The Recognition of Glycolate Oxidase Apoprotein with Flavin Analogs in Higher Plants

Wei-Jun WANG*, Jing-Quan HUANG, Chong YANG, Jiu-Jiu HUANG, and Ming-Qi LI

College of Life Science, South China Agricultural University, Guangzhou 510642, China

Abstract   The dependence of glycolate oxidase apoprotein (apoGO) activity on flavin analogs was surveyed in 9 higher plants from 7 families. Activities of all apoGOs depended not only on flavin mononucleotide (FMN) but also on flavin adenine dinucleotide (FAD), but not on riboflavin. The kinetic analysis showed that FMN was the optimum cofactor for apoGO from leaves of *Brassica campestris*. In plant kingdom, FMN, FAD and riboflavin are three flavin analogs with very similar structure, and they could coexist and be inter-converted from each other, so the question is how the apoprotein of glycolate oxidase (GO) recognized these flavin analogs. No inhibition effect of riboflavin on the activity of apoGO with FMN or FAD was found and no obvious quenching of riboflavin or apoGO protein fluorescence was detected with the addition of apoGO or riboflavin, respectively. These results indicated that riboflavin did not bind to apoGO tightly like FMN and FAD. Inorganic phosphate (Pi) did inhibit the activity of GO, and kinetic analysis revealed that this inhibition was caused by the competitive binding to apoGO between Pi and FMN. This competitive binding was further confirmed by the inhibition of Pi to the quenching of FMN and apoGO protein fluorescence with apoGO and FMN, respectively. It was suggested that the 5′-phosphate group of FMN or FAD may play a key role in the recognition and binding of riboflavin analog cofactors with apoGO.

Key words   glycolate oxidase in higher plant; flavin analogs; 5′-phosphate moiety; recognition

The net photosynthetic efficiency in C_3 plants (such as rice, wheat and other major crops) can be decreased by 30% due to the metabolism of photorespiration [1], in which glycolate oxidase (GO) serves as a key enzyme. It is known that GO, with flavin mononucleotide (FMN) as a cofactor, belongs to flavin oxidase [2]. But it differs from other flavoproteins in that FMN is loosely bound to its apoprotein and there exists a dissociation balance between them, which indicates that FMN probably regulates the GO activity at the level of cofactor [3]. Riboflavin, FMN and flavin adenine dinucleotide (FAD), three flavin analogs with similar structures, can coexist and be inter-converted in higher biosomes [4,5]. It is unknown whether the catalytic activity of GO in plants can also depend on FAD or riboflavin and how this apoprotein identifies

them, which are, therefore, dealt with in this study.

Materials and Methods

Materials

The plant samples were collected from the greenhouse or the campus sample garden except *Spinacia oleracea* and *Brassica campestris* L. ssp. *chinensis* var. *utilis*, which were purchased from the market. Macro-Prep ceramic hydroxyapatite (type II) was from Bio-Rad, Hercules, USA. FMN, FAD, and riboflavin were from Sigma, St. Louis, USA. Sephadex G-25 was from Amersham Pharmacia Biotech, Uppsala, Sweden, and DEAE-Cellulose from Whatman, New Jersey, USA. Other chemicals were of analytical grade.

Preparation of apoprotein of glycolate oxidase (apoGO)

300 g of fresh leaves from each plant were homogenized

in a blender with 500 ml phosphate buffer (20 mM, pH 8.0), filtered with ninon and then centrifuged at 15,000 *g* for 10 min. The supernatant was collected and its pH was adjusted to 5.6 with 10% acetic acid (*V/V*). After 30 min, the precipitate was separated by centrifugation (15,000 *g*, 10 min), and the supernatant was subjected to 20%–45% ammonium sulfate saturation fractionation. The precipitate was dissolved in a small volume of 20 mM phosphate buffer (pH 8.0). This solution was centrifuged (15,000 *g*, 10 min) and desalted through Sephadex G-25 column (2.5 cm×35 cm) with 20 mM phosphate buffer (pH 8.0). Subsequently the desalted solution was centrifuged (15,000 *g*, 10 min) and collected, from which the apoGO was prepared through Macro-Prep ceramic hydroxyapatite (Type II) chromatography by the method of Wang *et al.* [3]. To prepare purified apoGO of *Brassica campestris* L. ssp. *chinensis* var. *utilis*, the GO was first purified from leaves of *Brassica campestris* following the method of Peng *et al.* [6], and then the purified apoGO was prepared using the method of Wang *et al.* [3].

Enzyme activity assay

The glycolate oxidase reaction was coupled with the peroxidase reaction and assayed at 30 °C by monitoring H_2O_2 production [7]. The assay conditions were 0.1 M pH 8.0 Tris-HCl containing 5 mM glycolate, 1 mM antipyrine, 5 U horseradish peroxidase, 2 mM phenol, and 0.1 mM FMN in a final volume of 3.0 ml. Assays were initiated by the addition of glycolate and the absorbance increase at 520 nm was then monitored for 2 min. One unit of glycolate oxidase activity was defined as the amount of enzyme required to produce 1 µmol H_2O_2 per min.

Kinetic analysis of the binding between apoGO and FMN (or FAD)

The binding between apoGO and FMN (or FAD) can be designated as follows:

$$[cofactor] + [apoGO] \Leftrightarrow [GO \cdot cofactor]$$

where [*cofactor*], [*apoGO*], and [*GO·cofactor*] represent the concentration of cofactor, apoGO and holoenzyme, respectively. Because [*cofactor*] is far higher than [*apoGO*] in the assay solution, it can be regarded as a constant during the assay. We then have:

$$K_d' = \frac{[cofactor] \times [apoGO]}{[GO \cdot cofactor]}$$

$$[apoGO] = [GO]_{total} - [GO \cdot cofactor]$$

$$V = k_{cat} \times [GO \cdot cofactor]$$

$$V_{max} = k_{cat} \times [GO]_{total}$$

$$K_d' = \frac{[cofactor] \times ([GO]_{total} - [GO \cdot cofactor])}{[GO \cdot cofactor]}$$

$$= \frac{k_{cat} \times [cofactor] \times ([GO]_{total} - [GO \cdot cofactor])}{k_{cat} \times [GO \cdot cofactor]}$$

$$= \frac{[cofactor] \times (V_{max} - V)}{V}$$

From the above calculation, Eqn (1) and Eqn (2) can be obtained:

$$V = \frac{V_{max} \times [cofactor]}{[cofactor] + K_d'} \tag{1}$$

$$\frac{1}{V} = \frac{K_d'}{V_{max}} \times \frac{1}{[cofactor]} + \frac{1}{V_{max}} \tag{2}$$

Eqn (1) has a form similar to Michaelis-Menten equation. Eqn (2) is obtained from (1) and the apparent dissociation constant between apoGO and cofactor (K_d') can be determined by plotting $1/V$ vs. $1/[cofactor]$. The inhibition type of Pi on enzyme activity can also be determined by this plotting.

Fluorescence spectrometry

Fluorescence measurements were carried out with a Hitachi FL 4500 spectrofluorimeter. Flavin analog fluorescence emission was scanned from 460 nm to 660 nm (excitation at 460 nm) by the method of Mayhew and Wassink [8] and protein fluorescence emission was scanned from 290 nm to 450 nm (excitation at 280 nm) following the method of Tang *et al.* [9].

Results

Cofactor dependence of apoGO activity on various flavin analogs from several plant species

The dependence of apoGO activity on flavin analogs was surveyed in 9 plants from 7 families. It was shown that the activities of apoGOs from all samples depended on FMN, consistent with previous report [2]. However, it was further discovered that the activities of apoGOs could also depend on FAD but not on riboflavin. The activities of GOs with FAD were about 30%–70% of those with FMN from different plants (Table 1).

