

## EXISTENCE OF A NOVEL PROSTHETIC GROUP, PQQ, IN MEMBRANE-BOUND, ELECTRON TRANSPORT CHAIN-LINKED, PRIMARY DEHYDROGENASES OF OXIDATIVE BACTERIA

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Received 20 May 1981

### 1. Introduction

We have already purified membrane-bound, electron transport-linked, D-glucose dehydrogenase from *Pseudomonas fluorescens* [1], and found that the enzyme had an unknown prosthetic group differing from NAD(P) and flavin. Furthermore, it appears that alcohol [2], aldehyde [3] and D-glucose [4] dehydrogenases isolated in our laboratory from *Gluconobacter suboxydans* also have the same prosthetic group. This group's characteristics closely resembled those of glucose dehydrogenase purified from *Acinetobacter* [5] and of methanol dehydrogenases from *Pseudomonas* sp. M27 [6], *Rhodopseudomonas acidophila* [7] and *Hyphomicrobium* X [8]. Duine et al. [9] showed recently, that the novel prosthetic group was a pyrrolo-quinoline quinone (PQQ), the structure of which was determined using an X-ray analysis by Salisbury et al. [10].

To obtain more information on the prosthetic group, we isolated a mutant of *P. aeruginosa* having less glucose dehydrogenase activity than normal. This activity was shown to be restored by adding the prosthetic group extracted from the purified glucose dehydrogenase of *P. fluorescens*. Thus, the membrane of the mutant has an apo-glucose dehydrogenase and may be available for the identification of PQQ. Using the mutant membrane, we showed that alcohol, aldehyde and glucose dehydrogenases from *G. suboxydans* also had the same prosthetic group, PQQ.

### 2. Materials and methods

#### 2.1. Strains and materials

*P. aeruginosa* IFO 3445 and *G. suboxydans* IFO 12528 were used in this study. The purification of

D-glucose dehydrogenase from *P. fluorescens* [1], and of alcohol [2], aldehyde [3] and D-glucose [4] dehydrogenases from *G. suboxydans* have been described previously. Membranes were prepared by disruption of cells with a French press followed by an ultracentrifugation [11].

#### 2.2. Preparation of D-glucose dehydrogenase activity-less mutant and its cultivation

*P. aeruginosa* was inoculated on to a plate containing 20 g of D-glucose or glycerol, 5 g of polypeptone and 10 g of  $K_2SO_4$  in 1 liter of tap water and treated with acriflavine powder put on the plate. The search for mutant strains that did not produce acid was made on blue-colored plates containing 2% glucose as a sole carbon source and 0.4% bromothymol blue. The mutants could be detected as poorly growing colonies on the medium without change of color, while the parent strain formed yellowish colonies. A mutant used in this study, strain 108, was isolated as weakly acid producing and also devoid of  $N,N,N',N'$ -tetramethylphenylenediamine oxidase. To harvest cells of the parent and also the mutant strains, the organisms were grown aerobically in a medium consisting of 0.5% Na-D-gluconate, 0.1% polypeptone, 0.05% yeast extract, 0.075%  $K_2HPO_4$ , 0.025%  $KH_2PO_4$ , 0.03%  $MgSO_4$  and 0.005%  $FeSO_4$ . Cultivation was carried out at 30°C for 18 h with reciprocal shaking.

#### 2.3. Enzyme assay and activation methods

Activity of glucose dehydrogenase was measured using both phenazine methosulfate and 2,6-dichlorophenolindophenol as electron acceptors at pH 8.75 as described previously [1]. Enzyme concentration is expressed as  $\mu\text{mol}$  glucose oxidized per min at 25°C. Glucose dehydrogenase in the membrane of the

mutant was activated as follows. A membrane suspension (about 1 mg of protein) prepared from the mutant strain and the prosthetic group extracted from the several origins shown in table 1 were mixed in 0.01 M potassium phosphate buffer (pH 6.0) containing 5 mM  $\text{MgCl}_2$  in a final volume of 0.2 ml. After the mixture had been incubated at 25°C for 1 h, glucose dehydrogenase activity was measured with an aliquot withdrawn from the mixture.

### 3. Results and discussion

Purified glucose dehydrogenase of *P. fluorescens* showed no absorbance in the visible region, but a

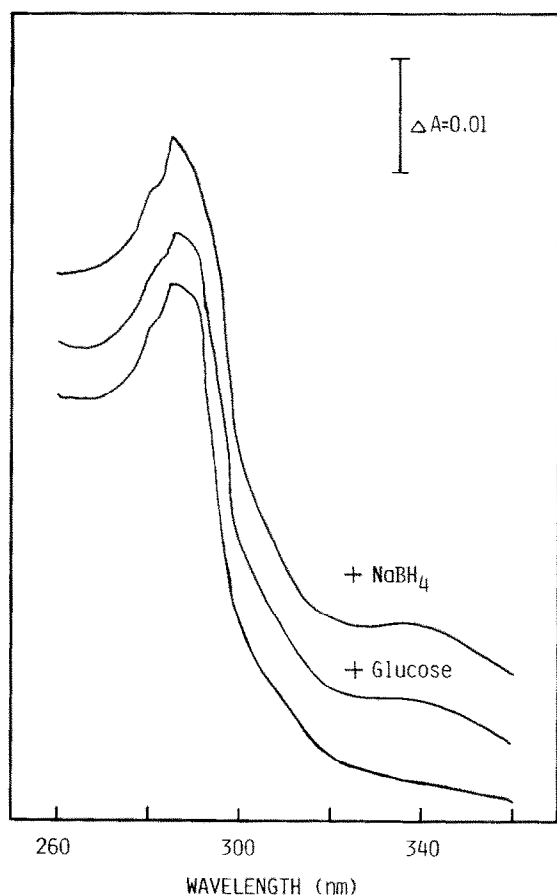


Fig.1. Absorbance spectra of D-glucose dehydrogenase from *P. fluorescens*. Purified enzyme (1 mg of protein) was precipitated with 40% ethanol to remove excess Triton X-100. The precipitate was solubilized by stirring overnight with 1% Brij 58. The supernatant (0.4 mg of protein/ml) was measured before and after the addition of a few grains of glucose or sodium borohydride.

characteristic absorbance appeared near at 340 nm by adding glucose or sodium borohydride (fig.1). The prosthetic group extracted from the purified enzyme showed an absorbance spectrum with a peak at 246 nm and shoulders at 275 and 326 nm (fig.2). The spectrum was consistent with those of the prosthetic groups from methanol dehydrogenase of methylotrophic bacteria [6,8] and glucose dehydrogenase of *Acinetobacter* [5,9]. By the addition of sodium borohydride, the peak at 246 nm was decreased and that

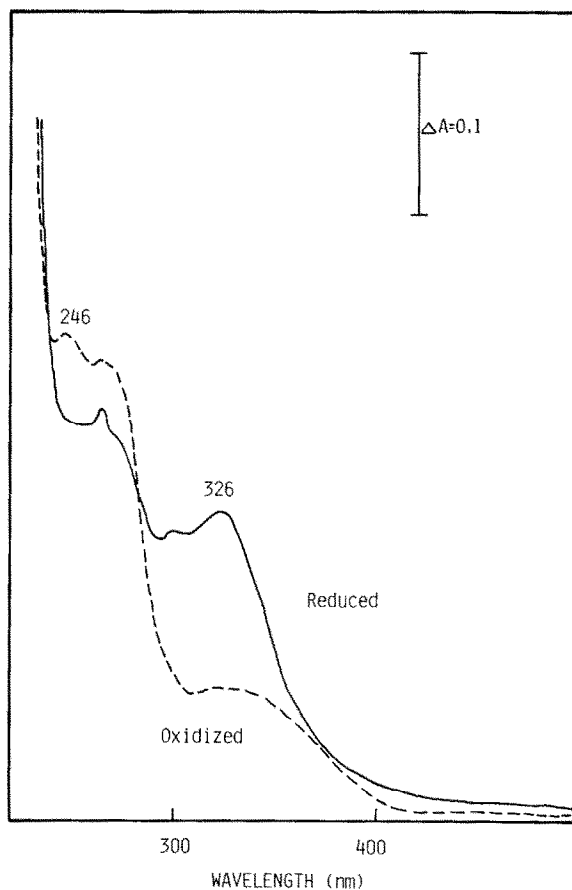


Fig.2. Absorbance spectra of the prosthetic group extracted from D-glucose dehydrogenase of *P. fluorescens*. Purified enzyme (1 mg of protein) was precipitated with 50% ethanol. The pellet was suspended in 1 ml of 0.01 M potassium phosphate buffer (pH 6.0) and boiled for 10 min. The supernatant obtained by centrifuging the suspension was washed with the same volume of chloroform to remove contaminated detergent. The upper phase was collected and measured with a Hitachi 557 dual wavelength spectrophotometer. The reduced form (—) was obtained by adding a few grains of sodium borohydride to the oxidized form (---).

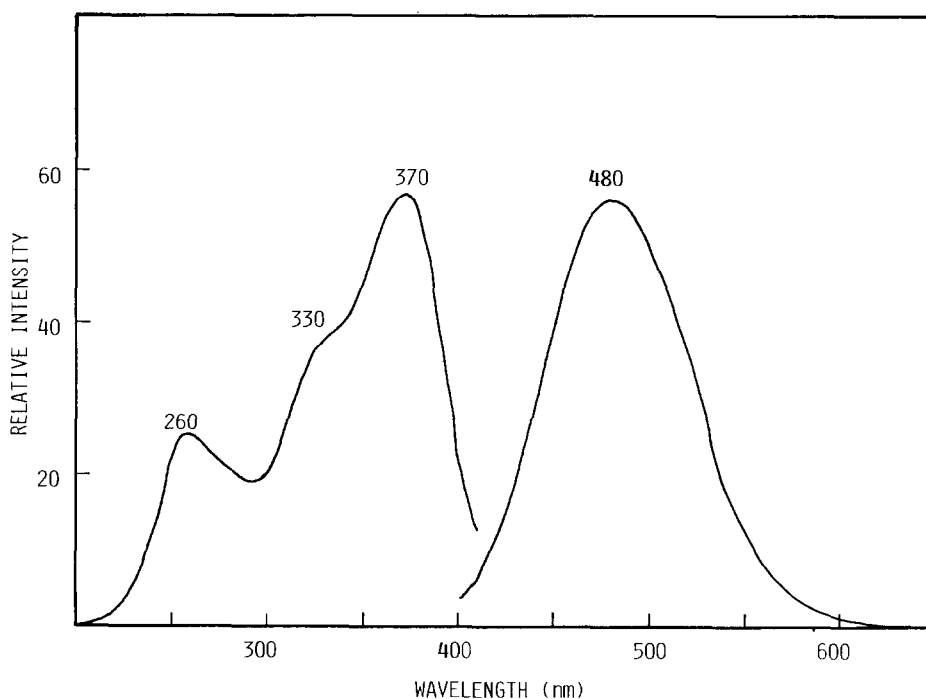


Fig.3. Fluorescence spectra of the prosthetic group extracted from D-glucose dehydrogenase of *P. fluorescens*. The sample was prepared as described in the legend of fig.2, and measured with an Aminco-Bowman spectrofluorometer J4-8962. The excitation spectrum was measured at 480 nm emission, and the emission spectrum at 370 nm excitation.

at 326 nm was increased. The same absorbance change has already been observed in the prosthetic group of glucose dehydrogenase from *Acinetobacter* [5] and in the cofactor of glucose dehydrogenase prepared from culture broth of *P. fluorescens* [12]. The prosthetic group from glucose dehydrogenase of *P. fluo-*

*rescens* showed a fluorescence spectrum with a maximum at 480 nm and an excitation maximum at 370 nm (fig.3). The spectrum was also identical with that of the prosthetic group from methanol dehydrogenase [6].

These spectral properties indicate that glucose

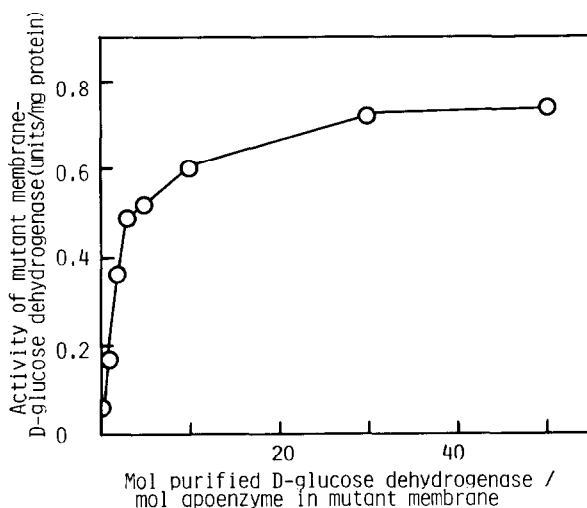


Fig.4. Activation of glucose dehydrogenase in the membrane of the glucose dehydrogenase activity-less mutant of *P. aeruginosa* the with prosthetic group extracted from D-glucose dehydrogenase of *P. fluorescens*. The prosthetic group was extracted from purified enzyme (1 mg of protein) with 90% methanol. The extract was evaporated to remove methanol, dissolved in 0.5 ml of 0.01 M potassium phosphate buffer (pH 6.0) containing 5 mM  $MgCl_2$ , and washed with 1 ml of chloroform. The prosthetic group extracted from 22 to 1100 pmol of the enzyme was incubated with the mutant membrane (0.98 mg of protein). The membrane contains 22 pmol of apoenzyme, calculating from the specific activity of the purified enzyme (370 units/mg protein). Enzyme activity is expressed as units per mg of the membrane protein. The horizontal axis shows the molar ratio of the enzyme used to extract the prosthetic group against the apoenzyme in mutant membrane.

dehydrogenase of *P. fluorescens* has PQQ [9] as the prosthetic group. The prosthetic groups from alcohol and aldehyde dehydrogenases of *G. suboxydans* (data not shown) and from glucose dehydrogenase of *G. suboxydans* [4] showed similar absorption and fluorescence spectra, suggesting that these dehydrogenases also have the same prosthetic group.

We isolated a mutant strain having little glucose dehydrogenase activity from *P. aeruginosa*. The membrane prepared from the mutant, Strain 108, had less than one-tenth the glucose dehydrogenase activity (0.04–0.08 units per mg of protein) compared with the 0.6–1.1 of that from the parent strain. Using the mutant membrane, we attempted to reactivate its glucose dehydrogenase with the prosthetic group

extracted from the parent strain, and further tested a cross-reactivation of the enzyme with those extracted from other species of enzymes or the membrane of other organism. The glucose dehydrogenase activity of the mutant membrane was increased 10 to 20-fold by adding the prosthetic group extracted from the purified glucose dehydrogenase of *P. fluorescens*. When the membrane of the parent strain was used instead of the mutant membrane, this activation was scarcely detected. This implies that glucose dehydrogenase is present as an apoenzyme lacking the prosthetic group in the mutant membrane. The activation was maximal when the prosthetic group extracted from over 10 mol of the purified enzyme was added per 1 mol of apoenzyme expected to be contained in

Table 1  
Activation of glucose dehydrogenase in the membrane of the glucose dehydrogenase activity-less mutant of *P. aeruginosa* with a prosthetic group fraction extracted from various enzymes and membranes

Origin of prosthetic group	Protein content <sup>a</sup> (mg)	Glucose dehydrogenase activity of the mutant membrane (units/mg protein)
None	—	0.04
Glucose dehydrogenase of <i>P. fluorescens</i>	0.05	0.85
Alcohol dehydrogenase of <i>G. suboxydans</i>	0.30	0.66
Aldehyde dehydrogenase of <i>G. suboxydans</i>	0.20	0.63
Glucose dehydrogenase of <i>G. suboxydans</i>	0.26	0.30
Membranes of <i>G. suboxydans</i>	36.5	0.81
Membranes of <i>G. suboxydans</i>	160	0.05
	2660	0.21

<sup>a</sup> Prosthetic groups added to the activation mixture in a total volume of 0.2 ml were prepared from samples having the protein content shown here

The prosthetic group was extracted from various purified dehydrogenases and membranes of two strains with 90% methanol. The methanol extracts from the membranes were purified by an Amberlyst column [8], in which a prosthetic group was eluted with methanol/water (1:1, v/v) containing 1 M NaCl, after washing the column with methanol/water (1:1, v/v) and then with the same solvent containing 0.1 M NaCl. The extracts from enzymes or the eluates from the column were evaporated to remove methanol, and then used for the activation, which was conducted as described in the text

the mutant membrane (fig.4). The glucose dehydrogenase of the mutant membrane was similarly activated with the prosthetic groups from alcohol, aldehyde and glucose dehydrogenases of *G. suboxydans* (table 1). Also, the extracts from the membranes of *P. aeruginosa* and *G. suboxydans* gave rise to activation of the enzyme in the mutant. Table 1 shows that *Gluconobacter* membrane contained much more PQQ than *Pseudomonas* membrane. This finding is consistent with the fact that several kinds of PQQ-dehydrogenases occur at high concentrations in *Gluconobacter* membrane whereas possibly only one PQQ-enzyme, glucose dehydrogenase, is found in *Pseudomonas* membrane.

Duine et al. [9] have shown that methanol and glucose dehydrogenases have a novel prosthetic group, PQQ, and they have proposed that the PQQ-containing dehydrogenase be classified as a 'quinoprotein'. Our present study showed that the glucose dehydrogenases of *P. fluorescens* and *G. suboxydans* were both a quinoprotein similar to the enzyme of *Acinetobacter* [9]. This suggests that electron transport-linked, NAD(P)-independent, glucose dehydrogenase is generally a quinoprotein like methanol dehydrogenase. Duine et al. [9] also suggested that the alcohol dehydrogenase of acetic acid bacteria was possibly a quinoprotein. We confirmed that alcohol, and further aldehyde, dehydrogenases of acetic acid bacteria had the same prosthetic group, PQQ, as its glucose dehydrogenase. These quinoproteins, glucose, alcohol and aldehyde dehydrogenases are involved in sugar oxidation that links to an electron transport system in the membrane of oxidative bacteria. It

should be noted that glucose dehydrogenase of *P. fluorescens* could reduce ubiquinone directly, unlike flavoprotein [13]. Thus, the quinoprotein may have a peculiar function in the electron transport chain of the oxidative bacteria.

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