FEMS Microbiology Letters 43 (1987) 195–200
Published by Elsevier

FEM 02844

The in vivo and in vitro substrate specificity of quinoprotein glucose dehydrogenase of *Acinetobacter calcoaceticus* LMD79.41

P. Dokter, J.T. Pronk, B.J. van Schie, J.P. van Dijken and J.A. Duine

Laboratory of Microbiology and Enzymology, Delft University of Technology, 2628 BC Delft, The Netherlands

Received 23 March 1987
Accepted 20 March 1987

Key words: *Acinetobacter calcoaceticus*; Glucose dehydrogenase; Quinoprotein; (Substrate specificity)

1. SUMMARY

Quinoprotein glucose dehydrogenase (GDH; EC 1.1.99.17) was partially purified from cell-free extracts of *Acinetobacter calcoaceticus* LMD79.41. The enzyme oxidized monosaccharides (D-glucose, D-allose, 2-deoxy-D-glucose, D-galactose, D-mannose, D-xylose, D-ribose and L-arabinose) as well as disaccharides (D-lactose, D-maltose and D-cellobiose).

Intact cells of *A. calcoaceticus* LMD79.41 also oxidized these monosaccharides, but not the disaccharides.

The difference in substrate specificity can not be explained by impermeability of the outer membrane for disaccharides, since right-side-out membrane vesicles did not oxidize disaccharides either. Destruction of the cytoplasmic membrane strongly affected the catalytic properties of GDH. Not only did the affinity towards some monosaccharides change substantially, but disaccharides also became good substrates upon solubilization of the enzyme. Thus, at least in *A. calcoaceticus* LMD79.41, the oxidation of disaccharides by GDH can be considered as an in vitro 'artefact' caused by the removal of the enzyme from its natural environment.

2. INTRODUCTION

Several microbial oxidoreductases are known which oxidize both monosaccharides and disaccharides. The D-glucoside-3-dehydrogenases of *Agrobacterium tumefaciens* [1] and *Flavobacterium saccharophilum* [2] for example oxidize both glucose and lactose to the corresponding 3-keto sugars. Indeed, *Agrobacterium* spp. can be characterized by the ability of whole cells to form 3-keto sugars [3]. Although disaccharides are excellent substrates for GDH (EC 1.1.99.17) from *A. calcoaceticus* LMD79.41 [4], whole cells of this organism do not oxidize disaccharides.

It has been reported that the outer membrane of *Pseudomonas aeruginosa* forms a permeability barrier for disaccharides [5]. The same phenomenon could occur in *A. calcoaceticus*, explaining the discrepancy in the catalytic properties of intact cells and purified enzyme.

On the other hand, it has been demonstrated that several forms of GDH exist in cell-free ex-

Correspondence to: J.A. Duine, Laboratory of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands.

tracts of *Bacterium anitratum* (*A. calcoaceticus*). The enzyme forms differed in their kinetic properties, as judged by the relative oxidation rates observed with glucose and xylose [6]. This observation suggests that the substrate-specificity pattern of GDH may be a function of its state of solubilization.

In this study an attempt was made to resolve the apparent discrepancy between purified GDH and intact cells with respect to their capacity to oxidize disaccharides. To this end, the kinetic constants of GDH for the oxidation of a variety of aldose sugars were determined for purified enzyme, intact cells and cytoplasmic membrane vesicles.

3. MATERIAL AND METHODS

3.1. Organism and culture conditions

A. calcoaceticus LMD79.41 was grown in acetate-limited chemostat cultures as described by Van Schie et al. [7].

3.2. Preparation of membrane vesicles

Cytoplasmic membrane vesicles from *A. calcoaceticus* were prepared with a procedure slightly modified from Stinnett et al. [8]. Cells from an acetate-limited continuous culture were harvested by centrifugation (10 min at 10 000 × *g*). The pellet was resuspended in an ice-cold solution containing 20% sucrose, 2.5% lithium chloride, 0.1% lysozyme, 10 mM potassium phosphate (pH 7.0) and 10 mM magnesium sulphate, to a density of 2.5 g dry weight · l^{-1}. The suspension was brought to 30 °C and then incubated for 90 min on a rotatory shaker at that temperature. This treatment turned cells into spheroplasts, which were collected by centrifugation (10 min at 10 000 × *g*). The pellet was resuspended using a hypodermic needle (1.5 mm) in the smallest volume possible of the above buffer without lysozyme. The suspension was slowly added to 50 vols. of ice-cold lysis buffer (10 mM potassium phosphate, pH 7.0, 1 mM magnesium sulphate) under vigorous stirring. DNase and RNase were added to a final concentration of 20 μg · ml^{-1} each. The mixture was incubated at 30 °C for 30 min. During this period the light-scattering of the suspension decreased due to cell lysis. The suspension was then centrifuged (10 min at 40 000 × *g*; 4 °C) and the pellet was resuspended in 50 ml of an ice-cold buffer containing 100 mM potassium phosphate (pH 7.0) and 10 mM magnesium sulphate. Whole cells and large fragments were removed by centrifugation (10 min at 3000 × *g*; 4 °C). The supernatant was carefully decanted, after which the centrifugation step was repeated. The supernatant, devoid of whole cells as judged by phase-contrast microscopy, was centrifuged (10 min at 40 000 × *g*; 4 °C) to collect membrane vesicles. The pellet was washed twice with 100 mM potassium phosphate (pH 7.0) containing 10 mM magnesium sulphate. The final pellet was resuspended in the same buffer to a final concentration of approx. 1 mg membrane protein · ml^{-1}. Membrane vesicles were stored in liquid nitrogen.

3.3. Enzyme purification

Partially purified GDH was prepared by applying a cell-free extract to a CM-Sepharose column and eluting GDH activity as described previously [4].

3.4. Enzyme assays

O_2-linked aldose oxidation by whole cells and membrane vesicles was assayed polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments, Inc., Yellow Springs, OH) at 30 °C in air-saturated 100 mM potassium phosphate buffer (pH 7.0) containing 10 mM magnesium sulphate. The initial reaction rates were determined with various substrate concentrations. The experimental data were plotted according to the method of Lineweaver and Burk [9].

The assays for dye-linked aldose oxidation by partially purified enzyme and by membrane vesicles were performed at 25 °C, measuring the rate of discoloration of Wurster's Blue at 610 nm of a mixture containing 80 μM Wurster's Blue, 1 mM KCN, enzyme or vesicles, 0.1 M Tris–HCl buffer (pH 7.0) and substrate in a final volume of 2 ml. The reaction was started by adding the substrate. One enzyme unit refers to 1 μmol of substrate converted per min under these conditions. The calculations were based on a molar absorption coefficient for Wurster's Blue of 12 400

$M^{-1} \cdot cm^{-1}$ at 610 nm [4]. An enzyme concentration of 1 nM was used, based on the specific activity value for homogeneous enzyme of 640 units · mg protein^{-1} and an M_r of 94000 [4]. The experimental data were plotted according to the method of Lineweaver and Burk [9].

3.5. Analytical procedures

Protein concentrations were determined by the method of Bradford [10], with bovine serum albumin as a standard. The presence of glucose in the aldose preparations was investigated by using a glucose assay (hexokinase/glucose-6-phosphate dehydrogenase test kit, Boehringer Mannheim) as well as by a gluconate assay, after the particular substrate had been converted into product (gluconate kinase/6-P-gluconate dehydrogenase test kit, Boehringer Mannheim).

3.6. Chemicals

Wurster's Blue (the free radical of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) was prepared as described previously [11]. All other chemicals were from commercial sources.