Specificity of cofactor dependence of apoGO activity

The purified apoGO was prepared from *Brassica*

Table 1   The cofactor dependence of apoGO activity on flavin analogs for several species plants

Plant species	Relative activity of apoGO with various flavin analogs (%)		
	FMN	FAD	Riboflavin
Gramineae			
Triticum aestivum	100	47.4	1.2
Oryza sativa L.	100	29.0	2.0
Zea	100	51.7	1.3
Olanaceae			
Nicotiana tabaccum	100	53.6	1.7
Cruciferae			
Brassica campestris	100	46.8	0.9
Cucurbitaceae			
Cucumis sativus	100	67.0	0.0
Chenopodiaceae			
Spinacia oleracea	100	71.1	1.1
Leguminosae			
Vigna unguiculatq	100	36.4	1.0
Caricaceae			
Carica papaya	100	45.5	0.0

campestris leaves, and its apparent dissociation constants with FMN and FAD were determined by the method of double-reciprocal plotting mentioned in "Kinetic analysis of the binding between apoGO and FMN (or FAD) ". The values were $K_{d}'_{(FMN)}=6.14\times10^{-7}$ M and $K_{d}'_{(FAD)}=2.93\times10^{-6}$ M (Fig. 1), indicating that apoGO binds more tightly to FMN than to FAD.

From Fig. 1, two other parameters in relation to co-factor-dependent specificity of enzyme activity on FMN or FAD were further determined for apoGO

from *Brassica campestris* leaves (Table 2). According

Table 2   The cofactor-dependent specificity of apoGO from Brassica campestris leaves

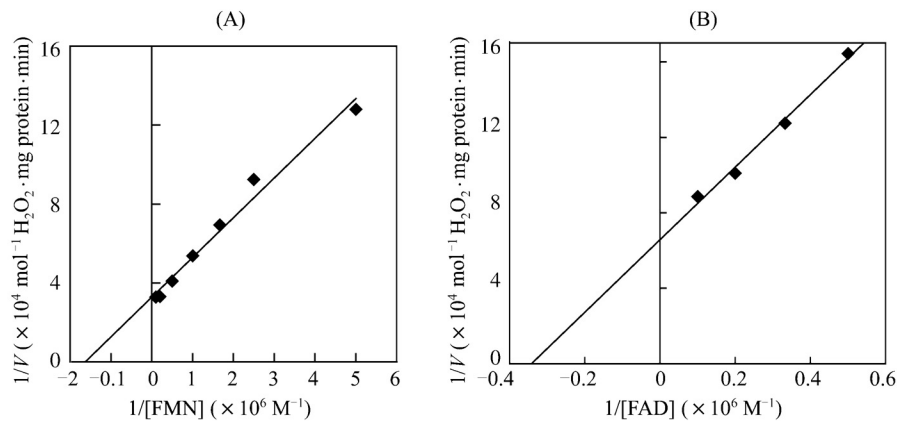| Cofactor	V_{max} (×10^{-5} mol H$_2$O$_2$ ·mg^{-1} protein·min^{-1})	K_{d}' (×10^{-7} M)	V_{max}/K_{d}'
FMN	3.05	6.14	49.60
FAD	1.52	29.30	5.19

Fig. 1   Kinetic plotting for determining the apparent dissociation constant between apoprotein of GO and FMN or FAD
(A) FMN vs. apoGO. (B) FAD vs. apoGO.

to the method used to evaluate the substrate specificity of enzyme activity [10], FMN could be judged as the optimum cofactor for GO activity based on the ratio of V_{max} to K_d'.

Influence of riboflavin on the binding between apoGO and FMN (or FAD)

The influences of riboflavin on the binding of apoGO with FMN or FAD were investigated by measuring the enzyme activity with riboflavin in assay solution. No inhibition by riboflavin was observed when riboflavin was added to assay solution even in concentration higher than that of FMN (or FAD) (Table 3), indicating that riboflavin could not bind to apoGO though its structure is similar to FMN and FAD.

