GLUCOSE DEHYDROGENASE FROM *ACINETOBACTER CALCOACETICUS*

A 'quinoprotein'

J. A. DUINE, J. FRANK, Jzn. and J. K. VAN ZEELAND
Laboratory of Biochemistry, Delft University of Technology, 67 Julianalaan 2628 BC Delft, The Netherlands

Received 17 October 1979

1. Introduction

Many *Pseudomonas* and *Acetobacter* species are capable of oxidizing aldoses to the corresponding lactone, by NAD(P)-independent dehydrogenases [1]. One of these enzymes, glucose dehydrogenase (glucose: (acceptor) oxidoreductase, EC 1.1.99.-), has been purified from *Acinetobacter calcoaceticus* and it appeared to contain a novel prosthetic group of unknown structure [2].

Here, the properties of the prosthetic groups of glucose dehydrogenase and methanol dehydrogenase (EC 1.1.99.8, an enzyme which is present in methylotrophic bacteria, grown on methane or methanol) are compared. Furthermore, a procedure for testing the activity of the prosthetic groups and similar compounds, using the apoenzyme of a glucose dehydrogenase, is described.

As the properties are similar, it is concluded that the prosthetic group of glucose dehydrogenase is, like that of methanol dehydrogenase [3–8], a 'pyrroloquinoline quinone' (PQQ). The name 'quinoproteins' is proposed for this distinct class of dehydrogenases.

2. Materials and methods

2.1. *Growth of the organisms*

Acinetobacter calcoaceticus LMD 70.9 and LMD 79.39 (formerly known as *Bacterium anitratum*, kindly provided by Professor Hauge) and *Acetobacter pasteurianus* s.s. *lovaniensis* LMD 31.2 were obtained from the Laboratory of Microbiology Culture Collection, Delft. They were grown on a mineral salt medium supplemented with 0.02 M Na succinate or 0.10 M ethanol at 22°C with good aeration. The cells were harvested at the end of the logarithmic phase and a wet-cell yield of ~4 g/l was obtained. *Gluconobacter oxydans* ATCC 621H cells, harvested in growth-phase 3 [9], were provided by Dr W. Olijve.

2.2. *Cell-free extract of* Acinetobacter calcoaceticus

Frozen cells (10 g) were thawed and mixed with 15 ml 36 mM Tris/39 mM glycine. After adding 6 mg lysozyme, the suspension was stirred at room temperature for 15 min and centrifuged for 10 min at 48 000 × *g*. The supernatant was discarded and the pellet extracted twice with 36 mM Tris/39 mM glycine, containing 1% Triton X-100. The supernatants of the centrifugation steps were combined and used immediately.

2.3. *Enzyme purification*

The cell-free extract was added to a DEAE–Sephacel column (13 × 2.2 cm), equilibrated with 36 mM Tris/39 mM glycine buffer, containing 1% Triton X-100 and the column was washed with the same buffer. The enzyme did not adhere to the column material and the combined active fractions were titrated with 2 M acetic acid to pH 6.0. This solution was added immediately to a column of CM-Sepharose CL-6 B (5 × 1 cm), equilibrated with 5 mM potassium phosphate (pH 6.0). After washing the column with the same buffer until no Triton X-100 was present in the eluate, the enzyme was eluted with 0.1 M potassium phosphate (pH 7.0).

2.4. *Apoenzyme preparation*

Glucose dehydrogenase was dialyzed against 0.1 M sodium acetate (pH 4.5), containing 3 M KBr at 4°C for 72 h. Then, it was dialyzed against 0.02 M potassium phosphate (pH 7.0) for 12 h.

2.5. *Enzyme assay*

Activity was determined in two ways:

1. Measuring the rate of oxygen consumption with a Clark electrode in a mixture of 2.8 mM phenazine methosulphate, 70 mM glucose, enzyme and 0.05 M Tris–HCl (pH 8.5).
2. Spectrophotometrically, measuring the rate of discoloration of Wurster's Blue at 600 nm (referred to as compound II in [3]) in a mixture of 20 mM glucose, 200 μM Wurster's Blue, enzyme and 0.05 M Tris–HCl (pH 8.5).

The apoenzyme test was performed in the same way except that before the measurement the apoenzyme was incubated with the same volume of a prosthetic group containing fraction at room temperature during 30 min. As a reference, apoenzyme incubated with an equal volume of buffer or water was used.

2.6. *Methods*

The prosthetic group was extracted with methanol from enzyme [3] and from whole cells [5] and purified on Amberlyst A21 ion exchanger [5].

High-performance liquid chromatography (HPLC) of the prosthetic group and its aldehyde/ketone adducts was performed as in [5]. The ESR hyperfine spectrum of the prosthetic group was measured in a solution of sodium salicylate/NaOH [6].

3. Results and discussion

The prosthetic groups of glucose and methanol dehydrogenase showed the same absorption spectrum (fig.1 and [3,5], respectively) and the same retention time by HPLC analysis (fig.2 and [5] respectively). Furthermore, the products formed with aldehydes and ketones were also identical, as judged by HPLC (fig.2 and [5]).

Although HPLC analysis is a very sensitive method to detect small differences between different compounds, yet another method was tried. The ESR hyperfine spectrum of a free radical in solution can be regarded as a 'fingerprint' of the molecule. As the hyperfine spectrum of the free radical from the prosthetic group of glucose dehydrogenase was identical with that of methanol dehydrogenase [6], it is concluded that they have the same chemical structure.

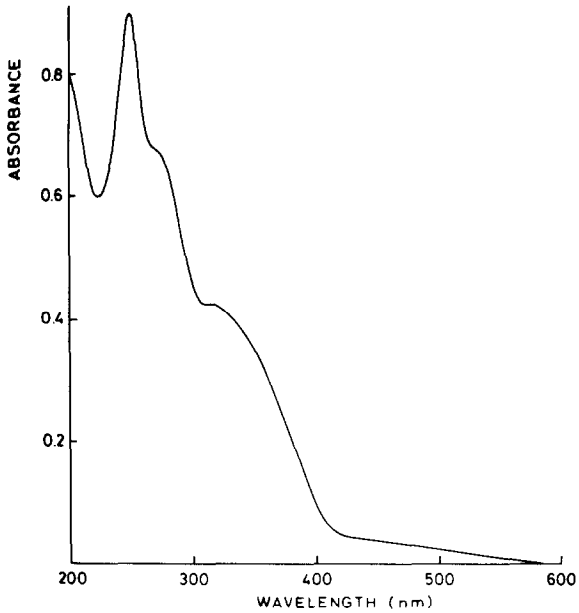
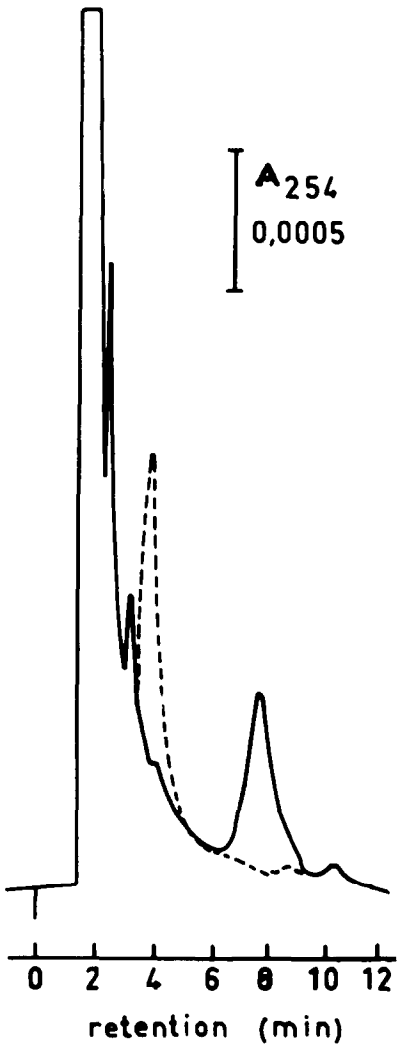


Fig.1. Absorption spectrum of the prosthetic group from glucose dehydrogenase. An enzyme solution, showing the same absorption spectrum as in [2], was extracted and further purified as described for methanol dehydrogenase [5]. The spectrum was recorded in 0.02 M potassium phosphate (pH 7.0).

