Rapid Paper

Microbial Production of Pyrrolo-quinoline Quinone

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The possibility of preparing pyrroloquinoline quinone (PQQ), a novel coenzyme, by a microbial method was proposed for the first time. PQQ was formed and accumulated in the culture medium of various microorganisms. Of the tested strains, some methylotrophs were most favorable for PQQ production and more than 10 $\mu\text{g/ml}$ of PQQ was found in the culture medium after 2 days incubation. An attempt at the isolation and purification of PQQ was made successfully and spectral evidence was obtained suggesting that the purified PQQ was identical with authentic PQQ. The spectral similarity of PQQ to the chromophores isolated from methylamine dehydrogenase and copper containing amine oxidases was also indicated.

In addition to the well-known pyridine nucleotide-dependent and flavin nucleotide-dependent dehydrogenases, it has become clear that there is a third class of dehydrogenases in which PQQ is involved as the coenzyme.¹⁾ Such a novel coenzyme was originally discovered and identified in methanol dehydrogenases of methylotrophic bacteria. Since the structural determination of PQQ by Salisbury *et al.*²⁾ who gave it the trivial name of methoxatin, there has been a rush of reports on PQQ. In addition to methanol dehydrogenases from various methanol-grown microorganisms,¹⁾ alcohol,³⁾ aldehyde³⁾ and glucose^{3,4)} dehydrogenases of acetic acid bacteria, and glucose dehydrogenases of *Acinetobacter calcoaceticus*⁵⁾ and *Pseudomonas fluorescens*³⁾ have been revealed to be PQQ-dehydrogenases. Methylamine dehydrogenase of *Pseudomonas* AM1 has also been shown to be a PQQ-enzyme in which PQQ is attached to the

enzyme protein *via* a covalent bond.⁶⁾ An enzymatic method for PQQ determination has also been established³⁾ and applied to routine assaying. In the meantime, several papers on organic synthesis of PQQ have appeared,^{7~10)} although no report is available on microbial production of PQQ. In our preliminary report,¹¹⁾ we indicated that acetic acid bacteria and some methylotrophs accumulate PQQ in the culture medium. Indeed, a detectable amount of PQQ is found in table vinegars. Microbial production of PQQ has further progressed with the use of some selected methylotrophs. In this paper, we should like to report on microbial production of PQQ and some spectral properties of the purified PQQ. At the same time, we should like to indicate the spectral similarity of PQQ to the chromophores isolated from methylamine dehydrogenase and copper containing amine oxidases from beef plasma, pig kidney and *Aspergillus niger*.

MATERIALS AND METHODS

Microorganisms. An obligate methylotroph, strain M5, was kindly supplied by Dr. N. Kato, Tottori University. *Pseudomonas* AM1 was a kind gift from Dr. S. Nagai, Hiroshima University. Some methylotrophic isolates obtained by the enrichment technique on methanol were also used. All methylotrophic bacteria were cultured on mineral salt-methanol (1%) medium aerobically at 30°C.

Chromatography of PQQ. Culture medium was separated from cells by centrifugation and applied to a DEAE-Sephadex A-25 column, which has been equilibrated with 2 mM potassium phosphate, pH 7.0. Elution of PQQ was done with a linear concentration gradient of KCl to 1.0 M. Elution was monitored by the optical density at 249 nm, fluorometry with excitation at 365 nm and emission at 465 nm, and measurement of PQQ content done by enzymatic analysis.

Enzymatic determination of PQQ. The PQQ contents of the culture medium and chromatographed samples were determined under essentially the same conditions as reported previously.³⁾ To a mutant membrane suspension of *P. aeruginosa*, which is unable to form PQQ but produces a normal level of apo-glucose dehydrogenase in its membrane, samples containing PQQ or authentic PQQ were added to convert the apo-enzyme to the holo-enzyme. Then the glucose dehydrogenase activity was assayed as

described previously.¹²⁾

Spectral measurements. Absorption spectra of PQQ were taken with a Hitachi model 200-10 spectrophotometer at 25°C. Fluorescence spectra were taken with a Hitachi model 650-10S fluorescence spectrophotometer at 25°C with excitation at 365 nm and emission at 465 nm.

Isolation of chromophores from methylamine dehydrogenase and copper containing amine oxidases. Methylamine dehydrogenase was purified from *Pseudomonas* AM1 according to the method described by Eady and Large,¹³⁾ and a highly purified enzyme preparation (about 3 units/mg) was used. Separation of the light subunit of the enzyme was performed as described by Shirai *et al.*¹⁴⁾ and about 40 mg of the light subunit was isolated. Beef plasma amine oxidase (700 mg, about 500 units/mg) was kindly prepared by Dr. K. Isobe, Amano Pharmaceutical Inc., according to the method by Yamada and Yasunobu.¹⁵⁾ Pig kidney diamine oxidase was purified according to the method by Yamada *et al.*¹⁶⁾ and the finally purified enzyme preparation (30 mg) showed about 1 unit/mg of specific activity. Fungal amine oxidase was prepared from *Aspergillus niger* as described previously¹⁷⁾ and a highly purified preparation (2 g, 9,500 units/mg) was obtained. These enzyme preparations in 2 mM potassium phosphate, pH 7.0, were heated in a boiling bath for 10 min. Pancreatic trypsin (P-L Biochemicals Inc.) and pronase (Kakenkagaku Inc.) were added to the cooled enzyme solution to a concentration of 2% of the heated proteins. After proteolytic digestion overnight at 37°C, the individual hydrolysates were lyophilized and passed through a Sephadex G-25 column (1 × 110 cm) in 2 mM potassium phosphate, pH 7.0. All samples gave two yellow peaks. They were further hydrolyzed in 6 N HCl under N₂ gas in sealed tubes at 110°C for 48 hr. Each sample was evaporated *in vacuo* and neutralized with alkali and then applied to a small column of DEAE-Sephadex A-25, which had been equilibrated with 2 mM potassium phosphate, pH 7.0. Elution of the chromophores was performed stepwise with 0.05 N HCl. Two yellow peaks were eluted separately as sharp peaks when monitored by optical density at 230 nm and 330 nm.

RESULTS AND DISCUSSION

Formation of PQQ by microorganisms

Productivity of PQQ was examined with a variety of microbial strains. Obligate methylotroph M5 and some isolated methylotrophs, which have not been characterized taxonomically, were found to be the most favorable of those so far examined and the PQQ content in the culture medium reached more than 10 µg/ml on 2 days incubation. This corre-

FIG. 1. Chromatographic Purification of PQQ on DEAE-Sephadex A-25.

Culture broth of strain M5 was separated from cells after 50 hr cultivation. An aliquot of the supernatant was applied to a DEAE-Sephadex column (1 × 5 cm) and washed thoroughly with 2 mM potassium phosphate, pH 7.0. Elution of PQQ was performed with a linear concentration gradient of KCl as indicated. Elution of PQQ was monitored by optical density at 249 nm (—●—), fluorescence intensity (—○—), and glucose dehydrogenase activity (----).

sponds to 1000 times as high as those reported in our preliminary report.¹¹⁾ A typical purification of PQQ on a DEAE-Sephadex A-25 column is shown in Fig. 1. PQQ was eluted as a pink colored band from the column at about 1 M KCl. When the elution was monitored by optical density, fluorescence intensity and enzyme activity, the three criteria completely coincided around the peak of PQQ. Purified PQQ was found to be homogeneous on HPLC on HW-40S (Toyo Pearl) and eluted as a sharp single peak as the authentic PQQ did.

Spectral properties of purified PQQ

The purified PQQ was subjected to some spectral examinations as shown in Fig. 2. Absorption spectra of PQQ at different pHs were identical with those of that isolated from

FIG. 2. Absorption Spectra (A) and Fluorescence Spectra (B) of the Purified PQQ at Different pHs.

The purified PQQ solution ($68 \mu\text{M}$) was adjusted to pH 7.0 with dilute sodium hydroxide (—). A tiny drop of concentrated HCl was added to the purified PQQ solution giving pH 1.0 (----). The purified PQQ solution was adjusted to pH 12.0 by the addition of concentrated sodium hydroxide (- - - -).

methanol dehydrogenase.¹⁸⁾ The concentrated PQQ solution had a pink color and it showed absorption maxima at 249 and 330 nm with a shoulder at 270 nm at neutral pH. A low and broad peak was also observed from 400 to 600 nm which is responsible for the pink color (Fig. 2-A). Of fluorescence spectra, excitation spectra at neutral pH showed three peaks at 260, 330 and 380 nm with emission at 465 nm. An emission peak at 485 nm was observed on excitation at 365 nm. The emission peak shifted to about 460 nm at acidic pH (Fig. 2-B). These spectral properties were identical with those reported¹⁾ and consistent with those of the authentic PQQ prepared by organic synthesis. Thus, the microbial production of PQQ was briefly mentioned and its identity with the authentic one was indicated. On request, we are ready to give our PQQ, even though only in small amounts, to investigators all over the world in the hope of further development in PQQ work.

A spectral study of PQQ led us to believe

that the copper containing amine oxidases might be PQQ-enzymes in which PQQ is attached to the enzyme protein covalently. The isolated and purified chromophores of amine oxidases from three sources were very similar to that from methylamine dehydrogenase, which has already been proved to contain a covalently bound PQQ (Fig. 3). It should also be recalled that such species of chromophores were isolated in the previous study¹⁷⁾ and at that time, they were tentatively concluded to be pyridoxal derivatives because no one knew of the novel coenzyme, PQQ. In Fig. 3, one of the two chromophors eluted first from the DEAE-Sephadex A-25 column with the individual enzyme hydrolysates is shown. An alternative chromophore (data not shown) also showed a similar fluorescence spectra having an excitation peak at 380 nm and an emission peak at 460 nm in 0.05 N HCl. Furthermore, these chromophores partially supported gluconate formation when added to a plate of the mutant of *P. aerugi-*

FIG. 3. Fluorescence Spectra of Chromophores Isolated from Methylamine Dehydrogenase from *Pseudomonas* AM1 (A) and Copper Containing Amine Oxidases from Beef Plasma (B), *Aspergillus niger* (C) and Pig Kidney (D).

One of the two chromophores eluted first from the DEAE-Sephadex A-25 column of the individual enzymes is shown in 0.05 N HCl.

nosa. More detailed information on PQQ production by microorganisms and results of further structural analysis of the chromophore from amine oxidases are being processed and will appear elsewhere separately.

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