Fluorescence analysis of interactions between apoGO and flavin analogs

The fluorescences of the solutions of FMN, FAD and riboflavin were detected after adding apoGO. It was shown that apoGO could obviously quench FMN fluorescence [Fig. 2(A)], but intensify FAD fluorescence [Fig. 2(B)]. This enhancement of FAD fluorescence might be due to its adenine group. The same phenomenon was also observed in FAD bound to apo-electron-transferring flavo-protein [11]. The above changes of flavin fluorescence implied the binding between apoGO and FMN (or FAD). However, the riboflavin fluorescence showed no apparent change after adding apoGO [Fig. 2(C)], indicating that no obvious binding occurred between apoGO and riboflavin. Furthermore, the protein fluorescence of apoGO was also measured to investigate the interaction between apoGO and flavin analogs. An obvious quenching of fluorescence of apoGO was observed when FMN or FAD was added to apoGO, while no evident quenching by riboflavin was observed. It was further confirmed that riboflavin did not bind to apoGO tightly but FMN and FAD did. FMN differs from riboflavin only in the 5′-phosphate

group, which is absent in riboflavin, implying that the 5′-phosphate group might play an important role in the binding between apoGO and cofactor.

The inhibitory effect of Pi on the activity of apoGO

When inorganic phosphate (pH 8.0, sodium phosphate and potassium phosphate) with different concentrations was added in the assay solution, obvious inhibition on GO activity depending on FMN was detected, while no inhibition by other inorganic salts such as NaCl and KCl was observed (Fig. 3), indicating that the inhibition by Pi was specific.

To understand the mechanism of such inhibition, the inhibition type of Pi was further investigated by the method of double-reciprocal plotting. It was shown that Pi was a noncompetitive inhibitor of GO activity versus substrate of glycolate [Fig. 4(A)] and was a typical competitive inhibitor versus FMN [Fig. 4(B)], indicating that Pi did compete with FMN for the cofactor-binding site on apoGO.

Influence of Pi on the fluorescence quenching of apoGO and FMN

Through fluorescence analysis, inhibitions of Pi on the quenching of FMN and apoGO protein fluorescence by apoGO and FMN, respectively, were observed. Both inhibitions were enhanced gradually with increasing Pi concentration in assay solution [Fig. 5(A,B)]. All these results further verified the competition of Pi with FMN for the cofactor-binding site on apoGO.

Discussion

Dependence of apoGO activity on flavin analog cofactors in higher plant

It was well known that the activity of GO was depen-

Table 3   The effect of riboflavin in assay solution to the activity of apoGO with FMN or FAD as cofactor

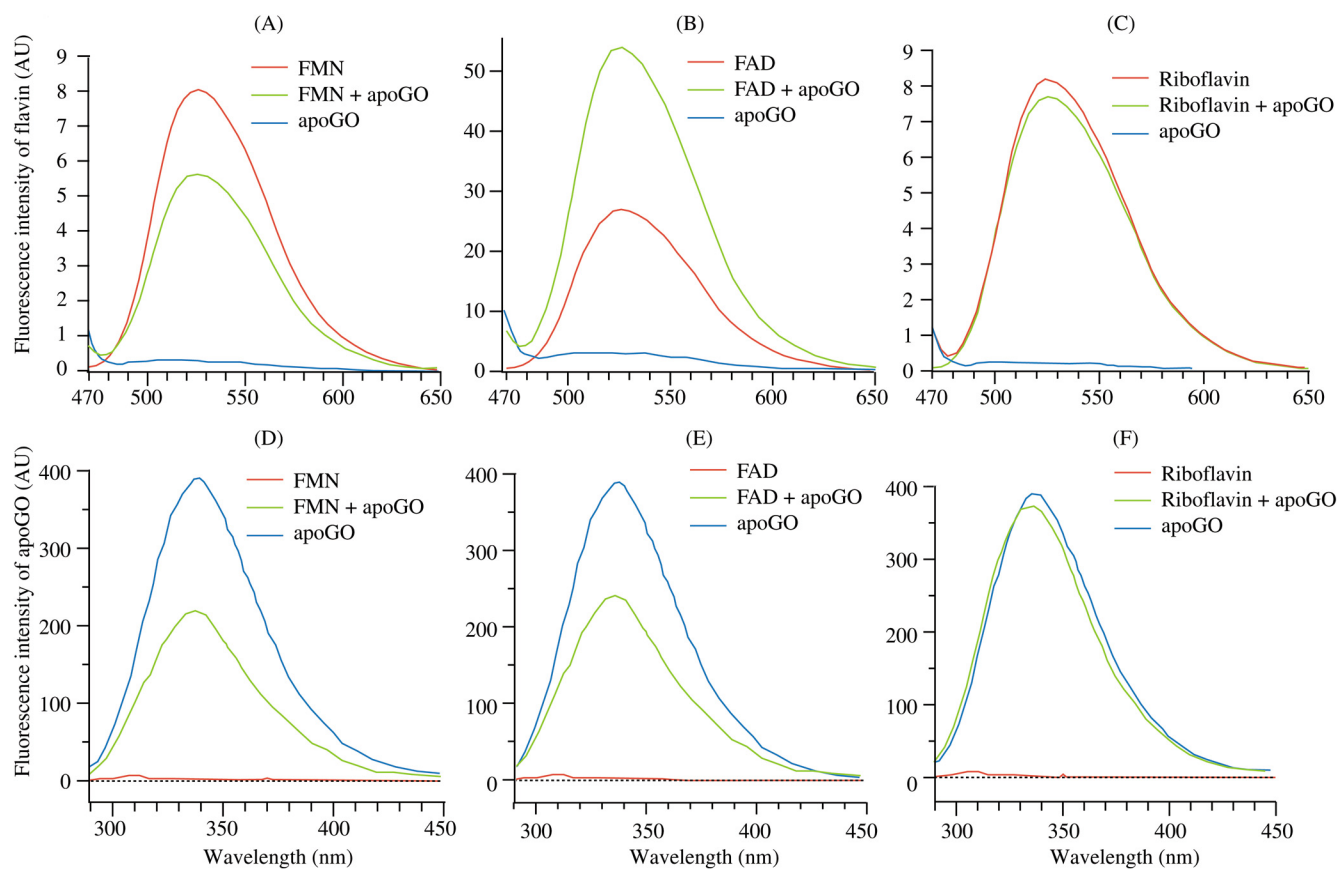| Cofactor	Cofactor concentration ($\times 10^{-5}$ M)	Relative activity (%)		
		The concentration of riboflavin in assay solution ($\times 10^{-5}$ M)		
		0	10	20
FMN	1.0	100	109.6	107.1
	5.0	100	101.2	102.3
FAD	1.0	100	112.5	102.8
	5.0	100	104.7	95.6

Fig. 2   **Effects of the bindings between apoGO and flavin analogs on the fluorescence of flavin and apoGO**

(A–C) Flavin fluorescence emissions were measured using excitation at 460 nm. (D–F) Protein fluorescence emissions were measured using excitation at 280 nm . The protein concentration of apoGO in all assays was 91 μg/ml and the concentration of FMN and FAD were 1.5×10^{-6} M, and that of riboflavin was 1.0×10^{-6} M. All measurements were carried out in 5.0×10^{-2} M Tris-HCl buffer (pH 8.0) at 25 °C.
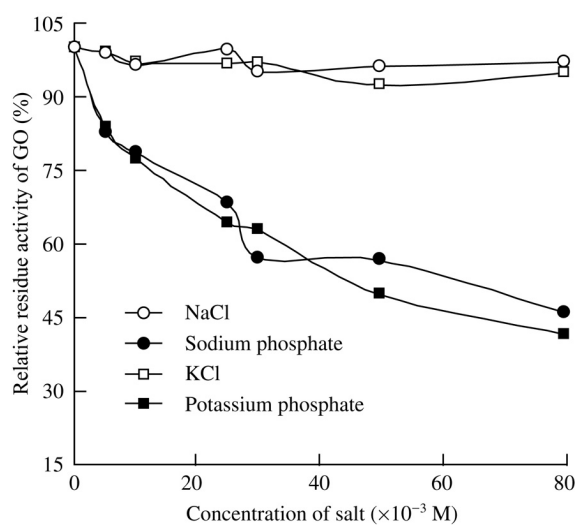

Fig. 3   **Inhibitory effects of inorganic phosphate on GO activity**

The concentration of FMN in assay solution was 1×10^{-4} M and that of apoGO protein was 14 μg/ml.

dent on FMN [2], but it was not clear whether the catalytic activity of GO in plants also depends on FAD or riboflavin. Through the survey of 9 higher plants from 7 families in this research it was further discovered that the activities of apoGO from all these samples also depend on FAD but not on riboflavin. The activities of GOs from different plants with FAD were lower than those with FMN (Table 1), furthermore FMN was judged as the optimum cofactor for activity of GO from *Brassica campestris* leaves.

The binding between apoGO and flavin analogs

FMN, FAD and riboflavin are all similar in structures with an isoalloxazine ring, which is responsible for electron transfer in redox reaction. But it is very interesting that the GO activity does not depend on riboflavin but on FMN or FAD. In the enzyme activity inhibition analysis (Table 3) and fluorescence quenching experiment (Fig. 2), it was shown that riboflavin did not bind to apoGO tightly
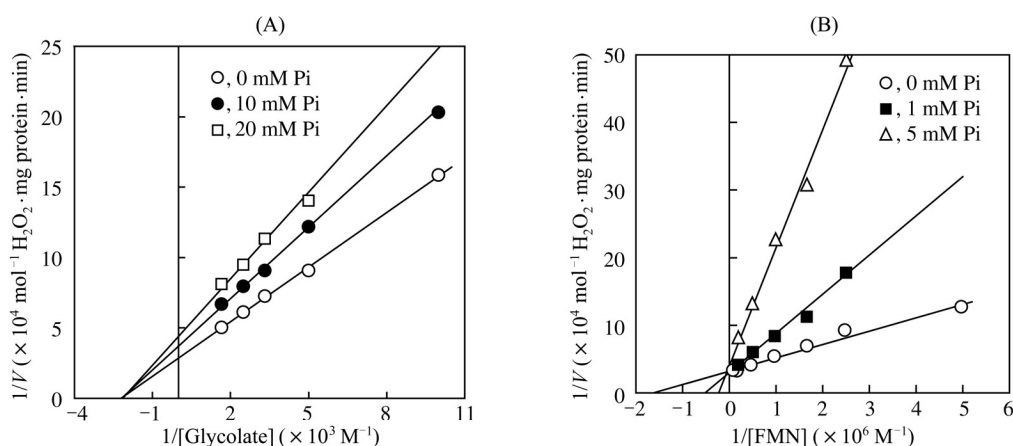
Fig. 4   Inhibitions on GO activity by Pi with different concentrations

Pi, sodium phosphate (pH 8.0). (A) The inhibition of Pi on GO activity versus substrate of glycolate. Concentration of FMN in assay was 5×10^{-5} M. (B) The inhibition of Pi on GO activity versus FMN. Concentration of glycolate in assay was 5 mM.
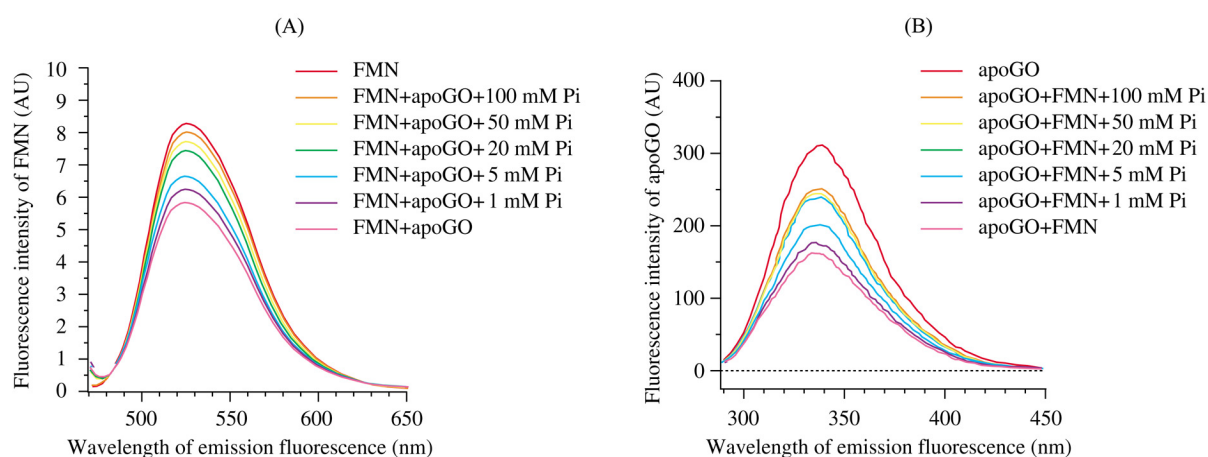

Fig. 5   The inhibition of Pi to the quenching of FMN and apoGO protein fluorescence

The concentration of apoGO in all assays was 91 mg/ml, and that of FMN was 1.5×10^{-6} M. The assay solution was 50 mM Tris-HCl buffer (pH 8.0). Pi, sodium phosphate (pH 8.0). (A) The inhibitions of Pi on the quenching of FMN fluorescence caused by apoGO. Pi inhibited the quenching effect of apoGO on FMN fluorescence in a dose dependent manner. (B) The inhibitions of Pi on the quenching of apoGO protein fluorescence caused by FMN. Pi inhibited the quenching effect of FMN on apoGO fluorescence in a dose dependent manner.

while FMN and FAD did, so riboflavin could not act as a cofactor of GO. FMN differs from riboflavin only in the 5′-OH which is replaced by Pi. It was thus presumed that 5′-phosphate in FMN and FAD might play a key role in the recognition between apoGO and its cofactors.

The key role of 5′-phosphate moiety of FMN and FAD in recognition between apoGO and cofactor

The three flavin analogs, FMN, FAD and riboflavin, can coexist and be converted into each other in plants. It was discovered in this study that Pi did inhibit the activity

of GO (Fig. 3) by competing with FMN for the same binding site on apoGO (Fig. 4). This inhibition was further confirmed by the inhibition of Pi on the quenching of FMN and apoGO protein fluorescence by apoGO and FMN respectively (Fig. 5), indicating that the 5′-phosphate moiety of FMN or FAD played an important role in the recognition and binding between apoGO and its cofactors. The crystal structure of spinach GO by X-ray showed that FMN was bound to the barrel structure at the carboxyl end of β-strands and there existed 22 polar contacts with a distance less than 3.3 Å between apoGO protein and FMN,

among them eight existed on the isoalloxazine ring of FMN, six existed on the ribitol chain and the remaining eight on the 5′-phosphate group [12]. Together with results from this study, we proposed that the eight polar contacts on 5′-phosphate should be most crucial in the recognition between apoGO and FMN or FAD. The important contribution of phosphate moiety in the binding between FMN and *Desulfovibrio vulgaris* flavodoxin was also observed by Murray *et al.* [13,14]. Does 5′-phosphate moiety initially bind to phosphate-binding site on apoGO surface and trigger the enzyme conformational change followed by the isoalloxazine ring insertion? Studies by Sandalova and Lindqvist [15] showed that the crystal structure of apoGO protein was slightly looser than that of holoenzyme. Details of FMN binding to apoGO need further investigations.

Acknowledgements

References

1   Douce R, Neuburgen M. Biochemical dissection of photorespiration. Curr Opin Plant Biol, 1999, 2(3): 214–222

2   Reumann S. The photorespiration pathway of leaf peroxisomes. In: Baker A, Graham IA eds. *Plant Peroxisomes—Biochemistry, Cell Biology and Biotechnological Applications.* Dordrecht, Netherlands: Kluwer Academic Publishers, 2002, 141–190

3   Wang WJ, Lin JQ, Li MQ. Binding between flavin mononucleotide and glycolate oxidase from higher plants. Acta Botanica Sinica, 2000, 42(3): 239–243

4   Jordan DB, Bacot KO, Carlson TJ, Kessel M, Viitanen PV. Plant riboflavin biosynthesis. Cloning, chloroplast localization, expression, purification, and partial characterization of spinach lumazine synthase. J Biol Chem, 1999, 274(31): 22114–22121

5   McCormick DB. A trail of research on cofactors: An odyssey with friends. J Nutr, 2000, 130(2): 323S–330S

6   Peng XX, Li MQ. Purification and crystallization of glycolate oxidase and effect of modification of tyrosine residue on its activity. Acta Phytophisiologica Sinica, 1989, 15(3): 257–262

7   Hall NP, Reggiani R, Pea PJ. Molecular weight of glycolate oxidase from C3 and C4 plants determined during early stages of purification. *Phytochem*, 1985, 24(8): 1645–1648

8   Mayhew SG, Wassink JH. Determination of FMN and FAD by fluorescence titration with apoflavodoxin. Methods Enzymol, 1980, 66: 217–220

9   Tang CK, Jeffers CE, Nichols JC, Tu SC. Flavin specificity and subunit interaction of *Vibrio fischeri* general NAD(P)H-flavin oxidoreductase FRG/FRase I. Arch Biochem Biophys, 2001, 392(1): 110–116

10   Dixon M, Webb EC, Thorne CJ, Tipton KF. Enzyme specificity. In *Enzyme*, 3rd ed. London: Longman Group Ltd., 1979, 231–270

11   Sato K, Nishina Y, Shiga K, Tojo H, Tada M. The existence of two different forms of apo-electron-transferring flavoprotein. J Biochem, 1991, 109(5): 734–740

12   Lindqvist Y. Structure and mechanism of spinach glycolate oxidase. In Müller F ed, *Chemistry and Biochemistry of Flavoenzymes*, Vol III. Boca Raton, Ann Arbor, London: CRC Press Inc., 1992, 367–387

13   Murray TA, Swenson RP. Mechanism of flavin mononucleotide cofactor binding to the *Desulfovibrio vulgaris* flavodoxin. 1. Kinetic evidence for cooperative effects associated with the binding of inorganic phosphate and the 5′-phosphate moiety of the cofactor. Biochemistry, 2003, 42(8): 2307–2316

14   Murray TA, Foster MP, Swenson RP. Mechanism of flavin mononucleotide cofactor binding to the *Desulfovibrio vulgaris* flavodoxin. 2. Evidence for cooperative conformational changes involving tryptophan 60 in the interaction between the phosphate- and ring-binding subsites. Biochemistry, 2003, 42(8): 2317–2327

15   Sandalova T, Lindqvist Y. Crystal structure of apo-glycolate oxidase. FEBS Lett, 1993, 327(3): 361–365

Edited by

You-Shang ZHANG