In view of the fact that the chemical structure is 4,5-dihydro-4,5-dioxo-1 H-pyrrolo [2,3-f] quinoline-2,7,9-tricarboxylic acid [7,8], in which the *o*-quinone groups appears to be the essential part of the molecule [7], and the fact that several dehydrogenases contain this prosthetic group (see discussion below), we propose the trivial name 'pyrrolo-quinoline quinone' (PQQ) for this prosthetic group.

Although the hyperfine ESR spectrum of the free radical detached from methanol dehydrogenase and that of the free radical induced in the purified prosthetic group are identical [6] (suggesting that PQQ is the real prosthetic group) further evidence is given here from reconstitution experiments. As shown in table 1, both prosthetic groups are active in the apoenzyme test, in accordance to the view that the



prosthetic groups are not modified during the isolation.

The apoenzyme is also most valuable for detecting the presence of PQQ in extracts of other microorganisms. In table 1 it is shown that an extract of *Acetobacter pasterianus*, s.s. *lovaniensis*, grown on ethanol, is active. As it is known that acetic acid bacteria have a NAD(P)-independent ethanol dehydrogenase in which no flavins can be detected [10], the presence of activity is not unexpected. The identity of PQQ was confirmed from the ESR hyperfine spectrum of the extract. The same applies to *Gluconobacter oxydans*, an organism containing NAD(P)-independent glucose and polyol dehydrogenases [9,11].

Besides the dehydrogenases discussed here, others have been found in which this prosthetic group is covalently bound to the protein part (unpublished results), suggesting a still wider occurrence. In view of the fact that a relevant property of the prosthetic group appears to be the *o*-quinonoid structure [7] and the current use of names like flavoproteins and haemoproteins, we propose to classify the PQQ-containing group of dehydrogenases as 'quinoproteins'.

Fig.2. HPLC chromatogram of a prosthetic group containing extract from glucose dehydrogenase. Conditions are described in [5]. Dashed line, after reaction with acetone. The prosthetic group and its acetone adduct had a retention time of 7.7 min and 3.8 min, respectively.

Table 1
Activity of extracts from enzymes and cells, tested with apoenzyme of glucose dehydrogenase

Extract from	Activity (nmol Wurster's Blue reduced/ min/ml)
Methanol dehydrogenase	41.3
Glucose dehydrogenase	37.6
Acetobacter pasteurianus s.s *lovaniensis*	40.5
Gluconobacter oxydans	36.9
Baker's yeast	< 0.1
Elution buffer	< 0.1

The extracts were prepared from methanol dehydrogenase of *Hyphomicrobium* X [3], glucose dehydrogenase (2 ml enzyme with A_{280} 0.60) and cells (4 g wet wt). Apoenzyme (A_{280} 0.5) (20 µl) were mixed with 20 µl prosthetic group-containing fraction (1 ml) which was eluted from the Amberlyst column [5]

Acknowledgement

We thank Dr W. Olijve, Organon, Oss, for providing the *Gluconobacter oxydans* cells.

References

[1] Hauge, J. G. (1966) Methods Enzymol. 9, 92–98.
[2] Hauge, J. G. (1964) J. Biol. Chem. 239, 3630–3639.
[3] Duine, J. A., Frank, J. and Westerling, J. (1978) Biochim. Biophys. Acta 524, 277–287.
[4] De Beer, R., Van Ormondt, D., Van Ast, M. A., Banen, R., Duine, J. A. and Frank, J. (1979) J. Chem. Phys. 70, 4491–4495.
[5] Duine, J. A. and Frank, Jzn, J. (1980) Biochem. J. in press.
[6] Westerling, J., Frank, J. and Duine, J. A. (1979) Biochem. Biophys. Res. Commun. 87, 719–724.
[7] Duine, J. A., Frank, Jzn, J. and Verwiel, P. E. J. (1980) Eur. J. Biochem. in press.
[8] Salisbury, S. A., Forrest, H. S., Crude, W. B. T. and Kennard, O. (1979) Nature 280, 843–844.
[9] Olijve, W. (1978) PhD Thesis, Groningen.
[10] Kersters, K. and De Ley, J. (1966) Methods Enzymol. 9, 346–354.
[11] Kersters, K. and De Ley, J. (1966) Methods Enzymol. 9, 170–179.