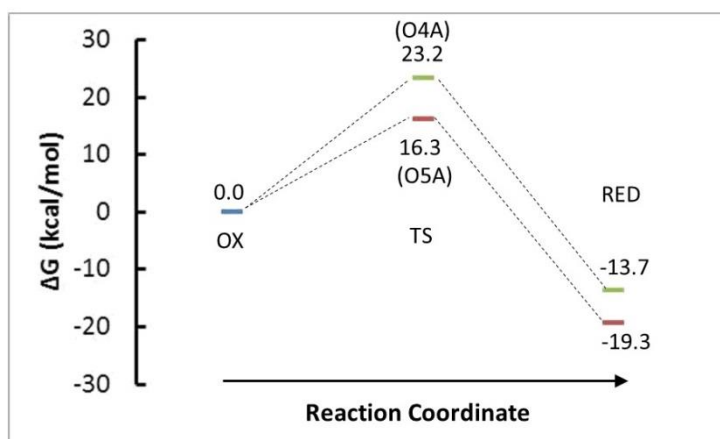


Figure 6. The Gibbs free energies of OX, transition state (TS) and RED.

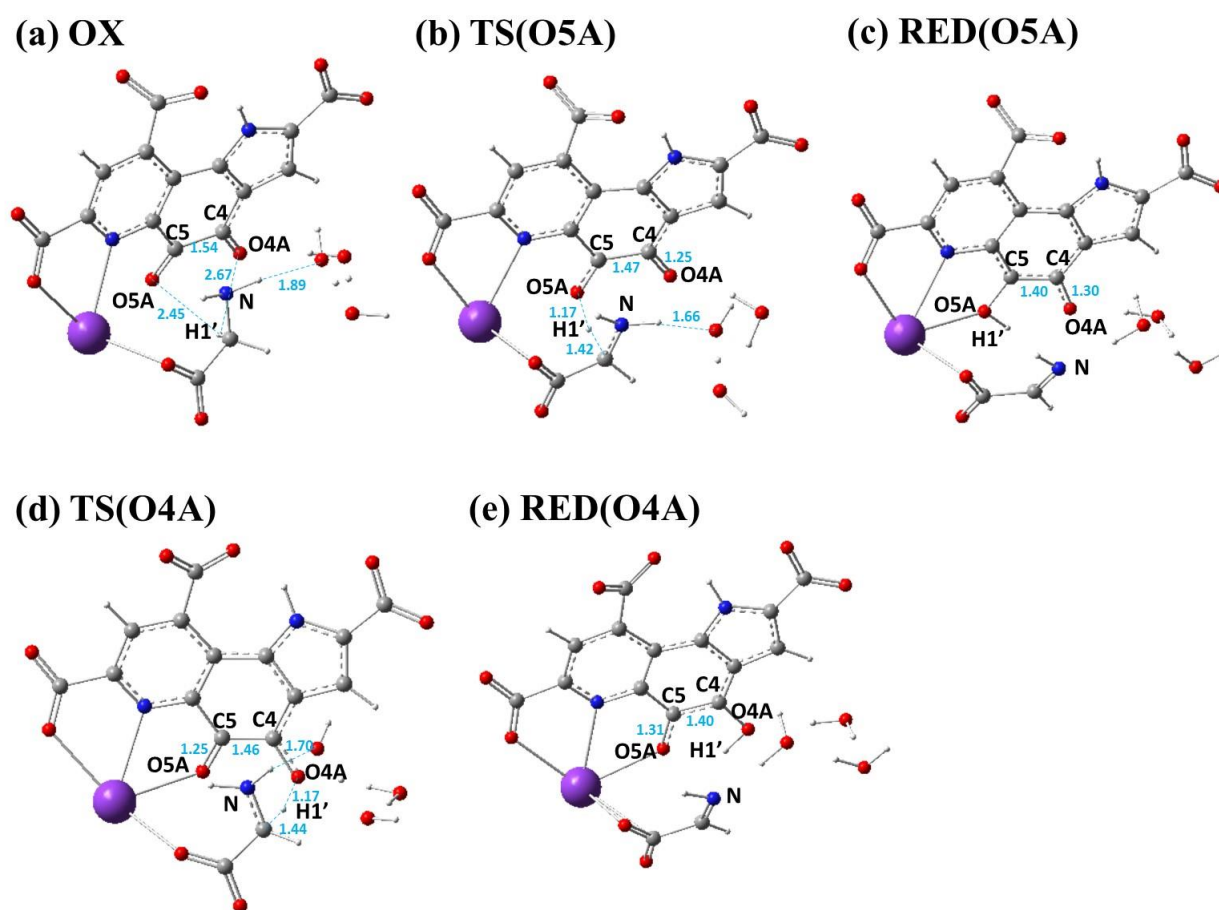


Figure 7. The optimized structures of (a) OX, (b) TS(O5A), (c) RED(O5A), (d) TS(O4A), and (e) RED(O4A). The distance is in Å. Figure 7(a) is the same structure as

Figure 3(a).

On the collision factor

We have so far presented the discussion based on the energy of the transition state for each elemental reaction. Reflecting experimental Arrhenius plot for rate constant k and reaction barrier E_a ,

$$k = Ae^{-\frac{E_a}{RT}},$$

it is also necessary to discuss pre-exponential factor A , which is the total number of collisions. Rigorous quantum level discussion of transition state theory including important tunneling effect as well as quantum treatment of proton are still rapidly advancing research area even after the instructive review appeared [50]. This is a future subject and is out of the scope of current study. Within the conventional transition state theory, we have roughly estimated the prefactors. The main conclusions, based on the energy barriers, are confirmed. The discussion is in SI (see Argument on the collision factor based on the transition state theory in SI).

Conclusion

A stepwise reaction, that is, a nucleophilic attack on C5 or C4 by glycine, and then proton and electron transfer from the glycine following a nucleophilic attack on C5 were investigated. A concerted reaction, in which proton transfers and electron transfer occur simultaneously, not including nucleophilic attack, was also studied. In the case of the stepwise reaction, the nucleophilic attack on C5 by the nitrogen atom of glycine is likely to occur. After the nucleophilic attack, the intermediate (INT(C5)) has the structure that facilitates the following proton transfer. The proton transfer of the H1' atom to O5A is most likely to occur. The electron transfer to the PQQ molecule also occurs. In the case of concerted reaction, the proton transfer of the H1' atom to O5A or O4A is difficult to occur.

Acknowledgements

All calculations were performed by using the RIKEN Integrated Cluster of Clusters (RICC) and the TSUBAME2.5 supercomputer in the Tokyo Institute of Technology. This work was supported by RIKEN Junior Research Associate Program.

Supplementary information

The detail of OX (Figure S1), the optimized structures in the additional one water around K^+ (Figure S2), the structures in the additional five water molecules (Figure S3), the optimized structures of different systems (Figure S4), the other optimized structure of TS(O5A), which is additional one water to that of TS(O5A) of Figure 7(b) (Figure S5), snapshot of proton transfer to O5A or O4A following a nucleophilic attack on C4 (Figure S6(a) or S6(b)), the Gibbs free energies of this study

and additional one water around K^+ (Table S1), the energies of this study and additional five water molecules (Table S2), the charge and distance of OX (Table S3), the charge on C4 and C5 in the different systems (Table S4), the energies of each state of Figures 2, 4 and 6 by using 6-31G(d,p) and 6-311++G(d,p) basis sets (Table S5-S7), the energies of each state of Figures 2, 4 and 6 by using B3LYP, B3LYP-GD3 and B3LYP-GD3BJ functional (Table S8-S10), the coordinate of each state in Figure 3, 5 and S5 (Table S11-S23), the mode with imaginary frequency at the TS on Figure 2, 4 and 6 (Movie 1-6), and argument on the collision factor (Argument).

Supplementary information

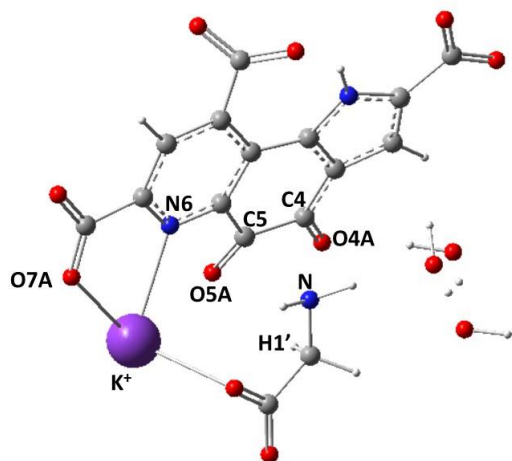


Figure S1. The optimized geometry of reactants with oxidized PQQ, which is abbreviated to OX. This structure is the same as that used in Figure 3a and 7a on this paper.

The distance between C5 and N atom of glycine was 2.39 Å, and that between C4 and N atom of glycine was 2.66 Å. And also, the distance between O5A and N atom of glycine was 2.94 Å, and that between O4A and N atom of glycine was 3.22 Å. The angle among O5A, C5 and N atom of glycine was 103.76 °, that among O4A, C4 and N atom of glycine was 105.73 °.

The distance between H1' and O5A was 2.45 Å, and that between H1' and O4A was 2.67 Å.

The distance between K⁺ and O5A, K⁺ and N6, and K⁺ and O7A were 2.88, 2.77, and 2.67 Å, respectively.

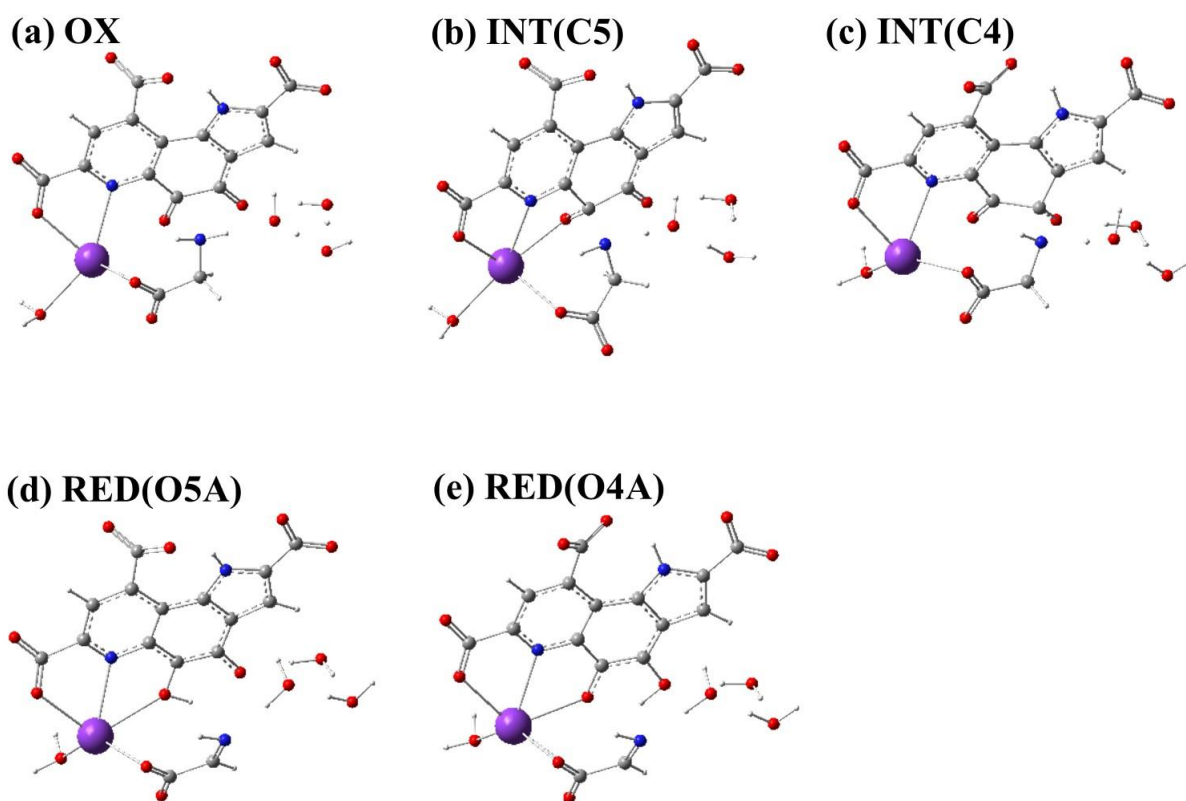


Figure S2. The optimized structures of OX, INT(C5), INT(C4), RED(O5A) and RED(O4A) in the system that is added one more explicit water molecule around K^+ cation to this study's system.

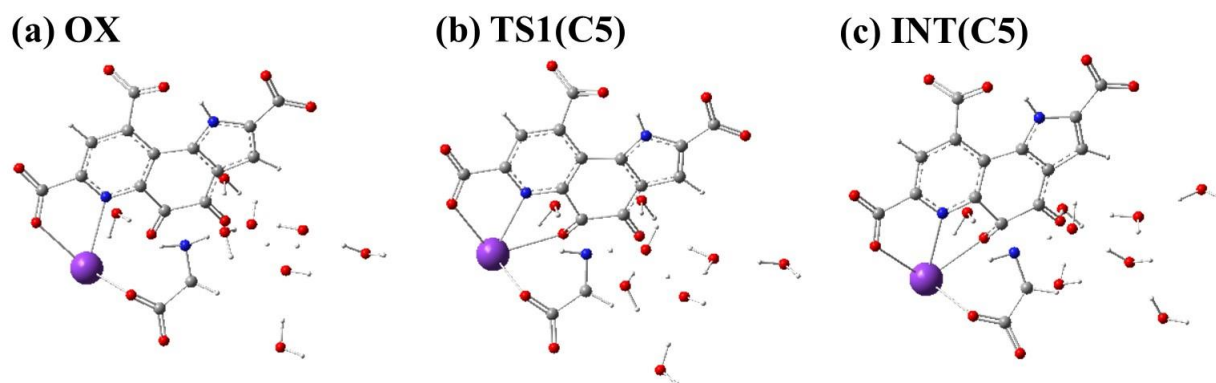


Figure S3. The structures of OX, TS1(C5) and INT(C5) in the system that is added five more explicit water molecules to this study's system.

Each water molecule is added around each H_2O or OH^- molecule of this study's system (, namely, three explicit water molecules are added) and furthermore, each water molecule is added around O5A and O4A (, namely, further two explicit water molecules are added. Total number of added water molecules is five.)

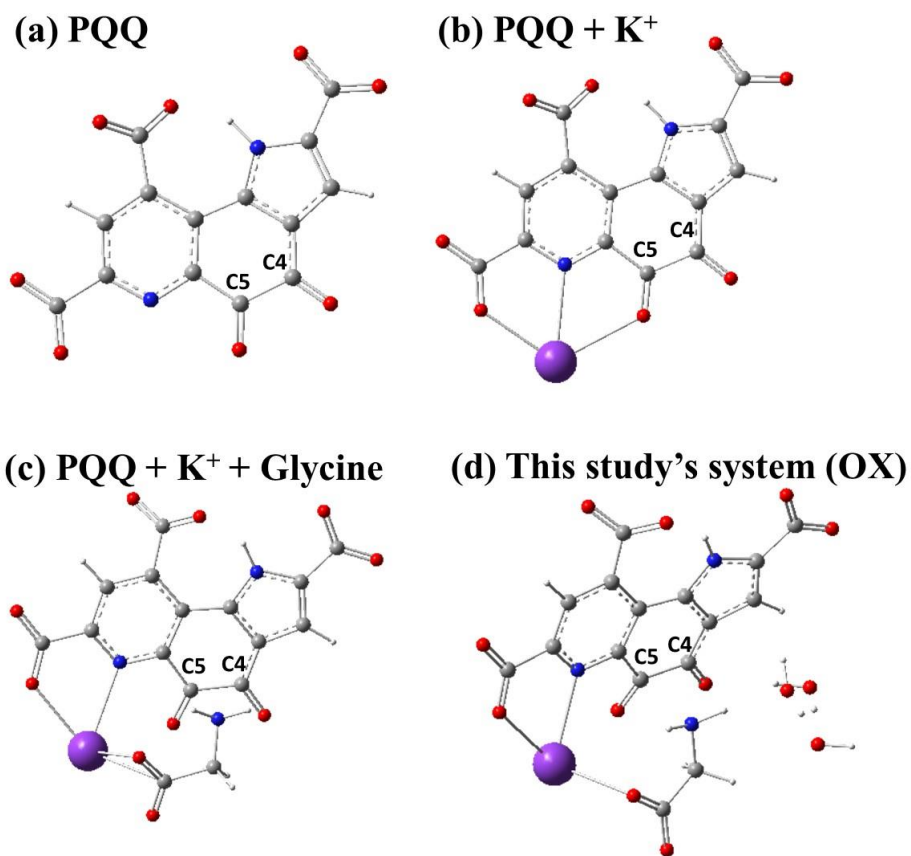


Figure S4. The optimized structures of different systems obtained at the same level of theory in this study. (a) only PQQ molecule, (b) one PQQ and one K⁺ cation, (c) one PQQ, one K⁺ cation and one glycine and (d) this study's system.

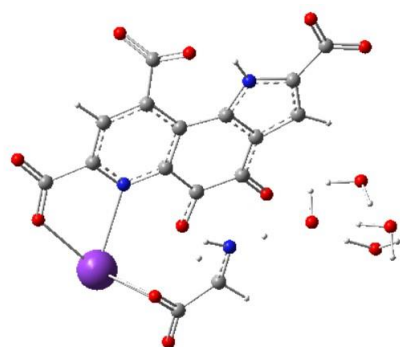
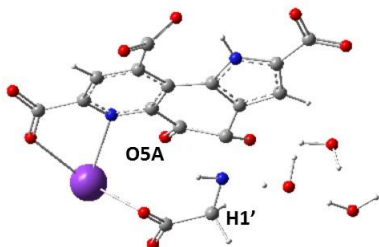
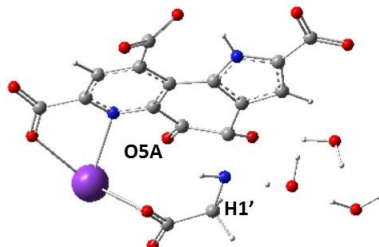


Figure S5. The other optimized structure of TS(O5A), which is added one more explicit water molecule to the structure of TS(O5A) of Figure 7(b).

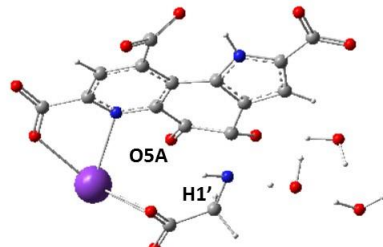
H1'-O5A : 2.94 Å



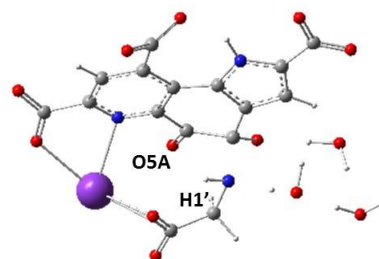
H1'-O5A : 2.64 Å



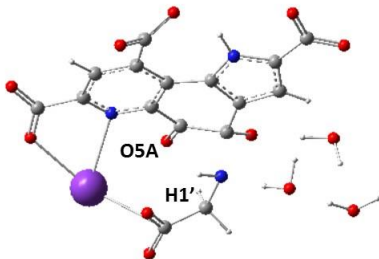
H1'-O5A : 2.34 Å



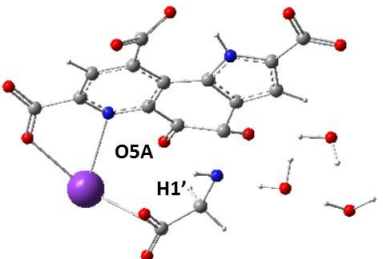
H1'-O5A : 2.14 Å



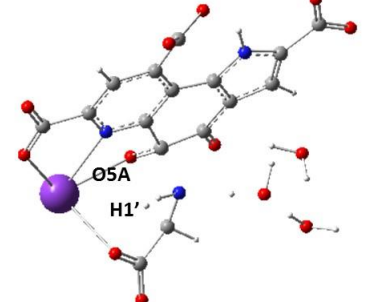
H1'-O5A : 1.94 Å



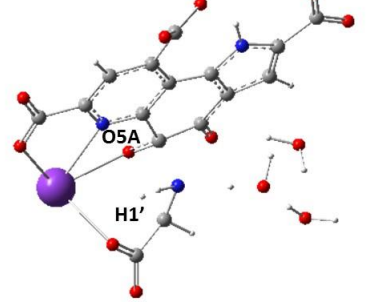
H1'-O5A : 1.74 Å



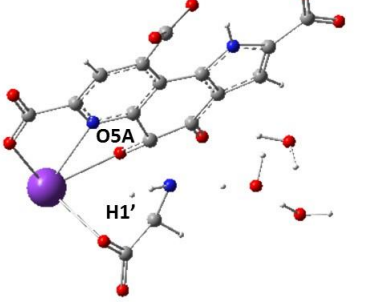
H1'-O5A : 1.59 Å



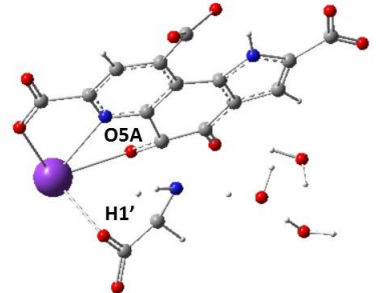
H1'-O5A : 1.44 Å



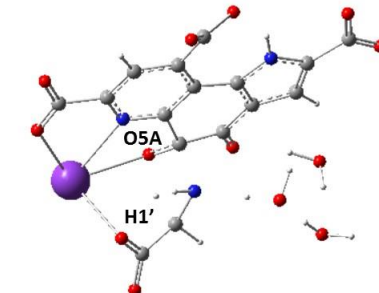
H1'-O5A : 1.34 Å



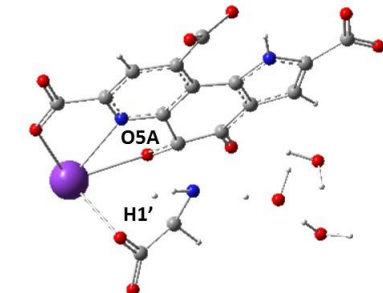
H1'-O5A : 1.32 Å



H1'-O5A : 1.30 Å



H1'-O5A : 1.29 Å



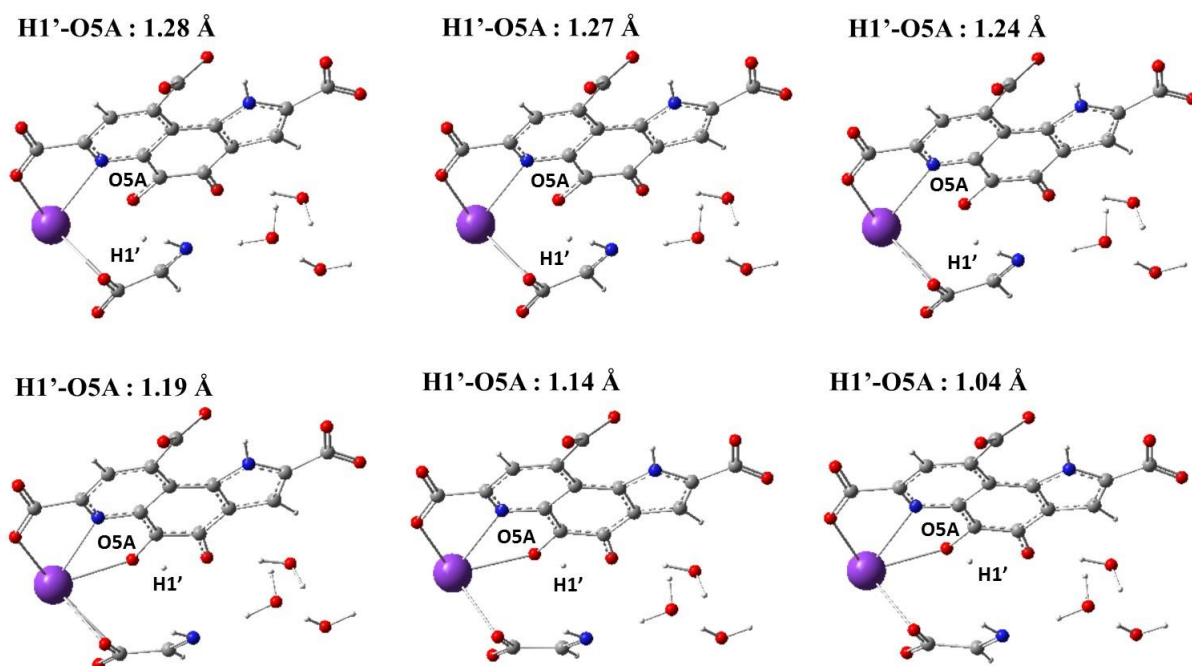


Figure S6(a). Snapshot of proton transfer to O5A following a nucleophilic attack on C4.

Same as the proton transfer following a nucleophilic attack on C5 case, there are two possible proton transfer from the α -carbon of glycine to PQQ (Figure 1(a)). At INT, the H atom on the α -carbon of glycine, which was nearest to PQQ, was labeled as H1' (Figure 3(e)). We calculate the proton transfer of H1' toward O5A at the same level calculations of this study. We optimize a structure which is fixed the distance between H1' and O5A and then reduce this distance step by step. We here report the snapshot.

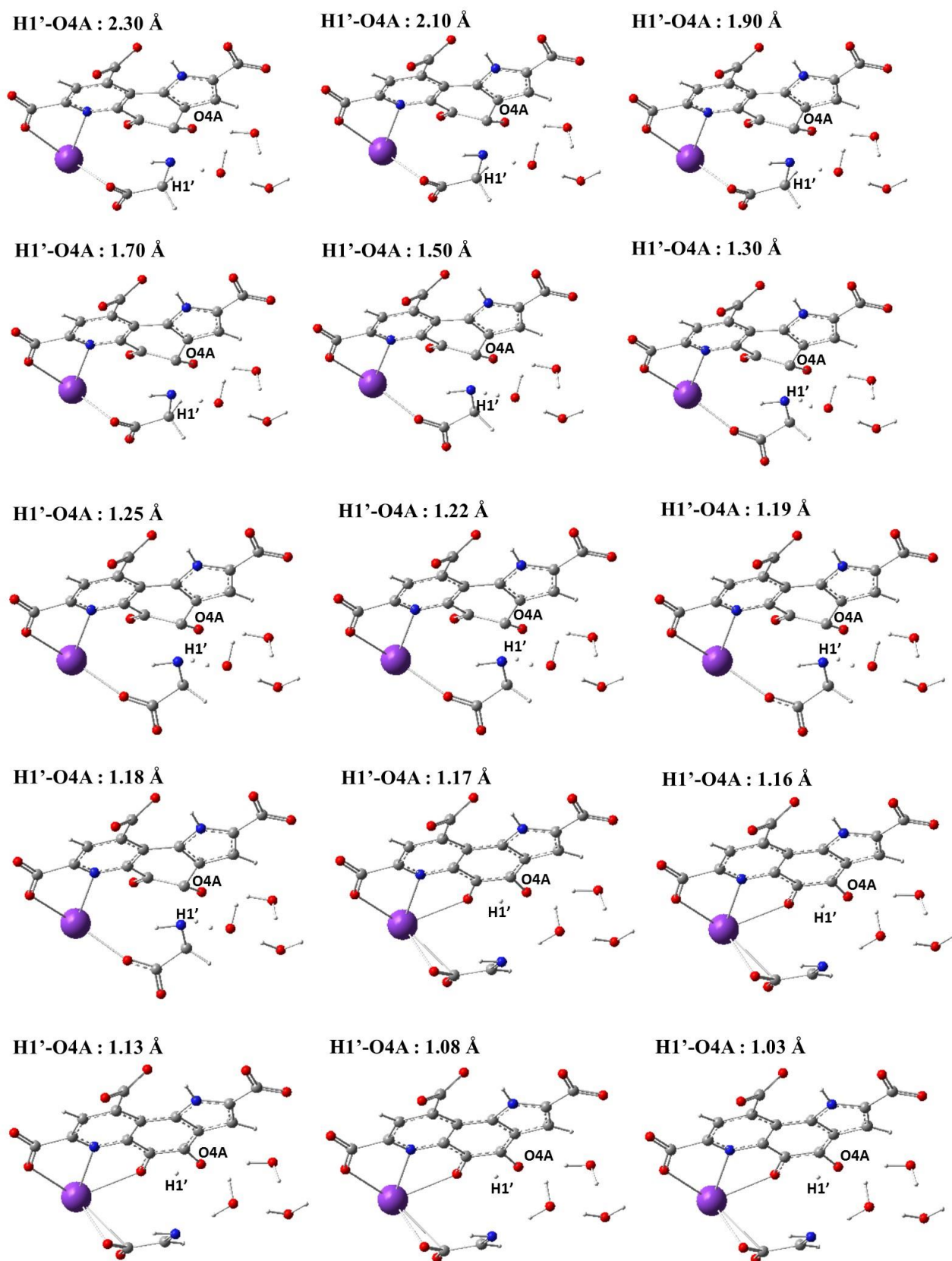


Figure S6(b). Snapshot of proton transfer to O4A following a nucleophilic attack on C4.

Same as the proton transfer following a nucleophilic attack on C5 case, there are two possible proton transfer from the α -carbon of glycine to PQQ (Figure 1(a)). At INT, the H atom on the α -carbon of glycine, which was nearest to PQQ, was labeled as H1' (Figure 3(e)). We calculate the proton transfer of H1' toward O4A at the same level calculations of this study. We optimize a structure which is fixed the distance between H1' and O4A and then reduce this distance step by step. We here report the snapshot .

Table S1. The Gibbs free energies of OX, INT(C5), INT(C4), RED(O5A) and RED(O4A) in the two different systems obtained at the same level of theory in this study. One is this study's system and another is the system that is added one more explicit water molecule around K^+ cation to this study's system.

ΔG (kcal/mol)	This study's system	Add water around K^+
OX	0.00	0.00
INT(C5)	-0.79	0.39
INT (C4)	3.05	5.11
RED (O5A)	-19.19	-17.02
RED (O4A)	-13.79	-13.36

Table S2. The energies of OX, TS1(C5) and INT(C5) in the system of this study and that is added five more explicit water molecules to this study's system.

The energies of OX, TS1(C5) and INT(C5) in the system of this study are obtained by optimizations at the same level calculation of this study. Those in the system that is added five more explicit water molecules to this study's system are obtained by single point calculations.

ΔE (kcal/mol)	This study	Add 5 water
OX	0.00	0.00
TS1(C5)	1.97	3.28
INT(C5)	-3.57	2.50

Table S3. The charge and distance of OX in Figure 3(a).

	Charge	Distance between N and C (Å)
C5	0.443	2.39
C4	0.360	2.66

Table S4. The charge on C4 and C5 in the different systems obtained at the same level of theory in this study.

	Charge (C5)	Charge (C4)
PQQ	0.374	0.296
PQQ + K ⁺	0.400	0.313
PQQ + K ⁺ + Glycine	0.442	0.352
This study's system	0.443	0.360

Table S5. The energies of OX, TS1(C5), INT(C5), TS1(C4) and INT(C4) (on Figure 2) by using 6-31G(d,p) basis set and 6-311++G(d,p) basis set. The energies of 6-31G(d,p) basis set were obtained by optimized structures. Those of 6-311++G(d,p) basis set were obtained by single point calculations using the optimized structures of 6-31G(d,p) basis set.

ΔE (kcal/mol)	6-31G(d,p)	6-311++G(d,p)
OX	0.00	0.00
TS1 (C5)	1.97	3.31
INT (C5)	-3.57	-0.92
TS1 (C4)	6.02	9.17
INT (C4)	0.74	4.43

Table S6. The energies of OX, TS1, INT, TS2(O5A), RED(O5A), TS2(O4A) and RED(O4A) (on Figure 4) by using 6-31G(d,p) basis set and 6-311++G(d,p) basis set. The energies of 6-31G(d,p) basis set were obtained by optimized structures. Those of 6-311++G(d,p) basis set were obtained by single point calculations using the optimized structures of 6-31G(d,p) basis set.

ΔE (kcal/mol)	6-31G(d,p)	6-311++G(d,p)
OX	0.00	0.00
TS1	1.97	3.31
INT	-3.57	-0.92
TS2 (O5A)	11.68	16.35
RED (O5A)	-18.12	-16.58
TS2 (O4A)	18.89	22.92
RED (O4A)	-12.67	-10.46

Table S7. The energies of OX, TS(O5A), RED(O5A), TS(O4A) and RED(O4A) (on Figure 6) by using 6-31G(d,p) basis set and 6-311++G(d,p) basis set. The energies of 6-31G(d,p) basis set were obtained by optimized structures. Those of 6-311++G(d,p) basis set were obtained by single point calculations using the optimized structures of 6-31G(d,p) basis set.

ΔE (kcal/mol)	6-31G(d,p)	6-311++G(d,p)
OX	0.00	0.00
TS (O5A)	19.87	23.51
RED (O5A)	-18.13	-16.60
TS (O4A)	25.61	26.67
RED (O4A)	-12.66	-10.45

Table S8. The energies of OX, TS1(C5), INT(C5), TS1(C4) and INT(C4) (on Figure 2) by using B3LYP, B3LYP-GD3 and B3LYP-GD3BJ functional. The energies of B3LYP functional were obtained by optimized structures. Those of B3LYP-GD3 and B3LYP-GD3BJ functional were obtained by single point calculations using the optimized structures of B3LYP functional.

ΔE (kcal/mol)	B3LYP	B3LYP-GD3	B3LYP-GD3BJ
OX	0.00	0.00	0.00
TS1 (C5)	1.97	1.88	1.15
INT (C5)	-3.57	-3.27	-4.05
TS1 (C4)	6.02	6.04	5.77
INT (C4)	0.74	1.05	0.87

Table S9. The energies of OX, TS1, INT, TS2(O5A), RED(O5A), TS2(O4A) and RED(O4A) (on Figure 4) by using B3LYP, B3LYP-GD3 and B3LYP-GD3BJ functional. The energies of B3LYP functional were obtained by optimized structures. Those of B3LYP-GD3 and B3LYP-GD3BJ functional were obtained by single point calculations using the optimized structures of B3LYP functional.

ΔE (kcal/mol)	B3LYP	B3LYP-GD3	B3LYP-GD3BJ
OX	0.00	0.00	0.00
TS1	1.97	1.88	1.15
INT	-3.57	-3.27	-4.05
TS2 (O5A)	11.68	11.96	11.39
RED (O5A)	-18.12	-14.28	-14.60
TS2 (O4A)	18.89	18.57	17.94
RED (O4A)	-12.67	-8.60	-8.85

Table S10. The energies of OX, TS(O5A), RED(O5A), TS(O4A) and RED(O4A) (on Figure 6) by using B3LYP, B3LYP-GD3 and B3LYP-GD3BJ functional. The energies of B3LYP functional were obtained by optimized structures. Those of B3LYP-GD3 and B3LYP-GD3BJ functional were obtained by single point calculations using the optimized structures of B3LYP functional.

ΔE (kcal/mol)	B3LYP	B3LYP-GD3	B3LYP-GD3BJ
OX	0.00	0.00	0.00
TS (O5A)	19.87	19.95	19.75
RED (O5A)	-18.13	-14.30	-14.61
TS (O4A)	25.61	25.32	24.75
RED (O4A)	-12.66	-8.60	-8.84

In the collision factor based on the transition state theory.

