Arch Microbiol (1990) 153:502–505

Archives of
Microbiology

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The role of magnesium and calcium ions in the glucose dehydrogenase activity of *Klebsiella pneumoniae* NCTC 418

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Received November 13, 1989/Accepted January 9, 1990

Abstract. Magnesium-limited chemostat cultures of *Klebsiella pneumoniae* NCTC 418 with 20 μM $CaCl_2$ in the medium showed a low rate of gluconate plus 2-ketogluconate production relative to potassium- or phosphate-limited cultures. However, when the medium concentration of $CaCl_2$ was increased to 1 mM, the glucose dehydrogenase (GDH) activities also increased and became similar to those observed in potassium- or phosphate limited cultures. It is concluded that this is due to Mg^{2+} and Ca^{2+} ions being involved in the binding of pyrroloquinoline quinone (PQQ) to the GDH apoenzyme. There seems to be an absolute requirement of divalent cations for proper enzyme functioning and in this respect Ca^{2+} ions could replace Mg^{2+} ions. The high GDH activity which has been found in cells grown under Mg^{2+}-limited conditions in the presence of higher concentrations of Ca^{2+} ions, is compatible with the earlier proposal that GDH functions as an auxiliary energy generating system involved in the maintenance of high transmembrane ion gradients.

Key words: *Klebsiella pneumoniae* – Chemostat culture – Glucose metabolism – Glucose dehydrogenase – Pyrroloquinoline quinone – Magnesium – Calcium

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The conversion of glucose to gluconate by *Klebsiella pneumoniae* NCTC 418 (formerly *Klebsiella aerogenes*) occurs in the periplasmic space via a membrane-bound pyrroloquinoline quinone (PQQ)-linked glucose dehydrogenase (GDH) (Neijssel et al. 1983) which oxidizes D-glucose to D-glucono-1,4-lactone and donates the reducing equivalents to the respiratory chain (Beardmore-Gray and Anthony 1986; Matsushita et al. 1987). D-Glucono-1,4-lactone is spontaneously hydrolysed to gluconate although an additional conversion by a lactonase, such as present in other microbial species (Jermyn 1960; Hucho and Wallenfels 1972), cannot be excluded. Gluconate subsequently can be oxidised to 2-ketogluconate by a membrane-bound, FAD-linked gluconate dehydrogenase (GaDH) (Matsushita et al. 1982).

Aerobic chemostat cultures of *K. pneumoniae* with glucose as the carbon source, are able to produce large amounts of gluconate and 2-ketogluconate when the residual glucose concentration in the culture fluid is high (Neijssel and Tempest 1975). Cultures of *K. pneumoniae* growing under potassium- or phosphate-limited conditions possess relatively high GDH activities as indicated by high specific gluconate plus 2-ketogluconate production rates in vivo and by high enzyme levels as measured in vitro. On the other hand, carbon-, nitrogen- or sulphate-limited cultures show relatively low GDH activities. On the basis of these results Hommes et al. (1985) proposed that GDH, together with GaDH, functions as an auxiliary energy generating system that is involved in the maintenance of a high transmembrane gradient of K^+ and phosphate ions, respectively, which tends to be dissipated by leakage of these ions through the cytoplasmic membrane. In this connection, Mulder et al. (1986) suggested that in potassium-limited cultures of *Escherichia coli* the presence of two potassium uptake systems with different affinities for potassium could result in a futile cycle of potassium ions across the membrane. The inward current of this ion would occur via the derepressed high affinity potassium uptake system and the outward current would be caused by leakage via the constitutive low affinity potassium uptake system.

Mg^{2+} ions are required in large intracellular concentrations [0.1–0.3% (w/w)] presumably to allow proper ribosome functioning (Tempest and Hunter 1965). Although the concentration of free Mg^{2+} ions in the cytoplasm is not known, it is to be expected that cells

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Abbreviations: PQQ, pyrroloquinoline quinone; GDH, glucose dehydrogenase (EC 1.1.99.17); GaDH, gluconate dehydrogenase (EC 1.1.99.3); CAP, chloramphenicol; WB, Wurster's Blue [1,4-bis-(dimethylamino)-benzene perchlorate]

grown under magnesium-limited conditions will have to maintain a high transmembrane magnesium gradient. Nelson and Kennedy (1972) have shown that *E. coli* possesses at least two magnesium uptake systems, a constitutive low affinity uptake system and a repressible high affinity one. If *K. pneumoniae* also possesses both these magnesium uptake systems, a high magnesium gradient across the cytoplasmic membrane could cause leakage of magnesium ions through the low affinity uptake system and would result in a futile cycle of magnesium ions. This futile cycle would increase the energy requirement of these cells and could therefore result in high GDH activities during magnesium-limited growth of *K. pneumoniae*. We therefore studied the regulation of glucose dehydrogenase activity in magnesium-limited chemostat cultures of this organism.

Materials and methods

Organism

Klebsiella pneumoniae NCTC 418 was maintained by monthly subculture on tryptic meat-digest agar slants.

Growth conditions

Cells were grown in a Bioflo New Brunswick fermenter (New Brunswick Scientific Co., Inc., Edison, NJ, USA) or in a 500 ml Modular Fermenter 500 Series II (LH Engineering Co. Ltd., Stoke Poges, Bucks, UK) at a dilution rate (D) of $0.30 \pm 0.02\ h^{-1}$. The culture pH value was set at 6.0 ± 0.1 and a temperature of 35°C. 2 N NaOH was used as titrant. The magnesium-limited medium was according to Evans et al. (1970) and contained 62 μM $MgCl_2$. Glucose (90 mM input) was used as carbon source. When higher concentrations of $CaCl_2$ (0.2 or 1 mM) were added to the medium the glucose concentration was increased to 140 mM, so as to compensate for the increased conversion of glucose into gluconate and 2-ketogluconate and to maintain the glucose dehydrogenase fully saturated with glucose (K_m for glucose = 5 mM; Neijssel et al. 1983).

Pulse procedures

The cultures were pulsed by injecting 10 ml of a sterilized stock solution into the culture vessel by means of a syringe. Samples were taken at regular time intervals, cells and supernatants were separated by centrifugation in an Eppendorf centrifuge. The supernatants were frozen for later analyses. Oxygen consumption and carbon dioxide production were recorded continuously throughout the experiment.

The increase in specific gluconate production rate ($q_{gla,extra}$) was calculated from the gluconate concentration ([gla]) at different time intervals after the pulse, the dilution rate (D) and the dry weight in steady state (dw_0) using the equation.

$$q_{gla,extra} = (d[gla]/dt) \cdot e^{Dt} \cdot dw_0^{-1}.$$

Stimulation of gluconate dehydrogenase (GaDH)-activity in vivo was never observed, because in steady state cultures gluconate was present in saturating amounts [$K_{m,gla}$ = 2 mM (Matsushita et al. 1982)]. Therefore the GDH-activity in vivo after the pulse (v_{GDH}) could be calculated from the specific gluconate and 2-ketogluconate production rates in steady state, $q_{gla,ss}$ and $q_{kgla,ss}$ respectively, and the increase in specific gluconate production rate:

$$v_{GDH} = q_{gla,ss} + q_{kgla,ss} + q_{gla,extra}.$$

Preparation of the cell-free extracts

An appropriate volume (60 – 80 ml) was withdrawn from the culture vessel and centrifuged at $6000 \times g$ for 10 min. Pellets were washed twice in a 50 mM phosphate buffer (pH 6.0). For preparation of cell free extracts the pellets were resuspended in approximately 8 ml of the same buffer and the suspensions were then sonically disrupted, using a Branson Sonifier B-12 Cell Disrupter, during 8 periods of 30 s at 75 W with intermittent 45 s cooling on ice. The sonified cell extracts were centrifuged for 10 min at $12000 \times g$ to remove intact cells. The supernatants were used in the glucose dehydrogenase assays.

Glucose dehydrogenase assay

The spectrophotometric assay of glucose dehydrogenase using Wurster's Blue (WB) as the electron acceptor was according to Hommes et al. (1985). The assay was carried out at 35°C in a Beckman DU40 spectrophotometer (Beckman Instruments Inc., Irvine, USA). NaCN (1.5 mM) was added to block electron transport chain activity when necessary. Protein was assayed by the biuret method using bovine serum albumin as standard (Gornall et al 1949).

Analyses

Bacterial dry weight was measured using the method of Herbert et al. (1971). Glucose, gluconate and 2-ketogluconate were determined by HPLC (LKB, Bromma, Sweden) with an Aminex A28 Column (Biorad, Richmond, USA), with 0.3 M formic acid adjusted to pH 5.5 with ammonia as eluent, using a 2142 Refractive Index Detector (LKB, Bromma, Sweden) and a SP 4270 Integrator (Spectra Physics, San Jose, USA), at 55°C. In order to determine acetate, pyruvate, 2,3-butanediol and lactate an Aminex HPX-87H Column (Biorad, Richmond, USA) was used with 0.01 N H_2SO_4 as eluent at 65°C. Oxygen consumption rates and carbon dioxide production rates were determined by gas analyses using an oxygen analyser (Taylor Servomex Type OA 272, Crowborough, Sussex, UK) and a carbon dioxide analyser (Servomex IR Gas Analyser PA 404, Crowborough, Sussex, UK). PQQ concentrations in the supernatant were determined according to Groen et al. (1986).

Results and discussion

Magnesium-limited cultures of *Klebsiella pneumoniae* were grown with the standard amount of 20 μM $CaCl_2$ (Evans et al. 1970) in the medium. The residual concentration glucose in the supernatant was maintained at more than 20 mM so as to saturate the glucose dehydrogenase (GDH) (Neijssel et al. 1983). These cultures showed very low gluconate and 2-ketogluconate production rates (4.5 mmol g dry weight^{-1} h^{-1}) when compared with those of potassium- or phosphate-limited cultures [22 and 14 mmol g dry weight^{-1} h^{-1}, respectively (Hommes et al. 1989b)]. The cells contained a small amount of GDH holoenzyme but high levels of the apoenzyme (Table 1).

In order to investigate whether this was caused by the low extracellular concentration of Mg^{2+} ions in the culture, the concentration of Mg^{2+} ions was instantaneously increased (a so-called "pulse-experiment") by injecting a $MgCl_2$ solution into the culture vessel. This resulted in an immediate increase in the gluconate production rate (Table 1). Enzymatic analyses showed this

Table 1. Response of *Klebsiella pneumoniae* NTCT 418, growing under magnesium-limited conditions (with 20 μM $CaCl_2$ in the medium) to pulses with different compounds. In vitro glucose dehydrogenase (GDH) activities were determined 45 min after the pulse, in vivo activities (which remained constant) after 20 min (D = 0.3; pH = 6.0; t = 35°C)

Pulsed compound	Glucose dehydrogenase activity		
	In vitro nmol WB · min^{-1} · mg $protein^{-1}$		In vivo mmol · gdw^{-1} · h^{-1}
	Holo-enzyme	Holo- + apoenzyme	
Steady state	28	336	4.5
$MgCl_2$	128	252	21
$CaCl_2$	136	377	13
PQQ	28	350	8
PQQ + $MgCl_2$	229	377	15
PQQ + $CaCl_2$	nd	nd	19
CAP + $MgCl_2$	195	295	nd
CAP + $CaCl_2$	222	350	nd

CAP, chloramphenicol; nd, not determined
Final concentrations in culture vessel: $MgCl_2$, 20 mM; $CaCl_2$, 20 mM; PQQ, 1.25 μM; CAP, 50 μg/ml

increase to be accompanied by an increase of the GDH holoenzyme level. Interestingly, a pulse of $CaCl_2$ into the culture vessel (Table 1) resulted also in a stimulation of GDH activity.

Variations of in vivo activities (i.e. the sum of gluconate- and 2-ketogluconate production rates) observed in these experiments were always reflected in GDH holoenzyme levels present in these cells, whereas the total amount of GDH enzyme protein did not fluctuate to a large extent. This can be explained by assuming that the main effect of Mg^{2+} and Ca^{2+} ions is either on the synthesis of PQQ or on the attachment of PQQ to the GDH apoenzyme. To discriminate between these two possibilities chloramphenicol (CAP, final concentration 50 μg/ml) was pulsed into the culture prior to a pulse of the relevant ion. Under these conditions again significant levels of holoenzyme were formed. Apparently, derepression of the enzymes involved in the biosynthesis of PQQ was not necessary. This latter conclusion was confirmed by the presence of trace amounts of PQQ in the supernatant of the steady state culture (data not shown) which proved that PQQ synthesis was not limiting the assembly of GDH holoenzyme.

Because of the stimulatory effect of a Ca^{2+}-pulse and the fact that experiments with washed suspensions of different organisms have shown that there is an absolute requirement for divalent cations extracellularly to allow binding of PQQ to the GDH (van Schie et al. 1987; Ameyama et al. 1985), *K. pneumoniae* was grown under steady state magnesium-limited conditions in the presence of various concentrations of Ca^{2+} ions in the medium. As shown in Table 2, the cells were not able to produce gluconate when no Ca^{2+} was present while a stimulatory effect of Ca^{2+} ions was observed. Saturation of the GDH activity in vivo occurred at a Ca^{2+} concentration of 1 mM.

To study whether elevated concentrations Mg^{2+} or Ca^{2+} could have exerted a stimulatory effect on the synthesis of PQQ, we investigated magnesium-limited cultures of *K. pneumoniae* without added Ca^{2+} ions, but with saturating concentrations of PQQ [200 nM (Hommes et al. 1989 a)] in the medium. In these cultures, no gluconate and 2-ketogluconate production was observed (Table 2) which is in agreement with the very weak stimulation of gluconate- and 2-ketogluconate production rates after a PQQ pulse. However, a small, but significant amount of GDH holoenzyme was found. This can be explained by the fact that GDH holoenzyme was formed during the preparation of the cell-free extract, because Mg^{2+} ions may have leaked out of the cell as soon as the carbon source was removed and bound contaminating amounts of PQQ to the GDH apoenzyme. On the other hand, a similar mechanism would apply to the enzyme in cell-free extracts prepared after a pulse of PQQ to the culture, but no increased amount of GDH holoenzyme was present in this latter case.

Our results can be explained by assuming that the major role of these divalent cations is an involvement in the attachment of PQQ to the GDH apoenzyme although we would like to point out that a direct stimulatory effect

Table 2. Steady state parameters of *Klebsiella pneumoniae* NCTC 418 growing in steady state under magnesium-limited conditions with various input concentrations $CaCl_2$ in the medium. Carbon recoveries were in between 90% and 100%. