Short Communication

An Enzyme Catalyzing Liberation of Pyrroloquinoline Quinone (PQQ) from PQQ-Adducts

Osao ADACHI, Kazuyuki OKAMOTO,
Kazunobu MATSUSHITA, Emiko SHINAGAWA
and Minoru AMEYAMA

Laboratory of Applied Microbiology,
Department of Agricultural Chemistry,
Faculty of Agriculture,
Yamaguchi University,
Yamaguchi 753 Japan

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Pyrroloquinoline quinone (PQQ) was liberated enzymatically from PQQ-adducts such as PQQ-2,4-dinitrophenylhydrazine (PQQ-DNP) or PQQ-serine, when incubated with a cell-free extract from *Pseudomonas* sp. VM15C, an auxotrophic strain for PQQ occurring symbiotically in polyvinyl alcohol degradation.^{1,2)} Since the strain cannot grow singly on polyvinyl alcohol in the absence of exogenous PQQ or a PQQ-donor, the growth extent is strictly dependent on PQQ fed in the culture medium.³⁾ It can be deduced that, if *Pseudomonas* VM15C could use only the free form of PQQ to grow on polyvinyl alcohol, the organism should have an enzyme generating PQQ from PQQ-adducts when grown on the growth substrate in the presence of PQQ-adducts. This study started to see whether the organism can grow under such conditions where some naturally occurring material, in which the presence of PQQ-adducts has been indicated,⁴⁾ is provided instead of authentic PQQ.

When the organism was cultured on several media including a medium of polyvinyl alcohol and yeast extract instead of PQQ, growth occurred as shown in Fig. 1. These observations suggest the possibility of occurrence of an enzyme that can catalyze the liberation of PQQ from PQQ-adducts. PQQ-DNP was reacted

with a cell-free extract prepared from cells grown as above, and the reaction mixture was then assayed enzymatically for PQQ using a quinoprotein apo-glucose dehydrogenase (GDH).⁵⁾ Appearance of GDH activity indicates formation of holo-GDH using PQQ generated from the PQQ-adducts, because no PQQ-adducts have any appreciable coenzyme activity. The enzyme activity obtained with PQQ-DNP is shown in Table I. It is interesting to see that the enzyme occurs in the cell-free extract from the cells grown on polypeptone and yeast extract excluding polyvinyl alcohol (medium A). In addition to PQQ-serine and PQQ-glycine, the organism also used PQQ-DNP, an artificial PQQ-adduct, and grew on polyvinyl alcohol as the sole carbon source

Table I. EFFECTS OF CULTURE MEDIA ON THE FORMATION OF PQQ-LIBERATING ENZYME

Pseudomonas sp. VM15C was grown on 100 ml of the media A through D, which were the same as used in Fig. 1, until the growth reached the late exponential phase. After the cells were harvested and washed with distilled water, they were suspended in 5 mM potassium phosphate, pH 7.0, containing 5 mM MgCl₂. Both DNase and RNase were included at 10 µg per ml. After two passages of the cell suspension through a French pressure cell press at 1,000 kg/cm², the cell homogenate was spun down at 5,000 × g for 30 min at 4°C to eliminate intact cells. Membrane fractions were further removed by centrifugation at 125,000 × g for 60 min and the supernatant was used as a cell-free extract to see the liberation of PQQ from PQQ-adducts. The reaction mixture composed of 0.2 ml of PQQ-adduct (500 nM PQQ-DNP), 0.2 ml of 50 mM glycine-NaOH, pH 8.5, and 0.1 ml of cell-free extract in a total volume of 0.5 ml. Incubation was done at 25°C for 30 min. Then, a sample (0.2 ml) of the reaction mixture was incubated with *E. coli* membrane under the standard assay conditions for PQQ quantification,⁵⁾ where one unit of enzyme activity was defined as the enzyme catalyzing 1 µmol of PQQ liberation in 30 min. Protein was measured as described by Lowry *et al.*⁷⁾ with bovine serum albumin as a standard.

Medium	Total activity (munits)	Specific activity (µunits/mg)
A	2.48	36.6
B	0.61	13.2
C	1.22	51.9
D	—*	—

* No growth.

Fig. 1. Growth of *Pseudomonas* sp. VM15C on Various Culture Media.

The organism was grown on 100 ml of various media in a 500-ml shaking flask to which a Klett tube was attached and the growth was measured periodically by reading the turbidity with a Klett-Summerson photoelectric colorimeter as mentioned previously.⁶⁾ The basic composition of culture media per liter were: A, 5 g polypeptone, 0.1 g yeast extract, minerals^{1,2)} including N, P, K, Mg, Ca, and so forth, and vitamins, pH 7.5; B, 5 g polyvinyl alcohol, 0.1 g yeast extract, minerals, pH 7.5; C, 5 g polyvinyl alcohol, minerals, vitamins, and 30 μ g PQQ, pH 7.5; D, 5 g polyvinyl alcohol, minerals, vitamins, and no PQQ, pH 7.5. The composition of the medium C was essentially the same as mentioned in our previous paper.³⁾ PQQ used was prepared as described in our previous paper.⁸⁾

(data not shown). The enzyme activity looks small. However, such enzyme activity may be reasonable, because PQQ exerts its biochemical activity even at the picomolar level in nature. Some discrepancy in that the enzyme activity (Table I) is not always proportional to the extent of growth (Fig. 1) may be because the data presented here were obtained with PQQ-DNP. Unlike the other substrates, the PQQ-DNP used was not contaminated with a trace of free PQQ which might disturb the assay. There also might be differences in substrate specificity among the enzymes prepared.

The extent of holo-GDH formation, which

is nearly equal to the amount of PQQ liberated, was proportional to the reaction period and enzyme content. PQQ-liberating reaction was largely dependent on pH, with pH optima at 6.5 to 10.0, and no holo-enzyme formation occurred in the absence of the action of the cell-free extract. The ability to liberate PQQ disappeared upon heating the cell-free extract at 70°C for 10 min.

When the enzyme was reacted with an isolated chromophore fraction from diamine oxidase of pig kidney, holo-GDH formation was largely promoted. More than 0.3 mol of PQQ was recovered per mole of diamine oxidase after 30 min incubation of the chromophore with the enzyme, while less than 0.1 mol of PQQ was detected with the isolated chromophore. This means that diamine oxidase contains a covalently bound PQQ and the prosthetic group became PQQ-adducts during isolation as has been indicated.⁴⁾ Identification of PQQ in diamine oxidase was briefly mentioned in our previous paper⁸⁾ and the results have been reconfirmed in this study using PQQ-liberating enzyme. Identification or measurement of PQQ can be done readily with the quinoproteins in which PQQ is involved noncovalently. On the other hand, there has been chaos among the quinoproteins that contain covalently bound PQQ, because of the lack of an absolute method. Therefore, the PQQ-liberating enzyme could be available to see whether an enzyme contains a covalently bound PQQ in its prosthetic group.

The PQQ-liberating enzyme also occurs in a PQQ deficient strain of *Acinetobacter calcoaceticus* AC3 as has been preliminarily reported.⁹⁾ Further studies on complete enzyme purification and examination in detail of enzymatic properties and reaction mechanisms are now under way.

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References

- 1) C. Sakazawa, M. Shima, Y. Taniguchi and N. Kato, *Appl. Environ. Microbiol.*, **41**, 261 (1981).
 - 2) M. Shima, H. Saimoto, N. Kato and C. Sakazawa, *Appl. Environ. Microbiol.*, **46**, 605 (1983).
 - 3) M. Shima, H. Yamamoto, K. Ninomiya, N. Kato, O. Adachi, M. Ameyama and C. Sakazawa, *Agric. Biol. Chem.*, **48**, 2873 (1984).
 - 4) M. Ameyama, "PQQ and Quinoproteins," ed. by J. A. Jongejans and J. A. Duine, Kluwer Academic Publishers, (1989), pp. 149-157.
 - 5) M. Ameyama, M. Nonobe, E. Shinagawa, K. Matsushita and O. Adachi, *Anal. Biochem.*, **151**, 263 (1985).
 - 6) M. Ameyama, E. Shinagawa, K. Matsushita and O. Adachi, *Agric. Biol. Chem.*, **48**, 3099 (1984).
 - 7) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
 - 8) M. Ameyama, M. Hayashi, K. Matsushita, E. Shinagawa and O. Adachi, *Agric. Biol. Chem.*, **48**, 561 (1984).
 - 9) O. Adachi, K. Okamoto, K. Matsushita, E. Shinagawa and M. Ameyama, *Proc. Annual Meeting Jpn. Soc. Biosci. Biotech.*, **64**, 401 (1990).
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