The equation of traditional transition state theory is

$$k(T) = \frac{k_B T}{h} e^{-\frac{\Delta G^\ddagger}{RT}} = \frac{k_B T}{h} e^{\frac{\Delta S^\ddagger}{R}} e^{-\frac{\Delta H^\ddagger}{RT}},$$

where $\Delta G^\ddagger$, $\Delta S^\ddagger$ and $\Delta H^\ddagger$ are Gibbs free energy of activation, entropy of activation and enthalpy of activation, respectively. k_B is Boltzmann constant, T is temperature (I use the temperature of 298.15 K), h is Plank constant and R is gas constant (1.987×10^{-3} kcal $\cdot$ K $^{-1}$ $\cdot$ mol $^{-1}$), respectively. When a reaction proceeds, reactants should meet with the proper orientation. $\Delta S^\ddagger$ is directly related to the steric factor. In the Gaussian09 program, the thermochemical data are calculated according to the standard results of thermodynamics (ref. "Molecular Thermodynamics" by McQuarrie and Simon, 1999). Calculated partition functions are used for the estimation of the entropies.

The ratio of reaction rate of the first step in the stepwise mechanism (k_{step1}) divided by that of the concerted one ($k_{\text{concerted}}$) is $k_{\text{step1}}/k_{\text{concerted}} = 3.6 \times 10^9$. It means the first step in the stepwise mechanism (nucleophilic attack of nitrogen atom of glycine on C5) is much faster than the concerted reaction. Compared INT with OX, entropy contribution in ΔG ($= -0.8$ kcal/mol), that is $-T\Delta S$, is 1.1 kcal/mol, which means when OX changes to INT, the degree of freedom of structure becomes lower. At INT, a covalent bond between nitrogen atom of glycine and C5 is formed, and as a consequent, hydrogen atom on α -carbon of glycine remains close to O5A in this comparatively steady structure. The ratio of reaction rate of the second step (proton and electron transfer) in the stepwise mechanism (k_{step2}) divided by that of the concerted one ($k_{\text{concerted}}$) is $k_{\text{step2}}/k_{\text{concerted}} = 1.6 \times 10^4$. At this time, from INT to TS2(O5A) in the stepwise mechanism, entropy contribution in $\Delta G^\ddagger$ ($= 10.5$ kcal/mol), that is $-T\Delta S$, is -0.7 kcal/mol, which means the system regains the degree of freedom and that

contributes as the reaction rate becomes faster. On the other hand, from OX to TS(O5A) in the concerted reaction, entropy contribution in $\Delta G^\ddagger$ (= 16.3 kcal/mol), that is $-T\Delta S$, is 0.5 kcal/mol, which means it contributes as the reaction rate becomes slower. The ratio of reverse reaction rate of the first step in the stepwise mechanism ($k_{-1 \text{ step1}}$) divided by that of reaction rate of the second step in the stepwise mechanism (k_{step2}) is $k_{-1 \text{ step1}}/k_{\text{step2}} = 5.7 \times 10^4$. Although there can be reverse path, from INT to OX, in the stepwise reaction, compared the reaction rate from INT to RED in the stepwise reaction with that from OX to RED in the concerted reaction, the former one is much faster.

It should be noted that the traditional transition state theory is based on some hypotheses. Specifically in proton transfers, relevant protons have to be considered in a quantized manner, and the tunneling effect will promote the reaction rates.

Movie 1. The mode with imaginary frequency at the TS1(C5) on Figure 2. (see TS1_C5.gif)

Movie 2. The mode with imaginary frequency at the TS1(C4) on Figure 2. (see TS1_C4.gif)

Movie 3. The mode with imaginary frequency at the TS2(O5A) on Figure 4. (see TS2_O5A.gif)

Movie 4. The mode with imaginary frequency at the TS2(O4A) on Figure 4. (see TS2_O4A.gif)

Movie 5. The mode with imaginary frequency at the TS(O5A) on Figure 6. (see TS_O5A.gif)

Movie 6. The mode with imaginary frequency at the TS(O4A) on Figure 6. (see TS_O4A.gif)

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Chapter 3.

Comparison of amino acid oxidation catalyzed by pyrroloquinoline quinone in aqueous solution

Abstract

The comparison of amino acids (glycine, alanine and valine) oxidation reaction by PQQ in aqueous solution was investigated. A stepwise mechanism of previous study, which is a nucleophilic attack on C5 by the nitrogen atom of glycine, and then proton and electron transfer to PQQ, was referred to calculate alanine and valine oxidation reactions on this study. And also, in preparation of above study, the importance of a cation on calculation system was studied.

Introduction

The basic introduction of Pyrroloquinoline quinone (PQQ) are described at chapter 1 and chapter 2.

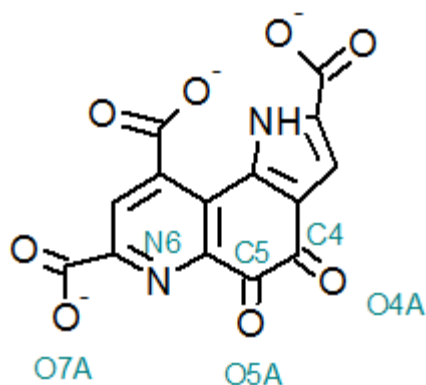
PQQ was found as a bacterial redox cofactor [1]. PQQ has reported antioxidant function, therefore, the understanding of redox mechanism proceeds to further development of catalyst-molecule. PQQ catalyzes effectively mediating the redox cycle, not only inside those proteins but also in the absence of proteins. PQQ can catalyze a repeated redox reaction in aqueous solution [2]. In an appropriate condition, PQQ catalyzes the repeated redox cycle with larger number of reaction than many bioactive quinones or enediols [2-4]. Under this appropriate environment, compared the reaction of PQQ with three amino acids which are glycine, alanine and valine, the reaction with glycine occurs better than that with alanine and valine, and compared that with alanine and valine, that with alanine occurs better than that with valine [5]. Therefore, at first, I focus on a mechanism of the PQQ-catalyzed glycine oxidation at the previous study (chapter 2) [6], and then on this study (at this chapter), refer to results of the previous study and focus on the difference of PQQ-catalyzed different amino acids oxidation. These results will lead to understand the effect of catalytic reaction by different amino acids and basis of further development of catalyst-molecule. And also, in preparation for calculations of three different amino acids oxidation reactions, the difference between calculation systems which include a K^+ cation and that does not include a K^+ cation is studied, because a cation was reported to need to keep the activity of redox cycle [7].

At the previous study (chapter 2) [6], it is concluded that one mechanism, a stepwise reaction, is likely to occur on the glycine oxidation by PQQ. This mechanism includes a nucleophilic attack, and a proton and electrons transfer. At first, a

nucleophilic attack on C5 by the nitrogen atom of glycine occurs, and then the proton (H1' atom) to O5A and electrons transfer from the glycine to the PQQ occurs. It is also gotten a knowledge that the structure of the intermediate (INT(C5)) after a nucleophilic attack facilitates the following proton transfer. At this chapter, based on these conclusions of previous study [6], I investigate here a study of comparison of three different amino acids oxidations by PQQ in aqueous solution and of role of K^+ cation by using quantum chemical calculations. I present here studies of molecular structures, electronic states, and relative Gibbs free energies of each point on the reaction pathway.

Computational details

Geometry optimizations and frequency analyses of all calculations were performed by using the density functional theory [8] with the B3LYP hybrid functional [9-11] and the 6-31G(d,p) basis set [12,13] on Gaussian 09 [14]. Gibbs free energies at 298.15 K were obtained by the frequency analyses which were calculated by using the optimized structures at each equilibrium geometry or transition state (TS). The effect of water solvent was considered by using the polarizable continuum model (PCM) [15,16]. Furthermore additional explicit water molecules were included in the calculation if needed.



The notation of atoms in PQQ as shown above are C4, C5, O4A, O5A, N6, and O7A, respectively. A calculation system was constructed from one PQQ molecule, one K^+ cation which coordinates with O5A, N6 and O7A atoms as shown above the labels, two explicit water molecules, one OH^- anion, and one amino acid (glycine, alanine or valine), as shown later. (In the section of study of role of K^+ , there is a little different components mentioned above) The position of K^+ cation in the initial structure of calculation system is supported by X-ray structures which include K^+ , other cation or metal ion [17-19] and other theoretical studies [6,20-24]. Furthermore, this cation was reported to need to keep the activity of redox cycle [7]. Therefore, in preparation for

calculations of three different amino acids oxidation reactions, the difference of energies between calculation systems which include a K^+ cation and that does not include a K^+ cation was monitored (see following the Results and Discussion section).

Two explicit water molecules and one OH^- anion in the initial structure of calculation system are referred to the previous study [6], which is considered the experiments in aqueous solution at alkaline pH condition [3,4]. The solvent effect was considered by using a PCM method, however, two H_2O and one OH^- molecules were incorporated explicitly in the calculation systems. That is because H_2O or OH^- molecule nearest to amino acid induces polarization and therefore induces a minus charge on the nitrogen atom of amino acid, and also H_2O or OH^- molecule nearest to amino acid enables a nucleophilic attack on C4 or C5 by the nitrogen atom at the first step of the reaction (Figure 1). On the previous study, it was found that it was important to treat these H_2O and OH^- molecules explicitly (i.e., not simply by PCM), which was H_2O or OH^- molecule nearest to one of the hydrogen atoms of the amino group was very significant [6].

Results and Discussion

(A) Role of a cation.

Mechanism of glycine oxidation, glycine oxidation added one H₂O around K⁺, and glycine oxidation with no K⁺ by PQQ in aqueous solution.

As referred the previous study [6], the mechanism of glycine oxidation have two modes, one is nucleophilic attack and other is proton and electron transfer (Figure 1(a), 1(b) and 1(c))

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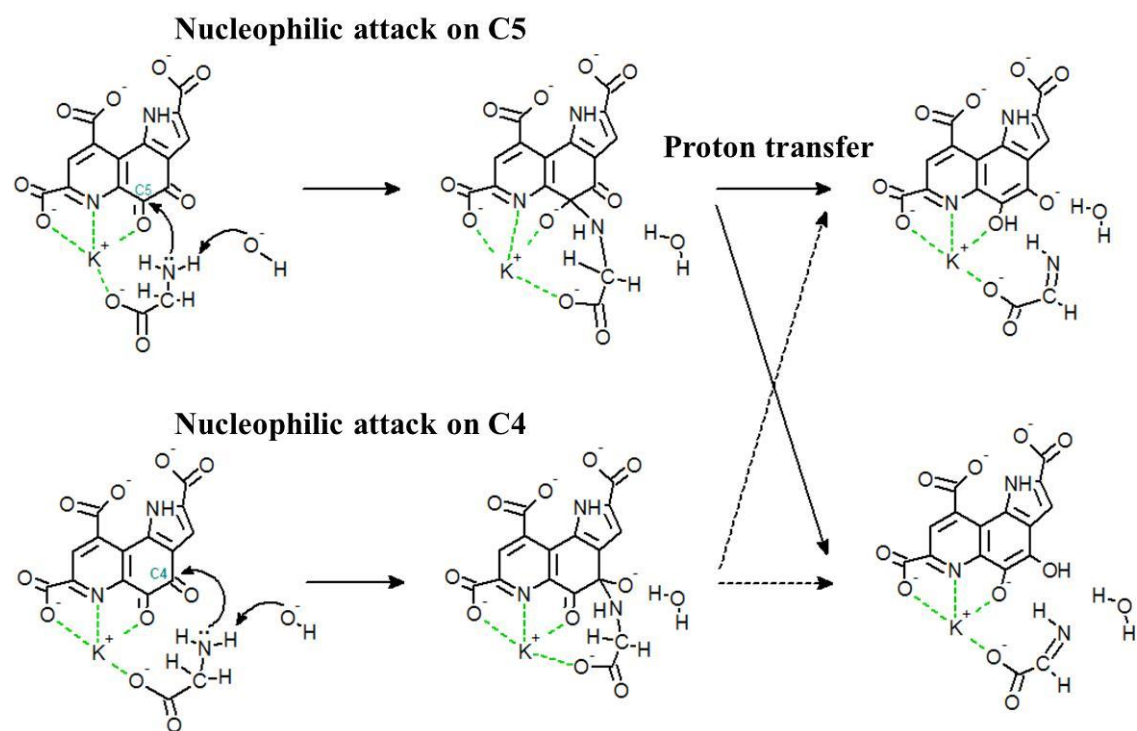
Nucleophilic attack

At the first reaction, a nucleophilic attack by a nitrogen atom of glycine on C5 (Figure 1(a)-, 1(b)- or 1(c)-upper) or C4 (Figure 1(a)-, 1(b)- or 1(c)-lower) of PQQ.

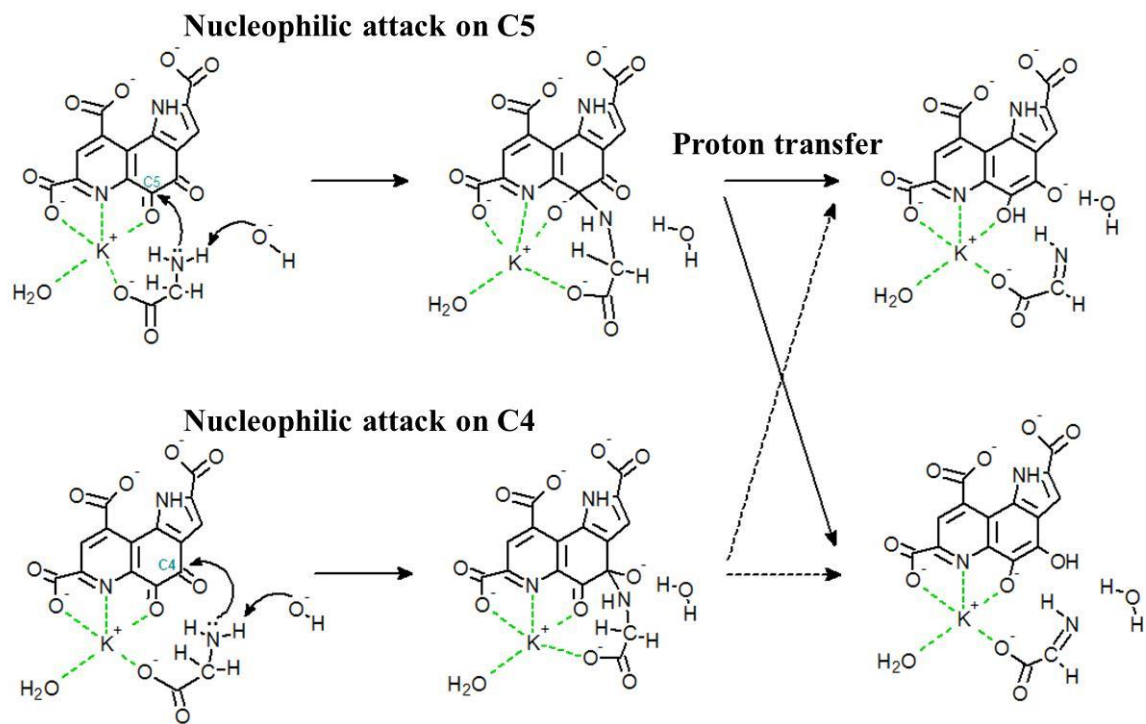
Proton transfer following nucleophilic attack

At the second reaction, proton and electron transfer [Figure 1(a), 1(b) or 1(c)] occur. For proton transfer, a hydrogen atom on the α -carbon of glycine is extracted as a proton by the lone pair of electrons of O4A or O5A. And while, for electron transfer, two electrons (which form the bond between the nitrogen atom of glycine and one of the carbon atoms) transfer to the PQQ molecule. As a result of this reaction, the single bond between C4 and C5 turns into a double bond. The nitrogen atom and the α -carbon of glycine thereby form a double bond. Thus, the amino acid glycine oxidation is completed. In a conventional manner, the reduced form of PQQ is described as both O4A and O5A are hydrogenated. After either O4A or O5A becomes a negative oxygen ion (O⁻), the PQQ can become the reduced form, since it easily receives one proton from one of the surrounding water molecules.

(a) Glycine oxidation



(b) Glycine oxidation added one H_2O around K^+



(c) Glycine oxidation with no K^+

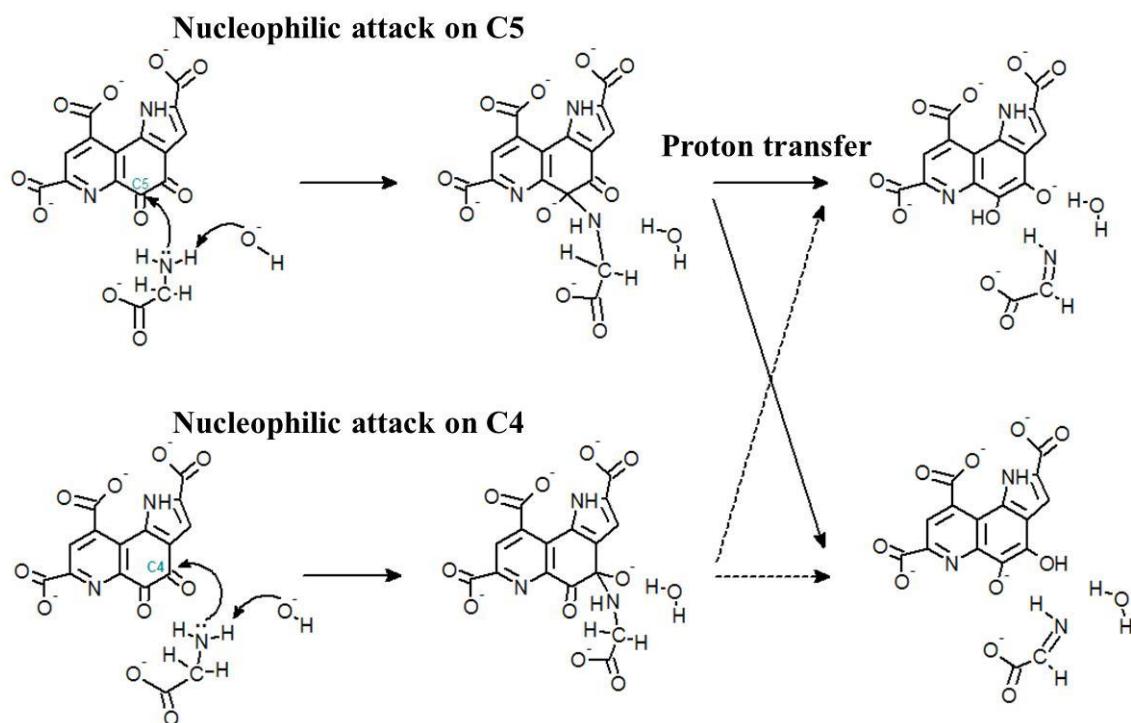


Figure 1. Three mechanisms of glycine oxidation (a), glycine oxidation added one H_2O around K^+ (b) and glycine oxidation with no K^+ (c). These three mechanisms have two modes of nucleophilic attack on C5 or on C4. The first step is nucleophilic attack by the nitrogen atom of glycine and the second step is proton transfer and elimination reaction.

Reactant structure

In case of glycine oxidation by PQQ, the geometry of the reactants with oxidized PQQ, which is here, this state is abbreviated to OX, is referred to the structure of previous study [6]. In case of glycine oxidation added one H_2O around K^+ and glycine oxidation with no K^+ , the OX geometries are also referred to the geometry (OX) of glycine oxidation by PQQ and are optimized first. As for the case of glycine oxidation added one H_2O around K^+ and glycine oxidation with no K^+ , from here, they are abbreviated to “added one H_2O around K^+ oxidation” and “no K^+ oxidation”,

respectively.

The details of OX on the calculation system of glycine, added one H₂O around K⁺ and no K⁺ oxidation are shown, respectively (see figures in the following discussions). The calculation system on glycine oxidation contains one PQQ molecule, one K⁺ cation, two explicit water molecules, one OH⁻ anion, and one glycine. And the calculation system on added one H₂O around K⁺ oxidation contains one more explicit water molecule around K⁺ and the component of glycine oxidation. According to a X-ray structure [25], a cation is placed on a fivefold coordination, that is why one more explicit water molecule around K⁺ is added in the calculation system. And also as for the calculation system on no K⁺ oxidation, K⁺ is eliminated from the component of glycine oxidation. If the K⁺ cation was included in calculation system, the K⁺ cation was placed stably among O7A, N6, and O5A by coordination bonds. Moreover the oxygen atom of the carboxyl group of glycine forms a coordination bond with K⁺. That provides the footing for glycine and makes the nucleophilic attack possible (Figure 1). Furthermore, the nitrogen atom of the amino group of glycine is placed very close to the C5 and C4 atoms, which allows the nitrogen atom to attack the C5 or C4 atoms as a nucleophilic attack reaction.

Glycine oxidation by PQQ

Nucleophilic attack on C5 or C4

Referred to the previous study [6], a nucleophilic attack on C5 by the nitrogen atom of glycine is likely to occur compared to that on C4, which is based on analyses of energies (Boltzmann distribution), Mulliken population analyses and distance between C5 or C4 and nitrogen atom.

Proton transfer following a nucleophilic attack on C5

Referred to the previous study [6], the structure of product after the nucleophilic attack on C5, which is the intermediate (INT(C5)), facilitates the following proton transfer. The proton transfer to O5A is reported to most likely to occur, which is based on the analyses of energies (Boltzmann distribution).

Glycine oxidation added one H₂O around K⁺

Nucleophilic attack on C5

The relative Gibbs free energies and optimized structures obtained by the calculated results of the nucleophilic attack reaction on C5 (Figure 1(b)-upper) are shown in Figures 2 and 3, respectively.

When the nitrogen atom of alanine attacks on C5, one of the hydrogen atoms of the amino group of glycine moves to the nearest oxygen atom of the H₂O or OH⁻ molecules [Figures 3(a), 3(b), and 3(c)]. The calculated result of transition state (TS) energy is 4.7 kcal/mol [Figure 2, TS1(C5)], which is relative to the reference energy; OX is the reference (0.0 kcal/mol). The imaginary frequency mode at TS1(C5) corresponds well to the mode that represents the nucleophilic reaction. The product after the nucleophilic attack on C5, which is INT(C5), of which the energy is 0.4 kcal/mol higher than that of the reactant OX (Figure 2). The electronic structure around C5 changed from sp² to sp³ hybridization. And the bond between the nitrogen atom of glycine and C5 is formed [C5-N=1.57Å; Figure 3(c)].

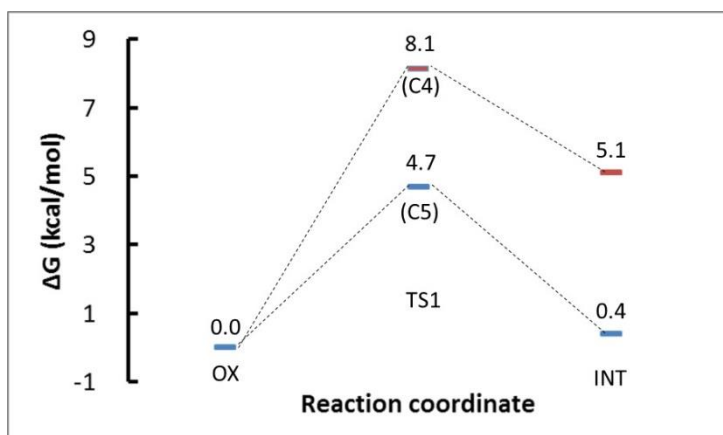


Figure 2. The Gibbs free energies of OX, transition state (TS) 1, and intermediate (INT), which are relative to OX (0.0 kcal/mol).

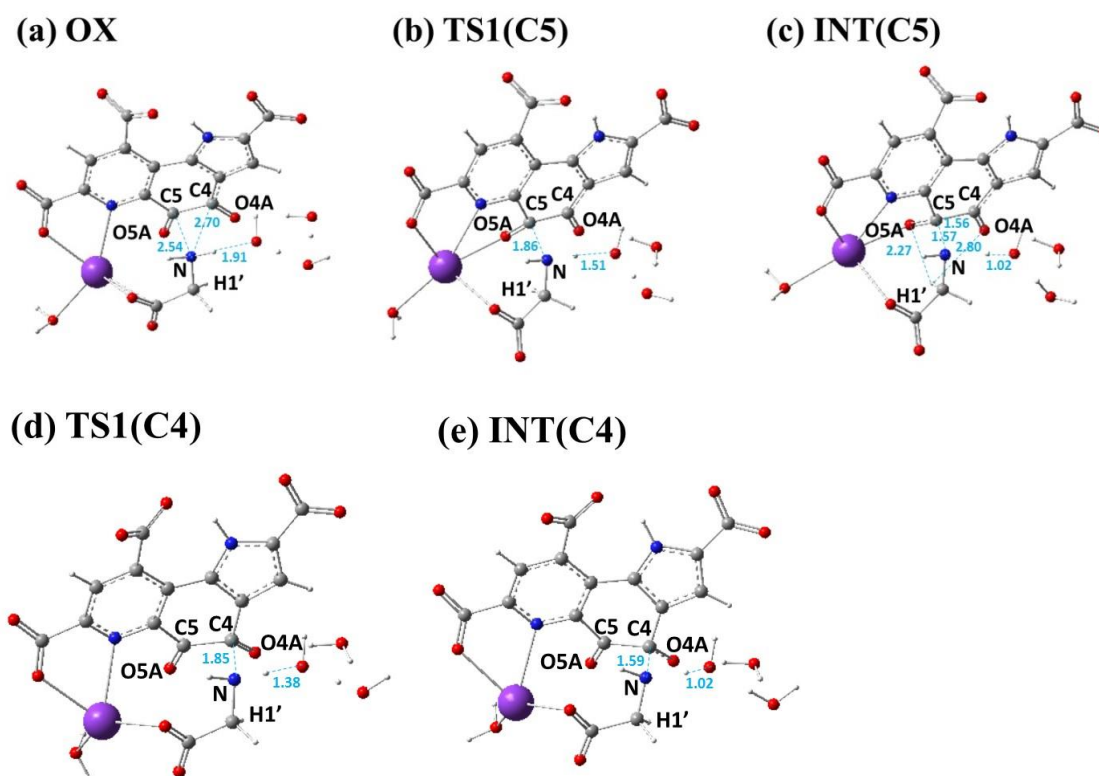


Figure 3. The obtained structures after optimization of (a) OX, (b) TS1(C5), (c) INT(C5), (d) TS1(C4), and (e) INT(C4). The distance is shown in Å.

Nucleophilic attack on C4

The relative Gibbs free energies and optimized structures obtained by the calculated results of the nucleophilic attack reaction on C4 (Figure 1(b)-lower) are shown in Figures 2 and 3, respectively.

When the nitrogen atom of glycine attacks on C4, one of the hydrogen atoms of the amino group of glycine moves to the nearest oxygen atom of the H₂O or OH⁻ molecules [Figures 3(a), 3(d), and 3(e)]. The TS energy is 8.1 kcal/mol [Figure 2, TS1(C4)]. The imaginary frequency mode at TS1(C4) corresponds well to the mode that represents the nucleophilic attack on C4 by the nitrogen atom of glycine. The product after the nucleophilic attack on C4, which is INT(C4), of which the energy is 5.1 kcal/mol higher than that of OX (Figure 2). The electronic structure around C4 changed from sp² to sp³ hybridization. The bond between the nitrogen atom of glycine and C4 is formed [C4-N=1.59Å; Figure 3(e)]. Compared the TS1 energies between TS1(C5) and TS1(C4), the TS1(C4) energy is 3.4 kcal/mol higher than TS1(C5) energy (Figure 2). The nucleophilic attack on C5 shown in Figure 1(b)-upper can be considered a major reaction because of the consideration of the Boltzmann distribution. This result is the same tendency of nucleophilic attack on glycine oxidation by PQQ.

Proton transfer following a nucleophilic attack on C5

The relative Gibbs free energies and optimized structures obtained by the calculated results of the proton transfer following a nucleophilic attack reaction on C5 (Figure 1(b)-upper) are shown in Figures 4 and 5, respectively.

The structure of product after the nucleophilic attack on C5, which is the intermediate (INT(C5)), facilitates the following proton transfer toward O5A or toward

O4A, because at this state, O5A, C5, the nitrogen atom of glycine, the α -carbon of glycine and one of the hydrogen on the α -carbon form a pentagon geometry, and also, O4A, C4, C5, the nitrogen atom of glycine, the α -carbon of glycine and one of the hydrogen on the α -carbon form a twisted hexagon geometry (Figure 3(c)). The closest hydrogen on the α -carbon of glycine to PQQ is H1' atom labeled in Figure 3(c).

The TS energy of proton (H1') transfer to O5A is 11.0 kcal/mol, while that to O4A is 19.5 kcal/mol (Figure 4, TS2). The activation energy from INT to TS2 in proton (H1') transfer to O5A is 10.6 kcal/mol, and while that from INT to O4A is 19.1 kcal/mol. Therefore, proton transfer to O5A is likely to occur considered the Boltzmann distribution. This result fall in line with that of proton transfer on glycine oxidation by PQQ. The imaginary frequency mode at TS2 corresponds well to the mode of proton transfer. From INT to TS2, in the case of the proton (H1') transfers to O5A, the bond between C5 and the nitrogen atom of glycine is lost (C5-N=1.57Å, Figure 3(c); C5-N=2.48Å, Figure 5(a)), and while in the case of proton (H1') transfer to O4A, it is the same reaction as that for H1' transfer to O5A (C5-N=1.57Å, Figure 3(c); C5-N=2.76Å, Figure 5(c)). Geometries show the electronic structure around the nitrogen atom shifts from sp³ to sp²-like, and that around C5 atom returns from sp³ to sp²-like (Figure 3(c), 5(a) and 5(c)).

After the proton (H1') transfer, INT changes to the reduced PQQ form (RED) [Figures 3(c), 5(b), and 5(d)]. The energy of RED after the proton (H1') transfer to O5A is -17.0 kcal/mol, and while, that correspond to O4A is -13.4 kcal/mol (Figure 4). The α -carbon of glycine changed from sp³ to sp², and then the bond between the nitrogen and α -carbon of glycine changed to a double bond [Figures 5(b) and 5(d)]. Either O4A or O5A shown here is a negative oxygen ion (O⁻) [Figures 5(b) and 5(d)], however, it

can be readily hydrogenated by the protonation from one of the surrounding water molecules. After this hydrogenation, the formation of RED becomes complete.

These phenomena in a sequence of reactions are consistent with that of proton transfer on glycine oxidation by PQQ.

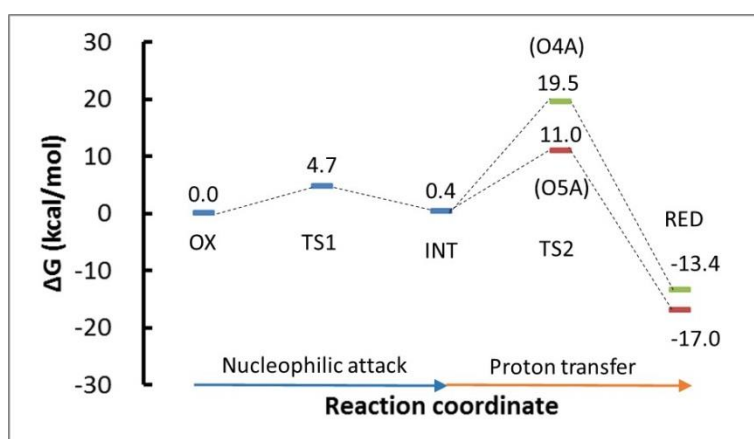
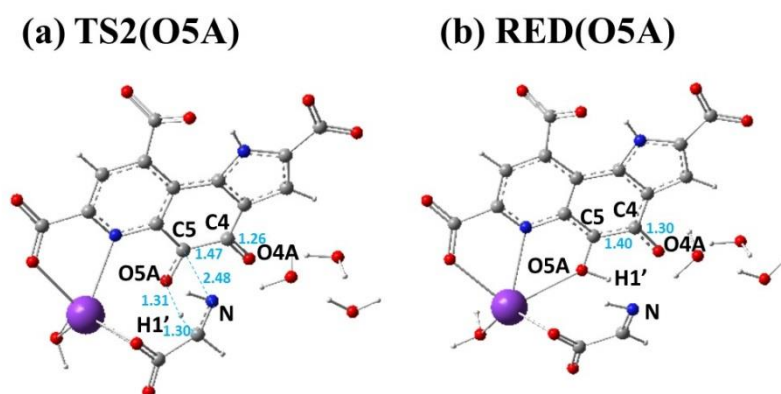
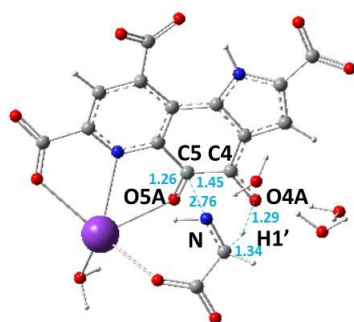


Figure 4. The Gibbs free energies of OX, transition state (TS) 1, intermediate (INT), TS2 and RED, which are relative to OX (0.0 kcal/mol). Those of OX, TS1 and INT are the same as shown in Figure 2 (blue).



(c) TS2(O4A)



(d) RED(O4A)

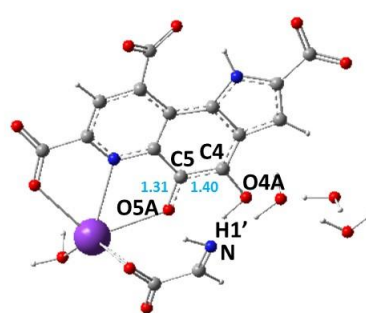


Figure 5. The obtained structures after optimization of (a) TS2(O5A), (b) RED(O5A), (c) TS2(O4A), and (d) RED(O4A). The distance is shown in Å.

Glycine oxidation with no K^+

Nucleophilic attack on C5

The relative Gibbs free energies and optimized structures obtained by the calculated results of the nucleophilic attack reaction on C5 (Figure 1(c)-upper) are shown in Figures 6 and 7, respectively.

When the nitrogen atom of alanine attacks on C5, one of the hydrogen atoms of the amino group of glycine moves to the nearest oxygen atom of the H_2O or OH^- molecules [Figures 7(a), 7(b), and 7(c)]. The calculated result of transition state (TS) energy is 6.9 kcal/mol [Figure 6, TS1(C5)]. The imaginary frequency mode at TS1(C5) corresponds well to the mode that represents the nucleophilic reaction. The product after the nucleophilic attack on C5, which is INT(C5), of which the energy is 2.9 kcal/mol higher than that of the reactant OX (Figure 6). The electronic structure around C5 changed from sp^2 to sp^3 hybridization. And the bond between the nitrogen atom of glycine and C5 is formed [C5-N=1.52Å; Figure 7(c)].

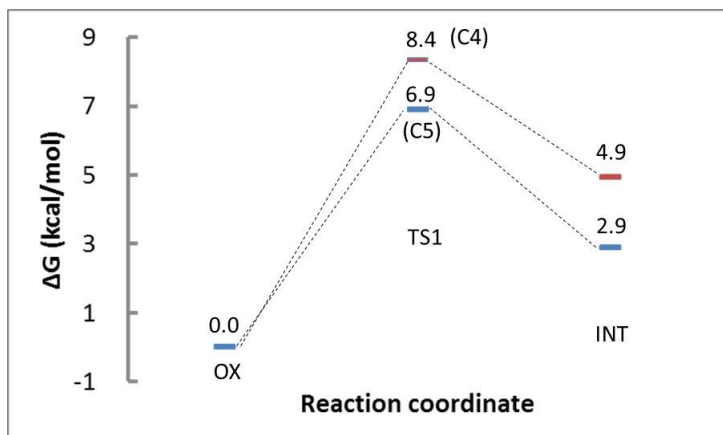


Figure 6. The Gibbs free energies of OX, transition state (TS) 1, and intermediate (INT), which are relative to OX (0.0 kcal/mol).

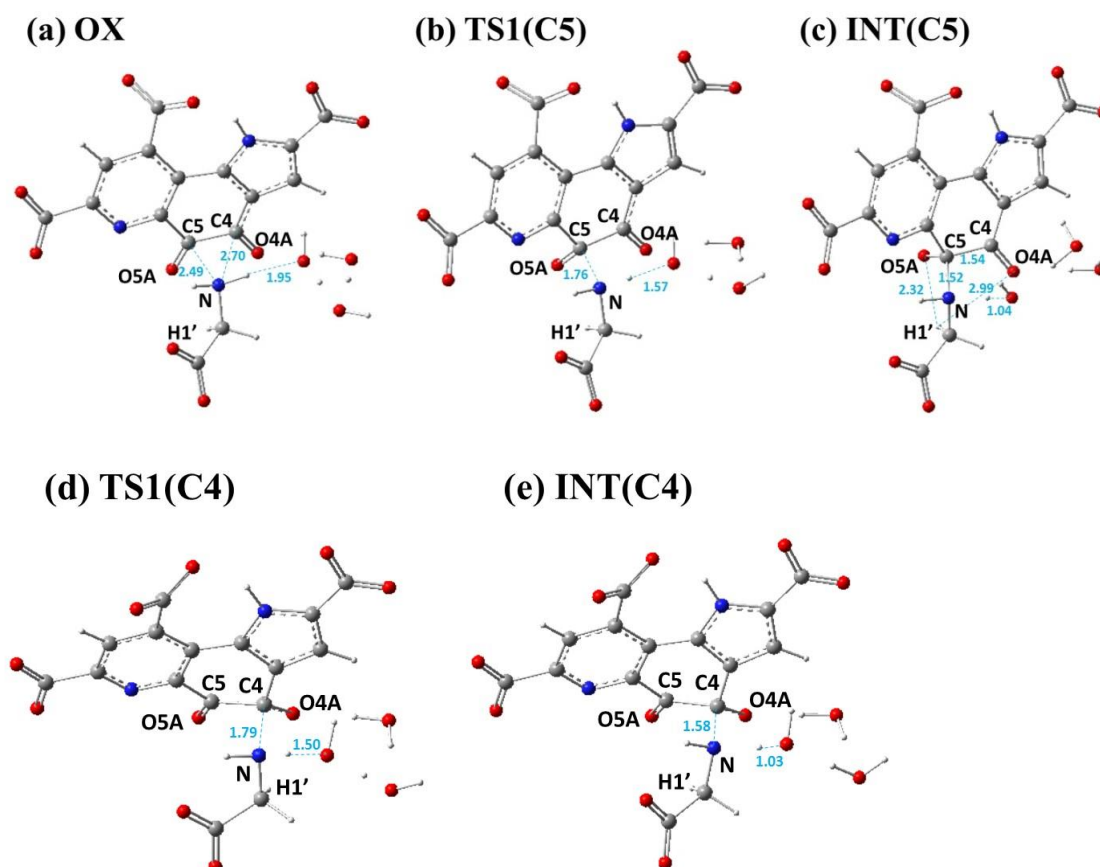


Figure 7. The obtained structures after optimization of (a) OX, (b) TS1(C5), (c) INT(C5), (d) TS1(C4), and (e) INT(C4). The distance is shown in Å.

Nucleophilic attack on C4

The relative Gibbs free energies and optimized structures obtained by the calculated results of the nucleophilic attack reaction on C4 (Figure 1(c)-lower) are shown in Figures 6 and 7, respectively.

When the nitrogen atom of glycine attacks on C4, one of the hydrogen atoms of the amino group of glycine moves to the nearest oxygen atom of the H₂O or OH⁻ molecules [Figures 7(a), 7(d), and 7(e)]. The TS energy is 8.4 kcal/mol [Figure 6, TS1(C4)]. The imaginary frequency mode at TS1(C4) corresponds well to the mode that represents the nucleophilic attack on C4 by the nitrogen atom of glycine. The product after the nucleophilic attack on C4, which is INT(C4), of which the energy is 4.9 kcal/mol higher than that of OX (Figure 6). The electronic structure around C4 changed from sp² to sp³ hybridization. The bond between the nitrogen atom of glycine and C4 is formed [C4-N=1.58Å; Figure 7(e)]. Compared the TS1 energies between TS1(C5) and TS1(C4), the TS1(C4) energy is 1.5 kcal/mol higher than TS1(C5) energy (Figure 6). The nucleophilic attack on C5 shown in Figure 1(c)-upper can be considered a major reaction because of the consideration of the Boltzmann distribution. This result is the same tendency of nucleophilic attack on glycine oxidation and glycine oxidation added one H₂O around K⁺ by PQQ.

Proton transfer following a nucleophilic attack on C5

The relative Gibbs free energies and optimized structures obtained by the calculated results of the proton transfer following a nucleophilic attack reaction on C5 (Figure 1(c)-upper) are shown in Figures 8 and 9, respectively.

The structure of product after the nucleophilic attack on C5, which is the

intermediate (INT(C5)), facilitates the following proton transfer toward O5A or toward O4A, because at this state, O5A, C5, the nitrogen atom of glycine, the α -carbon of glycine and one of the hydrogen on the α -carbon form a pentagon geometry, and also, O4A, C4, C5, the nitrogen atom of glycine, the α -carbon of glycine and one of the hydrogen on the α -carbon form a twisted hexagon geometry (Figure 7(c)). The closest hydrogen on the α -carbon of glycine to PQQ is H1' atom labeled in Figure 7(c).

The TS energy of proton (H1') transfer to O5A is 13.4 kcal/mol, while that to O4A is 20.1 kcal/mol (Figure 8, TS2). The activation energy from INT to TS2 in proton (H1') transfer to O5A is 10.5 kcal/mol, and while that from INT to O4A is 17.2 kcal/mol. Therefore, proton transfer to O5A is likely to occur considered the Boltzmann distribution. This result fall in line with that of proton transfer on glycine oxidation by PQQ. The imaginary frequency mode at TS2 corresponds well to the mode of proton transfer. From INT to TS2, in the case of the proton (H1') transfers to O5A, the bond between C5 and the nitrogen atom of glycine is lost (C5-N=1.52Å, Figure 7(c); C5-N=2.49Å, Figure 9(a)), and while in the case of proton (H1') transfer to O4A, it is the same reaction as that for H1' transfer to O5A (C5-N=1.52Å, Figure 7(c); C5-N=2.97Å, Figure 9(c)). Geometries show the electronic structure around the nitrogen atom shifts from sp³ to sp²-like, and that around C5 atom returns from sp³ to sp²-like (Figure 7(c), 9(a) and 9(c)).

After the proton (H1') transfer, INT changes to the reduced PQQ form (RED) [Figures 7(c), 9(b), and 9(d)]. The energy of RED after the proton (H1') transfer to O5A is -21.1 kcal/mol, and while, that correspond to O4A is -13.6 kcal/mol (Figure 8). The α -carbon of glycine changed from sp³ to sp², and then the bond between the nitrogen and α -carbon of glycine changed to a double bond [Figures 9(b) and 9(d)]. Either O4A

or O5A shown here is a negative oxygen ion (O^-) [Figures 9(b) and 9(d)], however, it can be readily hydrogenated by the protonation from one of the surrounding water molecules. After this hydrogenation, the formation of RED becomes complete.

These phenomena in a sequence of reactions are consistent with that of proton transfer on glycine oxidation and glycine oxidation added one H_2O around K^+ by PQQ.

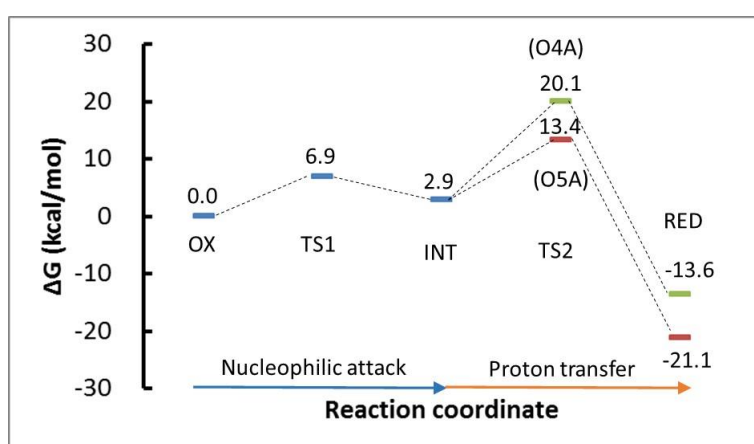
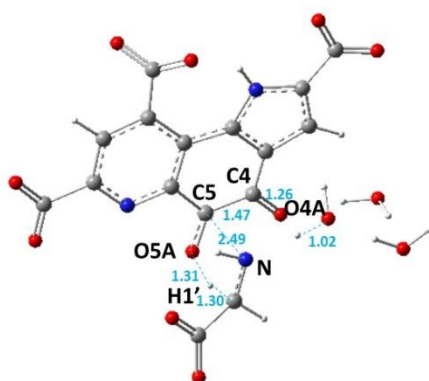
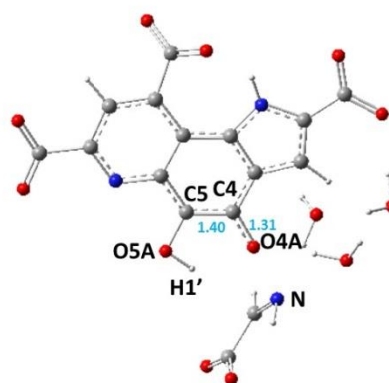


Figure 8. The Gibbs free energies of OX, transition state (TS) 1, intermediate (INT), TS2 and RED, which are relative to OX (0.0 kcal/mol). Those of OX, TS1 and INT are the same as shown in Figure 6 (blue).

(a) TS2(O5A)



(b) RED(O5A)



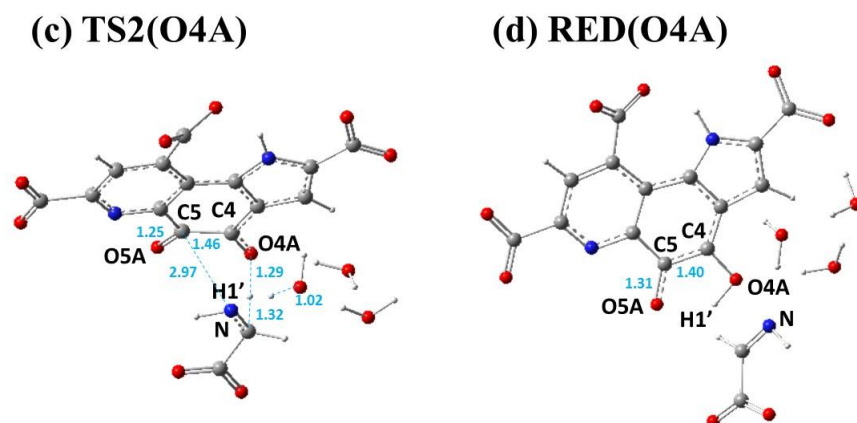


Figure 9. The obtained structures after optimization of (a) TS2(O5A), (b) RED(O5A), (c) TS2(O4A), and (d) RED(O4A). The distance is shown in Å.

Comparison of energies on glycine oxidation, glycine oxidation added one H₂O around K⁺ and glycine oxidation with no K⁺ by PQQ.

As for three different calculation systems, a nucleophilic attack on C5 by the nitrogen atom of glycine is likely to occur compared to that on C4. These structures of product after the nucleophilic attack on C5 (INT), facilitate the following proton transfer. The proton transfer to O5A is most likely to occur compared to that to O4A. The relative Gibbs free energies of above reaction pathway (Figure 1(a)-, 1(b)- and 1(c)-upper) are shown in Figure 10.

The TS energy which needs for the nitrogen atom of glycine attacks on C5 in the calculation system (glycine oxidation, added one H₂O around K⁺, or no K⁺) is 3.2 kcal/mol, 4.7 kcal/mol or 6.9 kcal/mol, respectively (Figure 10, TS1) [6]. Therefore, compared the calculation systems between including K⁺ (glycine oxidation or added one H₂O around K⁺) and not including K⁺ (no K⁺), a nucleophilic attack on C5 by the nitrogen atom of glycine in the calculation system of including K⁺ is easily to occur.

After the nucleophilic attack on C5, the proton transfer toward O5A occurs. The TS energy of proton transfer to O5A on each different calculation system (glycine oxidation, added one H₂O around K⁺, or no K⁺) is 9.7 kcal/mol, 11.0 kcal/mol or 13.4 kcal/mol, respectively (Figure 10, TS2) [6]. The activation energy from INT to TS2 in proton transfer to O5A is 10.5 kcal/mol, 10.6 kcal/mol or 10.5 kcal/mol, respectively. Therefore, a proton transfer to O5A on each calculation system is likely to occur equally considered only the viewpoint of energies, because the difference of energy of proton transfer among three different calculation systems is very small (less than 0.1 kcal/mol). That is why the difference of three different calculations is considered to most depend on the difference of energies of nucleophilic attack on C5 by glycine. However, I have to care about this proton transfer, because proton is treated classically on Gaussian calculation, which means proton is not quantized. In fact, the activation energies are considered to be lower than above-mentioned if proton is quantized because of tunnel effect. Here, the energies of first reaction, the TS energies of a nucleophilic attack, have the difference in energies, therefore, a nucleophilic attack reaction is considered to be a rate-limiting reaction, which means a nucleophilic attack affects on a speed of reaction.

Compared to the calculation systems between including K⁺ (glycine oxidation or added one H₂O around K⁺) and not including K⁺ (no K⁺), the tendency of difference of energies is consistent with the experiment which a cation located as mentioned above is needed to keep the activity of redox cycle [7]. In fact, the necessary energy of nucleophilic attack on C5 in the calculation system including K⁺ (glycine oxidation or added one H₂O around K⁺) is lower than that corresponding in the calculation system not including K⁺ (no K⁺). Therefore, a K⁺ is important for a nucleophilic attack on C5, and in other words, a K⁺ is important to work as a foothold of glycine on a nucleophilic

attack reaction. And also, compared to the calculation systems between glycine oxidation and added one H_2O around K^+ , the difference of energy is very small. Therefore, it is considered not to affect much on this reaction pathway whether one water coordinates with K^+ or not.

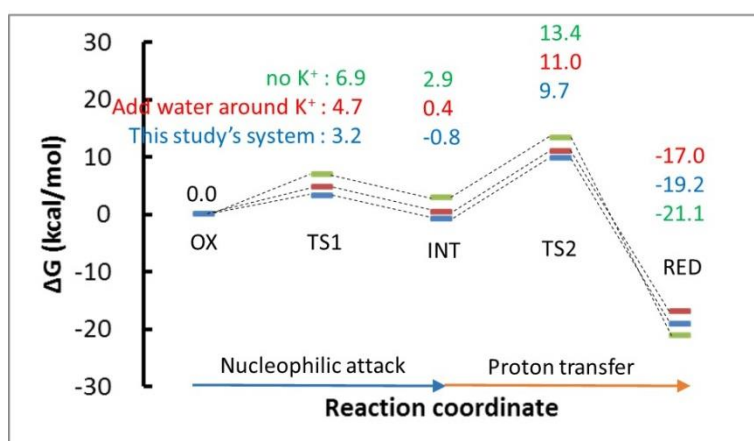


Figure 10. The Gibbs free energies of OX, transition state (TS) 1, intermediate (INT), TS2 and RED, which are relative to OX (0.0 kcal/mol). They are the same as shown in Figure 4, 8 and the result of previous study [6].

(B) Comparison of amino acid oxidation by PQQ in aqueous solution.

Mechanism of glycine, alanine and valine oxidation by PQQ in aqueous solution.

As referred the previous study [6], the mechanism of glycine, alanine or valine oxidation have two modes, one is nucleophilic attack and other is proton and electron transfer (Figure 11(a), 11(b) and 11(c)).

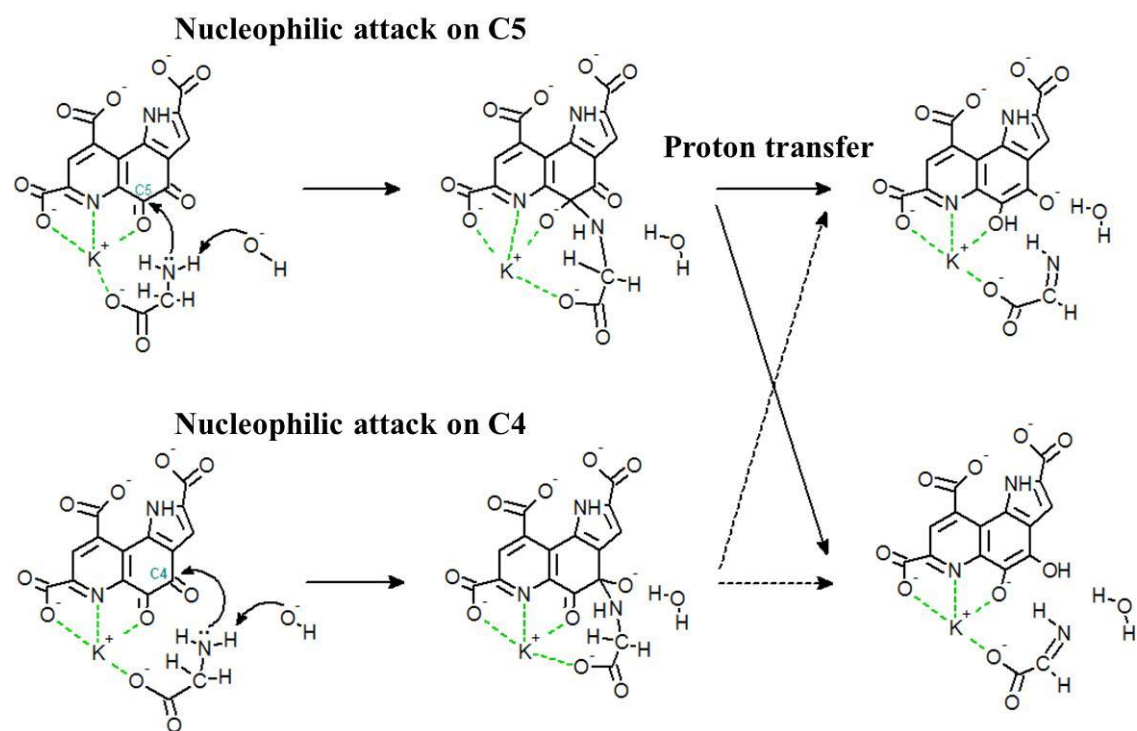
Nucleophilic attack

As for the first reaction, a nucleophilic attack by a nitrogen atom of amino acid (glycine, alanine or valine) on C5 (Figure 11(a)-, 11(b)- or 11(c)-upper) or C4 (Figure 11(a)-, 11(b)- or 11(c)-lower) of PQQ occurs.

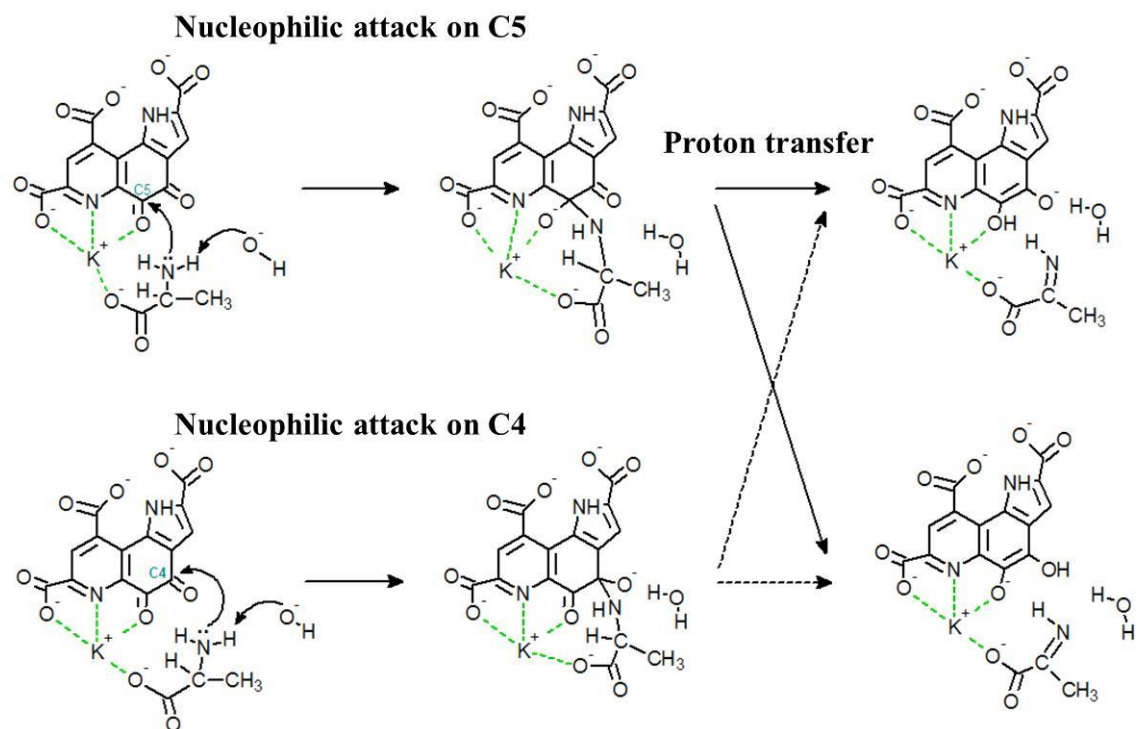
Proton transfer following nucleophilic attack

As for the second reaction, proton and electron transfer [Figure 11(a), 11(b) or 11(c)] occur. As for proton transfer, a hydrogen atom on the α -carbon of amino acid (glycine, alanine or valine) is extracted as a proton by the lone pair of electrons of O4A or O5A. At the same time, as for electron transfer, two electrons (which form the bond between the nitrogen atom of amino acid and one of the carbon atoms) transfer to the PQQ molecule. As a result of this reaction, the single bond between C4 and C5 turns into a double bond. The nitrogen atom and the α -carbon of amino acid (glycine, alanine or valine) thereby form a double bond. Thus, the amino acid (glycine, alanine or valine) oxidation is completed. In a conventional manner, the reduced form of PQQ is described as both O4A and O5A are hydrogenated. After either O4A or O5A becomes a negative oxygen ion (O^-), the PQQ can become the reduced form, since it easily receives one proton from one of the surrounding water molecules.

(a) Glycine oxidation



(b) Alanine oxidation



(c) Valine oxidation

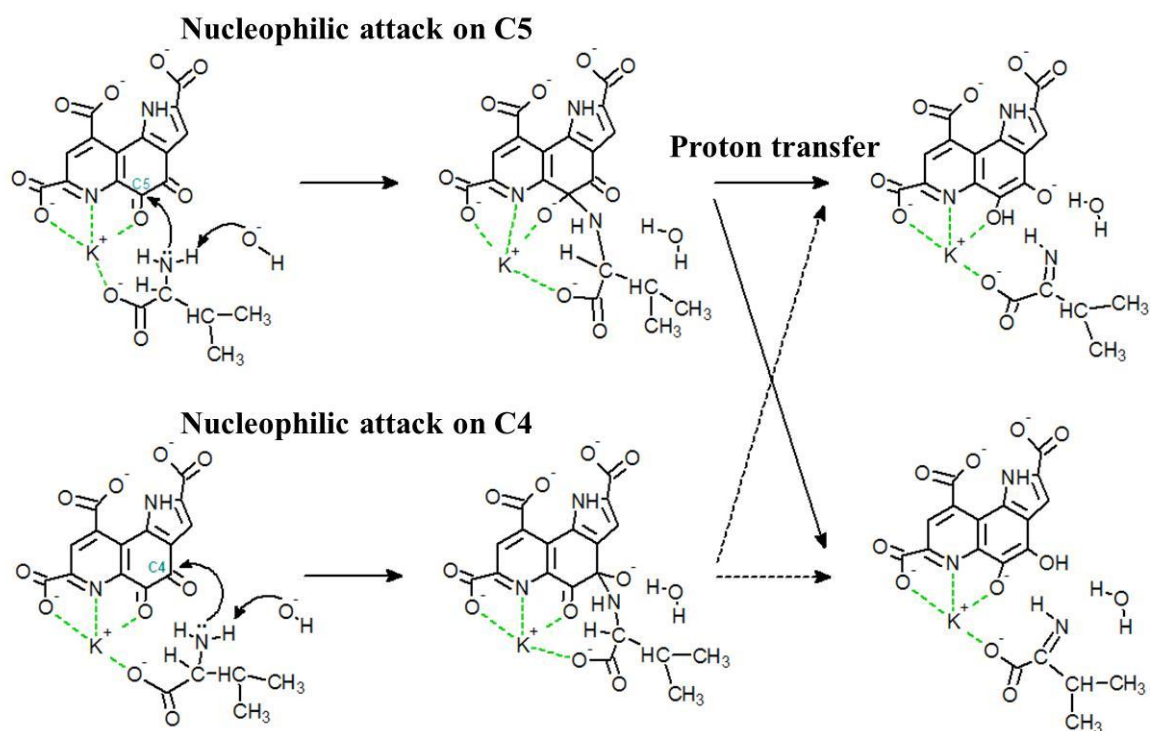


Figure 11. Three mechanisms of glycine oxidation (a), alanine oxidation (b) and valine oxidation (c). These three mechanisms have two modes of nucleophilic attack on C5 or on C4. The first step is nucleophilic attack by the nitrogen atom of glycine (a), alanine (b) or valine (c) and the second step is proton transfer and elimination reaction.

Reactant structure

In case of glycine oxidation by PQQ, the geometry of the reactants with oxidized PQQ, which is here, this state is abbreviated to OX, is referred to the structure of previous study [6]. In case of alanine and valine oxidation, the OX geometries are also referred to the geometry (OX) of glycine oxidation by PQQ and are optimized first.

The details of OX on glycine, alanine and valine oxidation by PQQ are shown in figures in the following discussions, respectively. Each calculation system contains one PQQ molecule, one K⁺ cation, two explicit water molecules, one OH⁻ anion, and

one amino acid (glycine, alanine or valine). The K^+ cation was placed stably among O7A, N6, and O5A by coordination bonds. Moreover, the oxygen atom of the carboxyl group of amino acid (glycine, alanine or valine) forms a coordination bond with K^+ , which provides the foothold for amino acid (glycine, alanine or valine) and makes the nucleophilic attack possible (Figure 11). Furthermore, the nitrogen atom of the amino group of amino acid (glycine, alanine or valine) is placed very close to the C5 and C4 atoms, which allows the nitrogen atom to attack the C5 or C4 atoms as a nucleophilic attack reaction.

Compared the structures of three OX, it is difficult to predict the difference of reaction, because the following two points are considered to work antagonistically. (i) One is the point that the α -carbon of amino acid (glycine, alanine or valine) become more positive by the effect of electronegativity of substituent. The difference of hydrogen, carbon and nitrogen atom is known as $H < C < N$, that is why the α -carbon of valine is most positive, and that of alanine is more positive by that of glycine (Table 1). The above effect induces the tendency which the H atom ($H1'$) of the α -carbon of amino acid becomes far from O5A. In fact, The distance between $H1'$ atom of valine and O5A is largest among three amino acids, and that between $H1'$ atom of alanine and O5A is larger than that between $H1'$ atom of glycine and O5A (Table 2). Therefore, the distance between the nitrogen atom of amino acid and C5 is also affected, that between the nitrogen atom of valine and C5 is largest among three amino acids, and that between the nitrogen atom of alanine and C5 is larger than that between the nitrogen atom of glycine and C5 (Table 2). These events cause unstable conformation. In other words, a nucleophilic attack cannot easily occur by this point. Therefore, this point prompts high barrier on a reaction (i.e., making the energy of TS high). (ii) The other is the point that

when the substituent is larger (i.e., total number of atoms of substituent is larger), the total number of electron increases, especially electron around the α -carbon of amino acid (glycine, alanine or valine) increases, since substituent is bond to α -carbon of amino acid (glycine, alanine or valine). This event induces that the nitrogen atom of amino acid pulls electrons around the α -carbon easily. As a result of this, the nitrogen atom becomes more negative when the substituent is larger. In fact, the nitrogen atom of valine is most negative among three amino acids, and that of alanine is more negative than that of glycine (Table 1). Therefore, this point prompts low barrier on a following nucleophilic attack reaction (i.e., making the energy of TS low). In other words, a nucleophilic attack can easily occur by this point. These two points, (i) and (ii), are considered to affect antagonistically on a reaction. Therefore, I actually calculated the energies on a reaction and investigated the comparison of three different amino acids oxidation by PQQ.

Table 1. The charge obtained by Mulliken population analysis of OX on glycine, alanine and valine oxidation by PQQ.

OX	charge (C α)	charge (N)	charge (O5A)	charge (C5)
Glycine	-0.129	-0.649	-0.571	0.443
Alanine	0.032	-0.653	-0.551	0.449
Valine	0.041	-0.661	-0.547	0.448

Table 2. The distance of OX on glycine, alanine and valine oxidation by PQQ. The distance is shown in Å.

OX	distance (O5A-H1')	distance (N-C5)
Glycine	2.45	2.39
Alanine	2.71	2.56
Valine	2.77	2.59

Glycine oxidation by PQQ

Nucleophilic attack on C5 or C4

Referred to the previous study [6], a nucleophilic attack on C5 by the nitrogen atom of glycine is likely to occur compared to that on C4, which is based on analyses of energies (Boltzmann distribution), Mulliken population analyses and distance between C5 or C4 and nitrogen atom.

Proton transfer following a nucleophilic attack on C5

Referred to the previous study [6], the structure of product after the nucleophilic attack on C5, which is the intermediate (INT(C5)), facilitates the following proton transfer. The proton transfer to O5A is reported to most likely to occur, which is based on the analyses of energies (Boltzmann distribution).

Alanine oxidation by PQQ

Nucleophilic attack on C5

The relative Gibbs free energies and optimized structures obtained by the calculated results of the nucleophilic attack reaction on C5 (Figure 11(b)-upper) are

shown in Figures 12 and 13, respectively.

When the nitrogen atom of alanine attacks on C5, one of the hydrogen atoms of the amino group of alanine moves to the nearest oxygen atom of the H₂O or OH⁻ molecules [Figures 13(a), 13(b), and 13(c)]. The calculated result of transition state (TS) energy is 5.7 kcal/mol [Figure 12, TS1(C5)], which is relative to the reference energy; OX is the reference (0.0 kcal/mol). The imaginary frequency mode at TS1(C5) corresponds well to the mode that represents the nucleophilic reaction. The product after the nucleophilic attack on C5, which is INT(C5), of which the energy is 0.6 kcal/mol higher than that of the reactant OX (Figure 12). The electronic structure around C5 changed from sp² to sp³ hybridization. The nitrogen atom of alanine forms a bond to C5 [C5-N=1.55Å; Figure 13(c)].

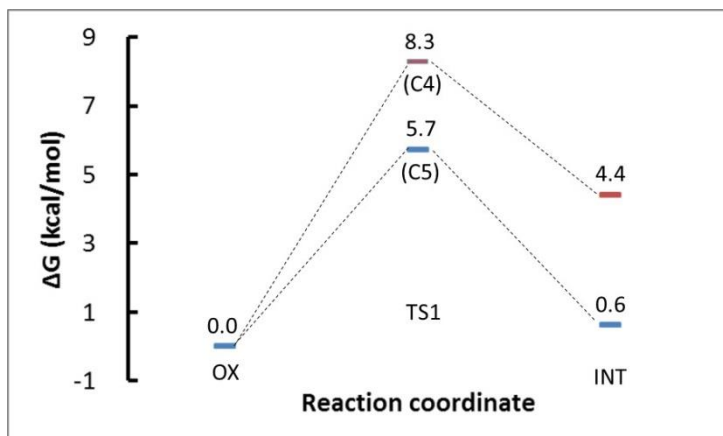


Figure 12. The Gibbs free energies of OX, transition state (TS) 1, and intermediate (INT), which are relative to OX (0.0 kcal/mol).

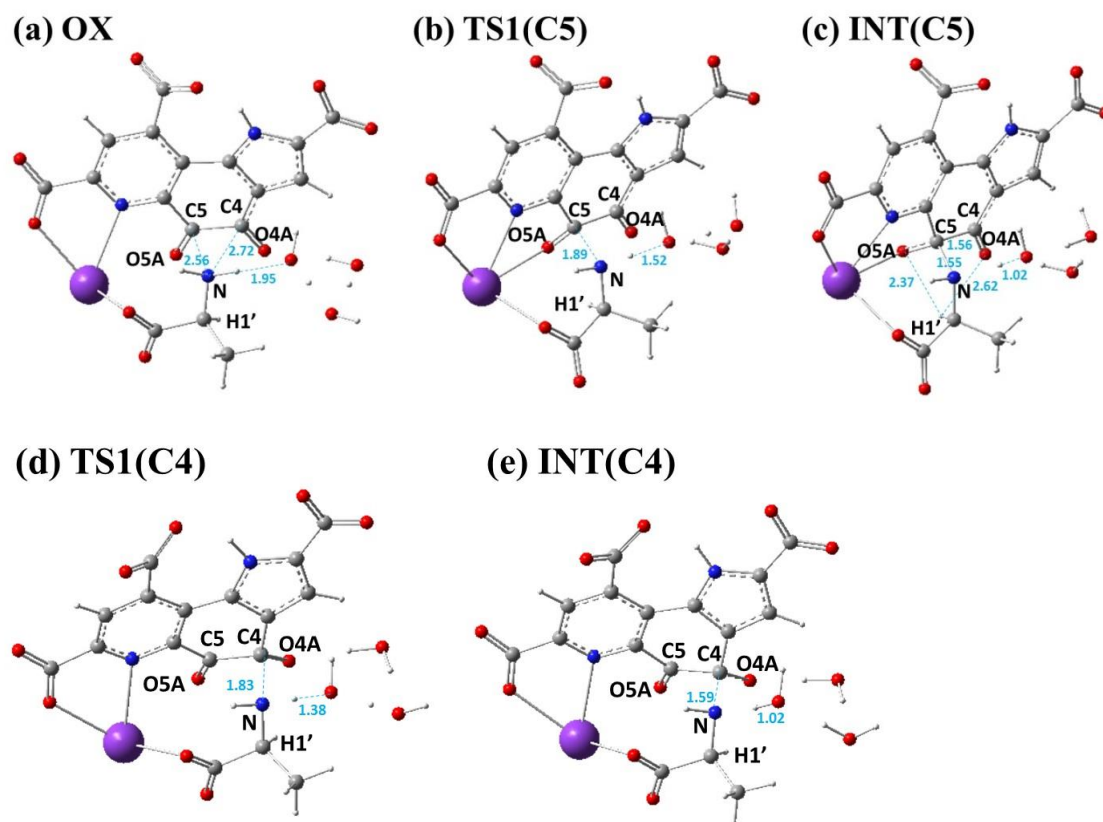


Figure 13. The obtained structures after optimization of (a) OX, (b) TS1(C5), (c) INT(C5), (d) TS1(C4), and (e) INT(C4). The distance is shown in Å.

Nucleophilic attack on C4

The relative Gibbs free energies and optimized structures obtained by the calculated results of the nucleophilic attack reaction on C4 (Figure 11(b)-lower) are shown in Figures 12 and 13, respectively.

When the nitrogen atom of alanine attacks on C4, one of the hydrogen atoms of the amino group of alanine moves to the nearest oxygen atom of the H₂O or OH⁻ molecules [Figures 13(a), 13(d), and 13(e)]. The TS energy is 8.3 kcal/mol [Figure 12, TS1(C4)]. The imaginary frequency mode at TS1(C4) corresponds well to the mode that represents the nucleophilic attack on C4 by the nitrogen atom of alanine. The product

after the nucleophilic attack on C4, which is INT(C4), of which the energy is 4.4 kcal/mol higher than that of OX (Figure 12). The electronic structure around C4 changed from sp² to sp³ hybridization. The nitrogen atom of alanine forms a bond to C4 [C4-N=1.59Å; Figure 13(e)]. Compared the TS1 energies between TS1(C5) and TS1(C4), the TS1(C4) energy is 2.6 kcal/mol higher than TS1(C5) energy (Figure 12). The nucleophilic attack on C5 shown in Figure 11(b)-upper can be considered a major reaction because of the consideration of the Boltzmann distribution. This result is the same tendency of nucleophilic attack on glycine oxidation by PQQ.

Proton transfer following a nucleophilic attack on C5

The relative Gibbs free energies and optimized structures obtained by the calculated results of the proton transfer following a nucleophilic attack reaction on C5 (Figure 11(b)-upper) are shown in Figures 14 and 15, respectively.

The structure of product after the nucleophilic attack on C5, which is the intermediate (INT(C5)), facilitates the following proton transfer toward O5A or toward O4A, because at this state, O5A, C5, the nitrogen atom of alanine, the α -carbon of alanine and one of the hydrogen on the α -carbon form a pentagon geometry, and also, O4A, C4, C5, the nitrogen atom of alanine, the α -carbon of alanine and one of the hydrogen on the α -carbon form a twisted hexagon geometry (Figure 13(c)). The closest hydrogen on the α -carbon of alanine to PQQ is H1' atom labeled in Figure 13(c).

The TS energy of proton (H1') transfer to O5A is 11.3 kcal/mol, while that to O4A is 20.9 kcal/mol (Figure 14, TS2). The activation energy from INT to TS2 in proton (H1') transfer to O5A is 10.7 kcal/mol, and while that from INT to O4A is 20.3 kcal/mol. Therefore, proton transfer to O5A is likely to occur considered the Boltzmann

distribution. This result fall in line with that of proton transfer on glycine oxidation by PQQ. The imaginary frequency mode at TS2 corresponds well to the mode of proton transfer. From INT to TS2, in the case of the proton (H1') transfers to O5A, the bond between C5 and the nitrogen atom of alanine is lost (C5-N=1.55Å, Figure 13(c); C5-N=2.50Å, Figure 15(a)), and while in the case of proton (H1') transfer to O4A, it is the same reaction as that for H1' transfer to O5A (C5-N=1.55Å, Figure 13(c); C5-N=2.82Å, Figure 15(c)). Geometries show the electronic structure around the nitrogen atom shifts from sp³ to sp²-like, and that around C5 atom returns from sp³ to sp²-like (Figure 13(c), 15(a) and 15(c)).

After the proton (H1') transfer, INT changes to the reduced PQQ form (RED) [Figures 13(c), 15(b), and 15(d)]. The energy of RED after the proton (H1') transfer to O5A is -22.6 kcal/mol, and while, that correspond to O4A is -17.7 kcal/mol (Figure 14). The α -carbon of alanine changed from sp³ to sp², and then the bond between the nitrogen and α -carbon of alanine became a double bond [Figures 15(b) and 15(d)]. Either O4A or O5A shown here is a negative oxygen ion (O⁻) [Figures 15(b) and 15(d)], however, it can be readily hydrogenated by the protonation from one of the surrounding water molecules. After this hydrogenation, the formation of RED becomes complete.

These phenomena in a sequence of reactions are consistent with that of proton transfer on glycine oxidation by PQQ.

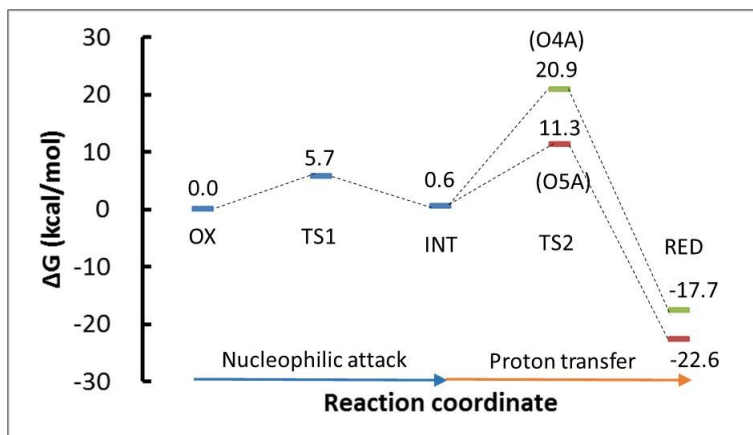
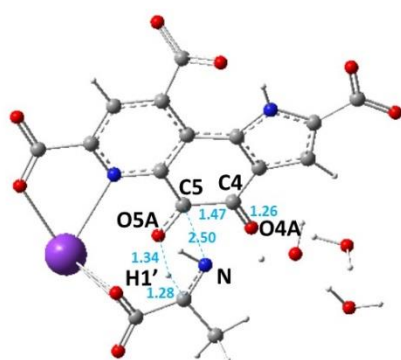
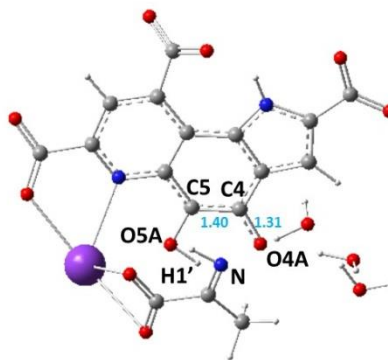


Figure 14. The Gibbs free energies of OX, transition state (TS) 1, intermediate (INT), TS2 and RED, which are relative to OX (0.0 kcal/mol). Those of OX, TS1 and INT are the same as shown in Figure 12 (blue).

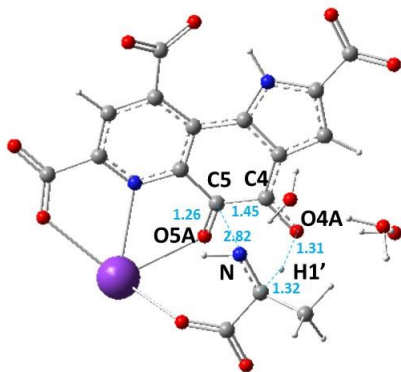
(a) TS2(O5A)



(b) RED(O5A)



(c) TS2(O4A)



(d) RED(O4A)

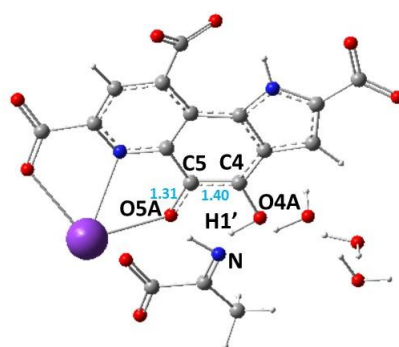


Figure 15. The obtained structures after optimization of (a) TS2(O5A), (b) RED(O5A), (c) TS2(O4A), and (d) RED(O4A). The distance is shown in Å.

Valine oxidation by PQQ

Nucleophilic attack on C5

The relative Gibbs free energies and optimized structures obtained by the calculated results of the nucleophilic attack reaction on C5 (Figure 11(c)-upper) are shown in Figures 16 and 17, respectively.

When the nitrogen atom of valine attacks on C5, one of the hydrogen atoms of the amino group of valine moves to the nearest oxygen atom of the H₂O or OH⁻ molecules [Figures 17(a), 17(b), and 17(c)]. The TS energy is 6.7 kcal/mol [Figure 16, TS1(C5)]. The imaginary frequency mode at TS1(C5) corresponds well to the mode that represents the nucleophilic reaction. The product after the nucleophilic attack on C5, which is INT(C5), of which the energy is 1.5 kcal/mol higher than that of the reactant OX (Figure 16). The electronic structure around C5 changed from sp² to sp³ hybridization. The nitrogen atom of valine forms a bond to C5 [C5-N=1.55Å; Figure 17(c)].

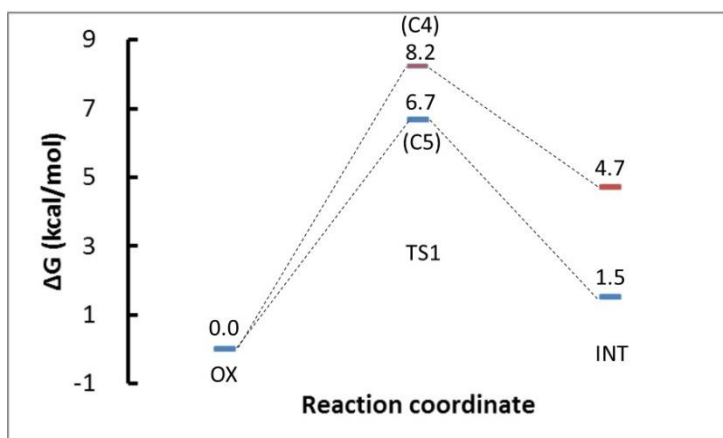


Figure 16. The Gibbs free energies of OX, transition state (TS) 1, and intermediate (INT), which are relative to OX (0.0 kcal/mol).

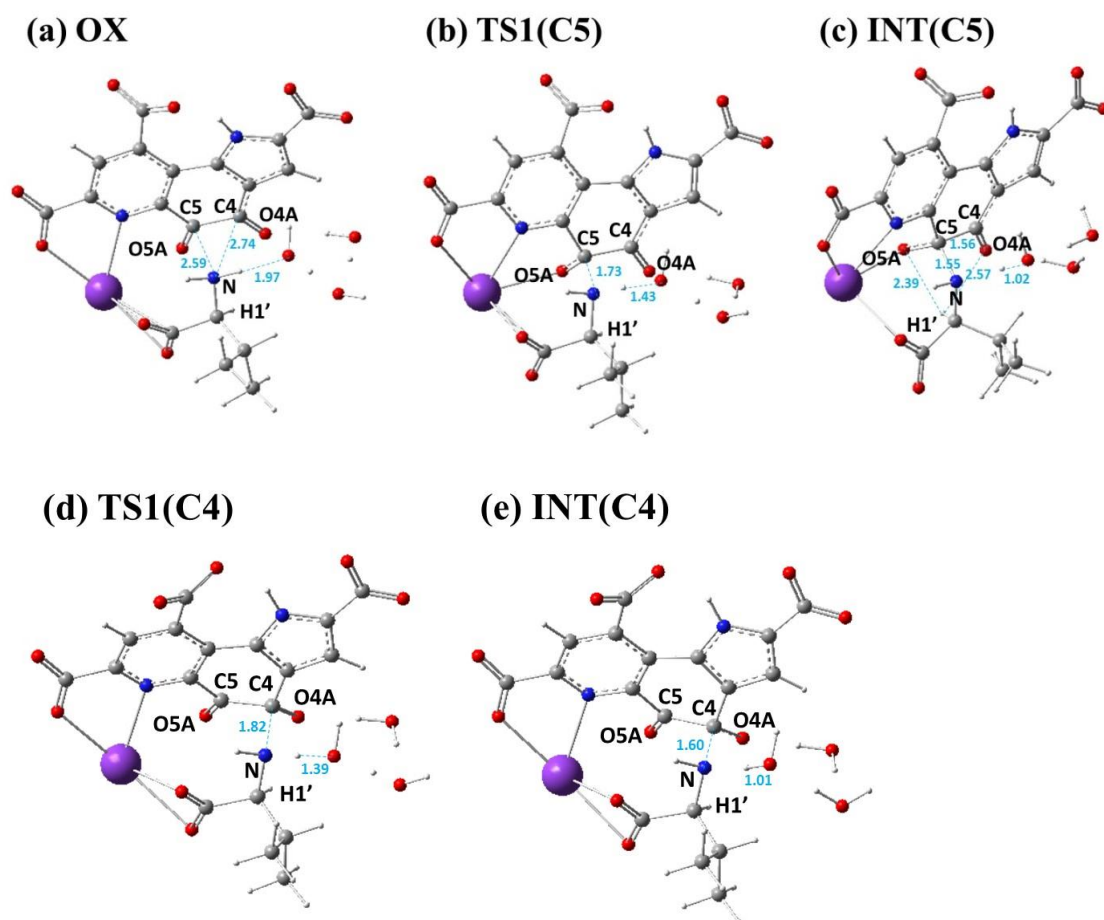


Figure 17. The obtained structures after optimization of (a) OX, (b) TS1(C5), (c)

INT(C5), (d) TS1(C4), and (e) INT(C4). The distance is shown in Å.

Nucleophilic attack on C4

The relative Gibbs free energies and optimized structures obtained by the calculated results of the nucleophilic attack reaction on C4 (Figure 11(c)-lower) are shown in Figures 16 and 17, respectively.

When the nitrogen atom of valine attacks on C4, one of the hydrogen atoms of the amino group of valine moves to the nearest oxygen atom of the H₂O or OH⁻ molecules [Figures 17(a), 17(d), and 17(e)]. The TS energy is 8.2 kcal/mol [Figure 16, TS1(C4)]. The imaginary frequency mode at TS1(C4) corresponds well to the mode that represents the nucleophilic attack on C4 by the nitrogen atom of valine. The product after the nucleophilic attack on C4, which is INT(C4), of which the energy is 4.7 kcal/mol higher than that of OX (Figure 16). The electronic structure around C4 changed from sp² to sp³ hybridization. The nitrogen atom of valine forms a bond to C4 [C5-N=1.60Å; Figure 17(c)]. Compared the TS1 energies between TS1(C5) and TS1(C4), the TS1(C4) energy is 1.5 kcal/mol higher than TS1(C5) energy (Figure 16). The nucleophilic attack on C5 shown in Figure 11(c)-upper can be considered a major reaction because of the consideration of the Boltzmann distribution. This result is the same tendency of nucleophilic attack on glycine and alanine oxidation by PQQ.

Proton transfer following a nucleophilic attack on C5

The relative Gibbs free energies and optimized structures obtained by the calculated results of the proton transfer following a nucleophilic attack reaction on C5 (Figure 11(c)-upper) are shown in Figures 18 and 19, respectively.

The structure of product after the nucleophilic attack on C5, which is the intermediate (INT(C5)), facilitates the following proton transfer toward O5A or toward O4A, because at this state, O5A, C5, the nitrogen atom of valine, the α -carbon of valine and one of the hydrogen on the α -carbon form a pentagon geometry, and also, O4A, C4, C5, the nitrogen atom of valine, the α -carbon of valine and one of the hydrogen on the α -carbon form a twisted hexagon geometry (Figure 17(c)). The closest hydrogen on the α -carbon of valine to PQQ is H1' atom labeled in Figure 17(c).

The TS energy of proton (H1') transfer to O5A is 12.6 kcal/mol, while that to O4A is 21.5 kcal/mol (Figure 18, TS2). The activation energy from INT to TS2 in proton (H1') transfer to O5A is 11.1 kcal/mol, and while that from INT to O4A is 20.0 kcal/mol. Therefore, proton transfer to O5A is likely to occur considered the Boltzmann distribution. This result falls in line with that of proton transfer on glycine or alanine oxidation by PQQ. The imaginary frequency mode at TS2 corresponds well to the mode of proton transfer. From INT to TS2, in the case of the proton (H1') transfers to O5A, the bond between C5 and the nitrogen atom of valine is lost (C5-N=1.55Å, Figure 17(c); C5-N=2.49Å, Figure 19(a)), and while in the case of proton (H1') transfer to O4A, it is the same reaction as that for H1' transfer to O5A (C5-N=1.55Å, Figure 17(c); C5-N=2.83Å, Figure 19(c)). Geometries show the electronic structure around the nitrogen atom shifts from sp³ to sp²-like, and that around C5 atom returns from sp³ to sp²-like (Figure 17(c), 19(a) and 19(c)).

After the proton (H1') transfer, INT changes to RED [Figures 17(c), 19(b), and 19(d)]. The energy of RED after the proton (H1') transfer to O5A is -21.8 kcal/mol, and while, that correspond to O4A is -16.9 kcal/mol (Figure 18). The α -carbon of valine changed from sp³ to sp², and then the bond between the nitrogen and α -carbon of valine

became a double bond [Figures 19(b) and 19(d)]. Either O4A or O5A shown here is a negative oxygen ion (O^-) [Figures 19(b) and 19(d)], however, it can be readily hydrogenated by the protonation from one of the surrounding water molecules. After this hydrogenation, the formation of RED becomes complete.

These phenomena in a sequence of reactions are consistent with that of proton transfer on glycine or alanine oxidation by PQQ.

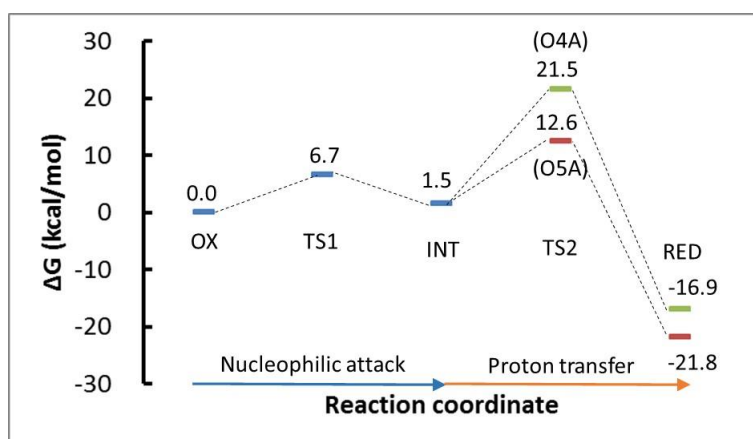
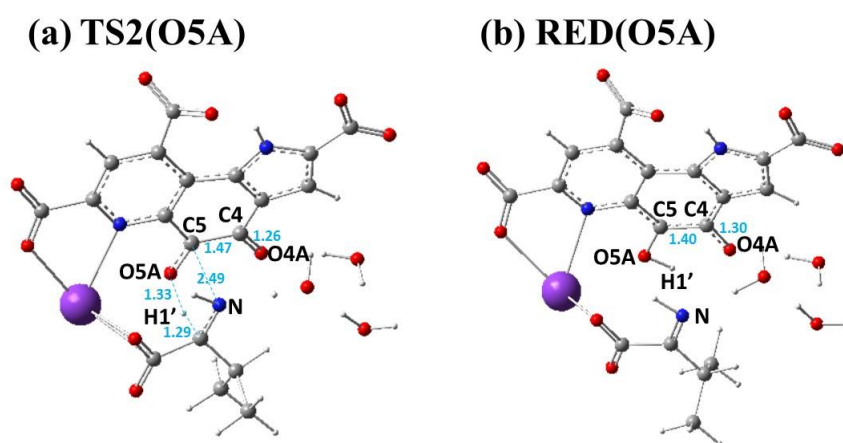


Figure 18. The Gibbs free energies of OX, transition state (TS) 1, intermediate (INT), TS2 and RED, which are relative to OX (0.0 kcal/mol). Those of OX, TS1 and INT are the same as shown in Figure 16 (blue).



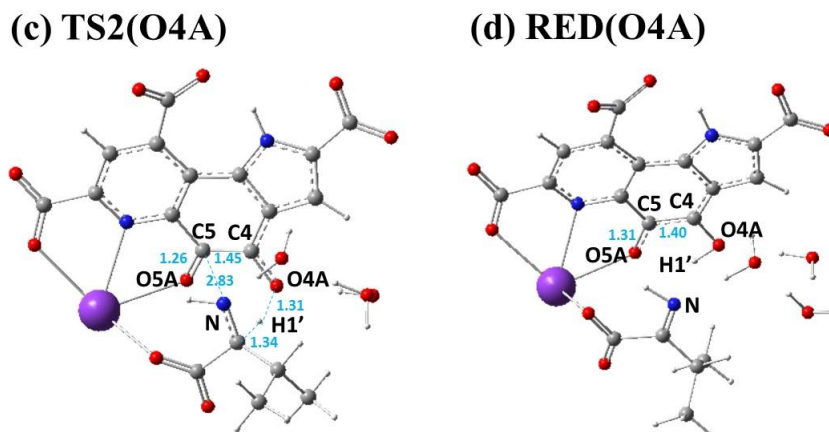


Figure 19. The obtained structures after optimization of (a) TS2(O5A), (b) RED(O5A), (c) TS2(O4A), and (d) RED(O4A). The distance is shown in Å.

Comparison of three amino acids (glycine, alanine and valine) oxidation by PQQ

As for three different amino acids (glycine, alanine or valine) oxidation by PQQ, a nucleophilic attack on C5 by the nitrogen atom of amino acid is likely to occur compared to that on C4. These structures of product after the nucleophilic attack on C5 (INT), facilitate the following proton transfer. The proton transfer to O5A is most likely to occur compared to that to O4A. The relative Gibbs free energies of above reaction pathway (Figure 11(a)-, 11(b)- and 11(c)-upper) are shown in Figure 20.

The TS energy which needs for the nitrogen atom of amino acid (glycine, alanine or valine) attacks on C5 is 3.2 kcal/mol, 5.7 kcal/mol or 6.7 kcal/mol, respectively (Figure 20, TS1) [6]. Therefore, a nucleophilic attack on C5 by the nitrogen atom of glycine is most easily to occur among three amino acids, and that by the nitrogen atom of alanine is likely to occur compared to that by the nitrogen atom of valine.

After the nucleophilic attack on C5, the proton transfer toward O5A occurs.

The TS energy of proton transfer to O5A on glycine, alanine or valine oxidation by PQQ is 9.71 kcal/mol, 11.26 kcal/mol or 12.55 kcal/mol, respectively (Figure 20, TS2) [6]. The activation energy from INT to TS2 in proton transfer to O5A is 10.51 kcal/mol, 10.66 kcal/mol or 11.05 kcal/mol, respectively. Therefore, a proton transfer to O5A on each amino acid oxidation is likely to occur equally from only the viewpoint of energies, because the difference of energy of proton transfer among three amino acids is very small (less than 0.5 kcal/mol). Therefore, the effect of three different amino acids oxidations by PQQ is considered to most depend on the difference of energies of nucleophilic attack on C5 by amino acids. However, I have to care about this proton transfer, because proton is treated classically on Gaussian calculation, which means proton is not quantized. In fact, the activation energies are considered to be lower than above-mentioned if proton is quantized because of tunnel effect. Here, the energies of first reaction, the TS energies of a nucleophilic attack, have the difference in energies, therefore, a nucleophilic attack reaction is considered to be a rate-limiting reaction, which means a nucleophilic attack affects on a speed of reaction.

The tendency of difference of energies is consistent with the experiment which is the reaction of PQQ with glycine occurs better than that with alanine and valine and also that with alanine occurs better than that with valine [5].

Considered these points, the effect of difference among three different amino acids oxidations by PQQ most depends on the difference of energies of nucleophilic attack on C5 by amino acid.

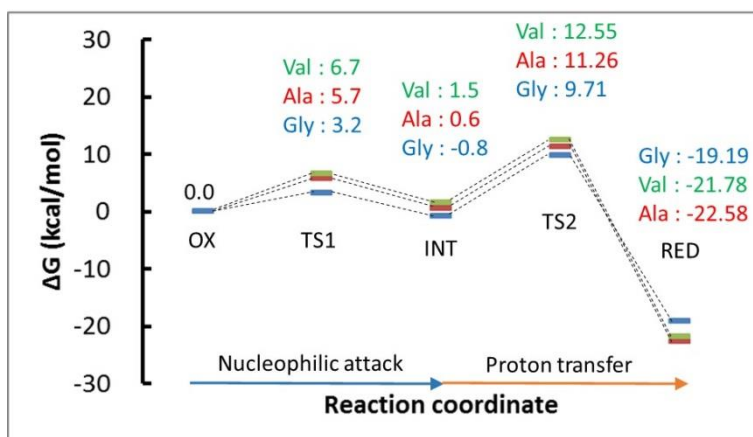


Figure 20. The Gibbs free energies of OX, transition state (TS) 1, intermediate (INT), TS2 and RED, which are relative to OX (0.0 kcal/mol). They are the same as shown in Figure 14, 18 and the result of previous study [6].

Comparison of three amino acids (glycine, alanine and valine) oxidation based on the transition state theory

Considered that the effect of difference among three different amino acids oxidations by PQQ most depends on the difference of energies of nucleophilic attack on C5 by amino acid, I compared the ratio of reaction rate of nucleophilic attack on C5 by amino acid.

The equation of traditional transition state theory is

$$k(T) = \frac{k_B T}{h} e^{-\frac{\Delta G^\ddagger}{RT}},$$

where $\Delta G^\ddagger$, k_B , T , h , and R are Gibbs free energy of activation, Boltzmann constant, temperature (I use the temperature of 298.15 K), Plank constant, and gas constant ($1.987 \times 10^{-3} \text{ kcal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$), respectively.

The ratio of reaction rate of the first step on glycine oxidation by PQQ ($k_{\text{step1(Gly)}}$) divided by that of the first step on valine oxidation by PQQ ($k_{\text{step1(Val)}}$) is

$k_{\text{step1(Gly)}}/k_{\text{step1(Val)}} = 333.5$. It means the first step on glycine oxidation (nucleophilic attack of nitrogen atom of glycine on C5) is much faster than that on valine oxidation (nucleophilic attack of nitrogen atom of valine on C5). And also, the ratio of reaction rate of the first step on alanine oxidation by PQQ ($k_{\text{step1(Ala)}}$) divided by that of the first step on valine oxidation by PQQ ($k_{\text{step1(Val)}}$) is $k_{\text{step1(Ala)}}/k_{\text{step1(Val)}} = 5.0$. It means the first step on alanine oxidation (nucleophilic attack of nitrogen atom of alanine on C5) is faster than that on valine oxidation (nucleophilic attack of nitrogen atom of valine on C5). The tendency of difference of ratio of reaction rate is consistent with the experiment which is the reaction of PQQ with glycine occurs better than that with alanine and valine and also that with alanine occurs better than that with valine [5].

Conclusion

(A) Role of a cation.

The role of K^+ was investigated on the three different calculation systems, glycine oxidation, glycine oxidation added one H_2O around K^+ , and glycine oxidation with no K^+ by PQQ in aqueous solution. Glycine oxidation by PQQ was referred to the previous study. In the three different calculation systems, a nucleophilic attack on C5 by the nitrogen atom of glycine is likely to occur compared to that on C4. These structures of product after the nucleophilic attack on C5 (INT), facilitate the following proton transfer. The proton transfer to O5A is most likely to occur compared to that to O4A. It is found that the effect of three different calculations depends on the difference of energies of nucleophilic attack on C5 by amino acids. As for the comparison between the calculation systems between including K^+ (glycine oxidation or added one H_2O around K^+) and not including K^+ (no K^+), the tendency of difference of energies is consistent with the experiment that a cation located as mentioned before is needed to keep the activity of redox cycle. It is found that a K^+ is important to work as a foothold of glycine on a nucleophilic attack reaction. And also, as for the comparison between the calculation systems between glycine oxidation and glycine oxidation added one H_2O around K^+ , it does not affect much on a reaction pathway whether one water coordinates with K^+ or not.

(B) Comparison of amino acid oxidation by PQQ in aqueous solution.

The comparison of amino acid (glycine, alanine or valine) oxidation reaction by PQQ in aqueous solution was investigated. Compared three OX, two points are found to affect antagonistically on a reaction, which prompt or does not prompt a nucleophilic attack. Glycine oxidation by PQQ was referred to the previous study.

Alanine and valine oxidation by PQQ were investigated. As for three different amino acid (glycine, alanine or valine) oxidation by PQQ, a nucleophilic attack on C5 by the nitrogen atom of amino acid is likely to occur compared to that on C4. These structures of product after the nucleophilic attack on C5 (INT), facilitate the following proton transfer. The proton transfer to O5A is most likely to occur compared to that to O4A. As for the comparison among three amino acids oxidations, the tendency of difference of energies among three amino acids oxidation is consistent with that of the experiment which is the reaction of PQQ with different amino acids. The effect of difference among three different amino acids oxidations by PQQ is considered to most depend on the difference of energies of nucleophilic attack on C5 by amino acid.

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Chapter 4.

Conclusion

Chapter 1.

Introduction

There are various organic functional molecules. I focus on an oxidation-reduction catalyst, especially for pyrroloquinoline quinone (PQQ) in my doctor thesis. PQQ is one of quinone compounds of biological system. It has not yet been settled whether PQQ is a vitamin or not. At this chapter, at first, I explained the detail of vitamins and vitamin analogous compounds. At second, I explained representative examples of quinone of biological system among vitamins and vitamin analogous compounds, which were vitamin K and ubiquinone. At last, I explained the detail of PQQ. PQQ has not yet been revealed much. The antioxidant function of PQQ is widely known and therefore gained considerable attention from many researchers. The understanding of its redox mechanism is a key to proceed further development.

The purpose of my doctor thesis is to understand the redox mechanism of PQQ. I investigated the mechanism of glycine oxidation catalyzed by PQQ in aqueous solution at the chapter 2. And then, I investigated the comparison of three amino acids (glycine, alanine and valine) oxidation catalyzed by PQQ in aqueous solution at the chapter 3. Moreover, the role of cation was investigated at the chapter 3.

Chapter 2.

Mechanism of glycine oxidation catalyzed by Pyrroloquinoline quinone in aqueous solution

As for the mechanism of glycine oxidation catalyzed by PQQ in aqueous

solution, two mechanisms were proposed, which were a stepwise reaction and a concerted reaction. A stepwise reaction includes two modes, which are a nucleophilic attack on C5 or C4 by glycine, and then proton and electron transfer from the glycine following a nucleophilic attack on C5. And while, a concerted reaction is that proton transfers and electron transfer occur simultaneously which does not include a nucleophilic attack.

In the stepwise reaction, it was found that the nucleophilic attack on C5 by the nitrogen atom of glycine is likely to occur compared to that on C4. After the nucleophilic attack, it was also found that the structure of the intermediate (INT(C5)) facilitates the following proton transfer. And also, it was found that the proton transfer of the H1' atom to O5A is most likely to occur compared to that corresponding to O4A and the electron transfer to the PQQ molecule also occurs. In the concerted reaction, it was found that the proton transfer of the H1' atom to O5A or O4A is difficult to occur.

Chapter 3.

Comparison of amino acid oxidation catalyzed by pyrroloquinoline quinone in aqueous solution

(A) Role of a cation

In the preparation for the comparison of three amino acids (glycine, alanine and valine) oxidation catalyzed by PQQ in aqueous solution, the role of K^+ was investigated on the three different calculation systems, glycine oxidation, glycine oxidation added one H_2O around K^+ , and glycine oxidation with no K^+ by PQQ in aqueous solution. Glycine oxidation by PQQ was referred to the chapter 2. In the three different calculation systems, it was found that a nucleophilic attack on C5 by the nitrogen atom

of glycine is likely to occur compared to that on C4. It was also found that these structures of product after the nucleophilic attack on C5 (INT), facilitate the following proton transfer. And also, it was found that the proton transfer to O5A is most likely to occur compared to that to O4A. These phenomena in a sequence of reactions are consistent with that corresponding to glycine oxidation by PQQ at the chapter 2. In this chapter, I realized the effect of three different calculations depends on the difference of energies of nucleophilic attack on C5 by glycine.

In the case of the comparison between the calculation systems between including K^+ (glycine oxidation or added one H_2O around K^+) and not including K^+ (no K^+), it was found that the tendency of difference of energies is consistent with the experiment that a cation located as mentioned at the chapter 3 is needed to keep the activity of redox cycle. It was also found that a K^+ is important to work as a foothold of glycine on a nucleophilic attack reaction. And while, in the case of the comparison between the calculation systems between glycine oxidation and glycine oxidation added one H_2O around K^+ , I found that it does not affect much on a reaction pathway whether one water coordinates with K^+ or not.

(B) Comparison of amino acid oxidation by PQQ in aqueous solution.

The comparison of amino acid (glycine, alanine or valine) oxidation reaction by PQQ in aqueous solution was investigated. Compared the structures of three OX, two points were found to affect antagonistically on a reaction, which prompt or does not prompt a nucleophilic attack. Glycine oxidation by PQQ was referred to the chapter 2. Alanine and valine oxidation by PQQ were investigated. As for three different amino acid (glycine, alanine or valine) oxidation by PQQ, it was found that a nucleophilic attack on C5 by the nitrogen atom of amino acid is likely to occur compared to that on

C4. It was also found that these structures of product after the nucleophilic attack on C5 (INT), facilitate the following proton transfer. And also, it was found that the proton transfer to O5A is most likely to occur compared to that to O4A. These phenomena in a sequence of reactions are consistent with that corresponding to glycine oxidation by PQQ at the chapter 2.

As for the comparison among three amino acids oxidations, I realized that the tendency of difference of energies among three amino acids oxidation is consistent with that of the experiment which is the reaction of PQQ with different amino acids. I also realized that the effect of difference of three different amino acids oxidations by PQQ is considered to most depend on the difference of energies of nucleophilic attack on C5 by amino acid.

Conclusion

I think that these results of my doctor thesis (chapter 2 and 3) are a basis for understanding the redox mechanism. In my doctor thesis, I focus on oxidation reaction and found valuable information. For further study of redox mechanism, I need to study about the reduction reaction in the future.

The list of my paper

The paper of No. 1 is described at the chapter 2 in my doctor thesis.

The paper of No. 2, 3 and 4 are not described in this doctor thesis. However, I studied these research subjects for mastering the quantum chemistry.

- 1. Mechanism of glycine oxidation catalyzed by pyrroloquinoline quinone in aqueous solution,**
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2. Photochromism of a Diarylethene Having an Azulene Ring,
JOURNAL OF ORGANIC CHEMISTRY, 77, 7, 3270-3276, 2012
3. Photochromism of 1,2-Bis(2-thienyl)perfluorocyclopentene Derivatives:
Substituent Effect on the Reactive Carbon Atoms,
JOURNAL OF PHYSICAL CHEMISTRY A, 116, 45, 10973-10979, 2012
4. A study of QM/Langevin-MD simulation for Oxygen-evolving center of photosystem II,
AIP Conf. Proc. 1568, 57, 2013

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