4. RESULTS

4.1. Substrate specificity of purified GDH

GDH, partially purified from *A. calcoaceticus* LMD79.41, exhibited a broad substrate specificity. The enzyme oxidized a variety of monosaccharides (hexoses, pentoses) and disaccharides (Table 1). The following compounds were not oxidized: D-glucosamine, glucoheptose, α-methyl-glucose, glucose-6-phosphate, D-fructose, L-rhamnose, D-lyxose, L-lyxose, D-arabinose, melezitose, raffinose, stachyose, D-mannitol and myo-inositol.

Enzyme specificity is illustrated by representing the apparent V_M/K_M values for the substrates as a percentage of that of glucose (Table 2). It is noteworthy that the disaccharide maltose is among the best substrate. Since commercial preparations of maltose and other sugars may be contaminated with glucose, the glucose content of the various sugars used was determined. Since the glucose content did not exceed 0.25% (not shown), it can be concluded that glucose contamination has no significant influence on the values mentioned in the tables.

4.2. Substrate specificity of GDH in intact cells

Intact cell of *A. calcoaceticus* LMD79.41 also oxidized a variety of aldose sugars. The apparent affinities of intact cells for the various monosaccharides showed a pattern similar to that of purified GDH (Tables 1 and 2). Notable exceptions were 2-deoxyglucose and xylose which were much

Table 1

Apparent kinetic parameters of whole cells, vesicles and partly purified GDH for oxidation of mono- and disaccharides

Substrate	GDH		Cells		Vesicles (O_2 uptake)		Vesicles (dye reduction)	
	K'_m (mM)	V'_M (units/mg protein)	K'_s (mM)	V'_M (μmol O_2/min/mg dry weight)	K'_s (mM)	V'_M (μmol O_2/min/mg protein)	K'_M (mM)	V'_M (units/mg protein)
D-Glucose	1.7	24.0	1.7	0.97	0.8	8.58	0.5	2.5
D-Allose	1.5	14.0	1.8	0.72	n.d.	n.d.	0.5	2.1
2-Deoxy-D-glucose	13.6	8.0	3.1	0.98	n.d.	n.d.	0.5	1.8
D-Galactose	3.5	8.9	6.6	0.71	n.d.	n.d.	2.2	2.0
D-Mannose	19.0	12.0	35.0	0.94	24.5	8.29	21.3	2.6
D-Xylose	5.5	7.8	4.2	1.25	n.d.	n.d.	1.0	2.6
D-Ribose	40.0	8.9	37.0	1.09	12.0	6.04	8.5	1.1
L-Arabinose								
disaccharides	4.8	7.5	8.1	0.72	4.9	7.66	2.1	2.0
Lactose	4.2	14.2	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
Maltose	3.2	27.0	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
Cellobiose	2.7	18.2	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.

n.d., not determined.

n.m., not measurable below a substrate concentration of 50 mM.

Table 2

GDH substrate specificities following from relative V'_M/K'_m values of whole cells, vesicles and partly purified GDH

Substrate	GDH (%) V'_M/K'_m	Cells (%) V'_M/K'_s	Vesicles (%)	
			(O_2 uptake) V'_M/K'_s	(dye reduction) V'_M/K'_m
D-Glucose	100	100	100	100
D-Allose	66	70	n.d.	83
2-Deoxy-D-Glucose	4	56	n.d.	74
D-Galactose	18	19	n.d.	17
D-Mannose	4	5	3	3
D-Xylose	10	52	n.d.	54
D-Ribose	1	5	5	3
L-Arabinose disaccharides	11	16	15	20
Lactose	24	n.m.	n.m.	n.m.
Maltose	60	n.m.	n.m.	n.m.
Cellobiose	48	n.m.	n.m.	n.m.

n.d., not determined.
n.m., not measurable below a substrate concentration of 50 mM.

better substrates for intact cells than for the purified enzyme.

The most striking difference between the kinetics of sugar oxidation by purified GDH and intact cells was the inability of the latter to oxidize the disaccharides lactose, maltose and cellobiose. The inability of intact cells to oxidize lactose was also noted by Kleber et al. [12] for a different strain of *A. calcoaceticus*.

4.3. Substrate specificity of GDH in membrane vesicles

An obvious explanation for the inability of intact cells to oxidize disaccharides would be a transport barrier. Restrictions in the catalytic activity may be imposed via two permeability barriers, namely the cell wall and the cytoplasmic membrane. The latter possibility can be excluded in this case, since GDH is located at the outside of the cytoplasmic membrane [13]. Indeed it has been shown that the rate of sugar oxidation by inside-out membrane vesicles of *A. calcoaceticus* is much lower than the oxidation rates observed with right-side-out vesicles [14].

To circumvent a possible barrier of the cell wall, right-side-out membrane vesicles seemed an attractive system to probe in vivo substrate specificity. The membrane vesicles, isolated as described in MATERIALS AND METHODS, exhibited a high rate of glucose oxidation and are capable of glucose-energized active uptake of solutes [15]. These properties are consistent with a right-side-out orientation.

The kinetic properties of GDH in membrane vesicles, measured with oxygen or Wurster's Blue as electron acceptors, follow the same pattern as observed with intact cells (Table 2). Most significantly, like whole cells, membrane vesicles were unable to oxidize disaccharides.

These results do not exclude the possibility that the outer membrane constitutes a permeability barrier for disaccharides in *A. calcoaceticus*. It is evident, however, that the observed differences between purified enzyme and whole cells must be caused by other factors. Furthermore, membrane vesicles are unable to oxidize disaccharides, neither with oxygen nor with Wurster's Blue as an electron acceptor. Thus, the possibility can also be excluded that the different substrate specificity patterns observed with purified GDH and intact cells are caused by the electron acceptors used.

4.4. Effects of solubilization on the substrate specificity of GDH

The results presented above clearly show that, upon purification, GDH gains the capacity to oxidize disaccharides. This property, however, does not result from the purification as such, but rather reflects the removal of the enzyme from its natural environment. For example, when cells are cultured

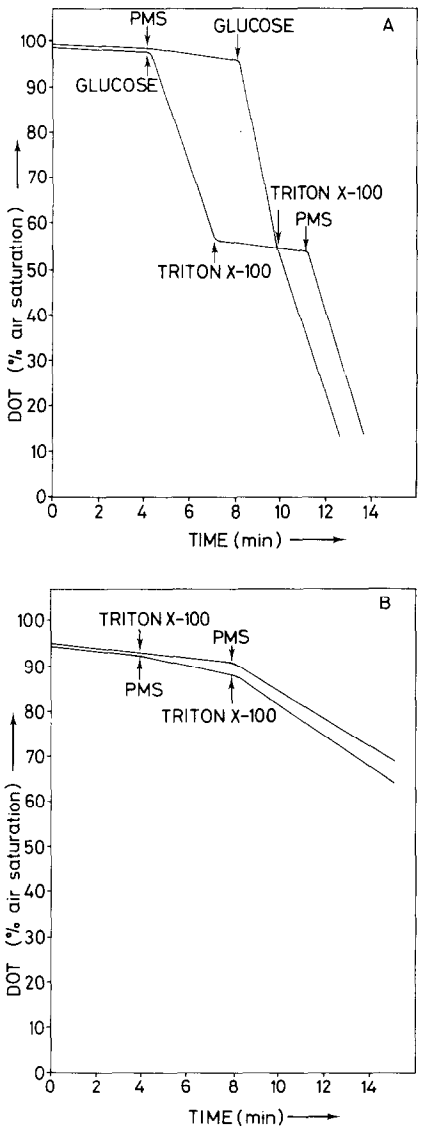

Fig. 1. Recorder tracings of oxygen consumption by membrane vesicles of *A. calcoaceticus* LMD79.41. (A) Effect of Triton X-100 and PMS on glucose oxidation. The initial reaction mixture contained 100 mM potassium phosphate (pH 7.0), 10 mM magnesium sulphate, 3000 $U \cdot ml^{-1}$ catalase and membrane vesicles (8.8 μg membrane protein $\cdot ml^{-1}$). Addition of glucose (20 mM), Triton X-100 (0.01% v/v) and PMS (0.3 mM) is indicated by arrows. (B) Effect of Triton X-100 and PMS on lactose oxidation. The initial reaction mixture contained 100 mM potassium phosphate (pH 7.0), 10 mM magnesium sulphate, 20 mM lactose, 3000 $U \cdot ml^{-1}$ catalase and membrane vesicles (8.8 μg membrane protein $\cdot ml^{-1}$). Addition of Triton X-100 (0.01% v/v) and PMS (0.3 mM) is indicated by arrows. Note that a small but significant lactose oxidizing activity was detectable after addition of PMS (lower curve). This activity, however, is not associated with the membrane vesicles, as it remained in the soluble fraction after centrifugation (10 min at 40000 × *g*; not shown). We therefore conclude that this activity is caused by GDH dissociated from the vesicles during resuspension or freezing and thawing.

in the presence of Triton X-100, GDH is found in the growth medium [13]. Also after disruption of the cells in a French pressure cell, GDH can be detected in the soluble fraction (not shown). In both enzyme preparations, GDH shows high activity with disaccharides. This suggests that the substrate specificity of the enzyme changes when the architecture of the cytoplasmic membrane is destroyed. The ability to convert disaccharides is acquired in a very rapid process (Fig. 1). Addition of a low concentration of Triton X-100 to *A. calcoaceticus* membrane vesicles results in an instantaneous inhibition of glucose-dependent oxygen consumption (Fig. 1A). The capacity to convert glucose is retained, however, as is revealed in the assay measuring oxygen consumption in the presence of phenazine methosulphate (PMS), indicating that inhibition by Triton X-100 is caused by damage of the electron transport chain, rather than from an effect of the detergent on the enzyme itself. As mentioned above, membrane vesicles are unable to oxidize disaccharides, measured either by oxygen uptake or Wurster's Blue reduction. In agreement with this, lactose-dependent oxygen consumption was not observed, neither in the absence nor in the presence of PMS (Fig. 1B). A drastic change occurred as a result of the addition of Triton X-100, provided that PMS was present. This observation clearly demonstrates that alteration of the substrate specificity of GDH occurs when the cytoplasmic membrane structure is disrupted.

5. DISCUSSION

Oxidation of disaccharides by GDH from *A. calcoaceticus* LMD79.41 can only be observed when the enzyme is detached from the cytoplasmic membrane. Not only the kinetic properties for disaccharide oxidation, but also the kinetic

parameters for some monosaccharides, for example 2-deoxyglucose and xylose, were affected (Tables 1 and 2). The change may take place via several steps since differences in substrate specificity between 'soluble' and 'particulate' enzymes were noted by Hauge [6,16]. To know whether the process is reversible, it would be of interest to study the catalytic properties of purified GDH after incorporation into liposomes.

So far, little is known about the changes in catalytic properties of membrane-bound enzymes upon solubilization. Although it has been shown that activity of membrane-bound enzymes may be dependent on the presence of lipids [17], little attention has so far been paid to the possibility of changing substrate specificity.

Our results with GDH purified from *A. calcoaceticus* LMD79.41 show that the in vitro properties of enzymes which are membrane-bound in vivo must be interpreted with caution. Oxidation of disaccharides by *A. calcoaceticus* LMD79.41 may be regarded as an in vitro 'artefact'. A similar conclusion may hold for GDH isolated from *Escherichia coli* [18] and *Gluconobacter suboxydans* [19], which have also been reported to oxidize disaccharides in vitro. However, the inability of GDH to oxidize disaccharides in vivo may vary with the type of enzyme. For example, it has been known for a long time that intact cells of various *Pseudomonas* spp. can oxidize disaccharides to the corresponding bionic acids [20–22]. Since the quinoprotein GDH is widespread among *Pseudomonas* spp. [23], the possibility exists that the enzyme in these organisms, in contrast to the GDH of *A. calcoaceticus* LMD79.41, is capable of oxidizing disaccharides in the membrane-bound state.

ACKNOWLEDGEMENTS

We are grateful to B. Vink for his technical assistance and to R. Rouwenhorst for many stimulating discussions. This work was supported by a grant from the Dutch Ministry of Economic Affairs.

REFERENCES

[1] Nishizuka, Y. and Hayaishi, O. (1962) J. Biol. Chem. 237, 2721–2728.
[2] Takeuchi, M., Ninomiya, K., Kawabata, K., Asono, N., Kameda, Y. and Matsui, K. (1986) J. Biochem. 100, 1049–1055.
[3] DeLey, J., Bernaerts, M., Rassel, A. and Guimot, J. (1966) J. Gen. Microbiol. 43, 7–17.
[4] Dokter, P., Frank, J.Jzn. and Duine, J.A. (1986) Biochem. J. 239, 163–167.
[5] Yoneyama, H., Akatsuka, A. and Nakae, T. (1986) Biochem. Biophys. Res. Commun. 134, 106–112.
[6] Hauge, J.G. (1960) Biochim. Biophys. Acta 45, 263–269.
[7] Van Schie, B.J., Van Dijken, J.P. and Kuenen, J.G. (1984) FEMS Microbiol. Lett. 24, 133–138.
[8] Stinnett, J.D., Guymon, L.F. and Eagon, R.C. (1973) Biochem. Biophys. Res. Commun. 52, 284–290.
[9] Lineweaver, H. and Burk, D.J. (1934) J. Am. Chem. Soc. 56, 658–666.
[10] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[11] Duine, J.A., Frank, J.Jzn. and Westerling, J. (1978) Biochim. Biophys. Acta 524, 277–287.
[12] Kleber, H.P., Haferburg, D., Asperger, O., Schmidt, M. and Aurich, H. (1984) Z. Allg. Mikrobiol. 24, 691–701.
[13] Dokter, P., Van Kleef, M.A.G., Frank, J.Jzn. and Duine, J.A. (1885) Enzyme Microb. Technol. 7, 613–616.
[14] Kitagawa, K., Tateishi, A., Nakano, F., Matsumoto, T., Morohoshi, T,. Tanino, T. and Usui, T. (1986) Agric. Biol. Chem. 50, 1453–1457.
[15] Pronk, J.T., Van Schie, B.J., Van Dijken, J.P. and Kuenen, J.G. (1985) Antonie van Leeuwenhoek 51, 560.
[16] Hauge, J.G. (1966) Methods Enzymol. 9, 92–98.
[17] Ingledew, W.J. and Poole, R.K. (1984) Microbiol. Rev. 48, 222–271.
[18] Ameyama, M., Shinagawa, E., Matsushita, K., Takimoto, K. and Adachi, O. (1986) Agric. Biol. Chem. 50, 49–57.
[19] Ameyama, M., Shinagawa, E., Matsushita, K. and Adachi, O. (1981) Agric. Biol. Chem. 45, 851–861.
[20] Bentley, R. and Slechta, L. (1960) J. Bacteriol. 79, 346–355.
[21] Kluyver, A.J., DeLey, J. and Rijven, A. (1951) Antonie van Leeuwenhoek, 17, 1–14.
[22] Stodola, F.H. and Lockwood, L.B. (1947) J. Biol. Chem. 171, 213–221.
[23] Duine, J.A., Frank, J.Jzn. and Jongejan, J.A. (1986) FEMS Microbiol. Rev. 32, 165–178.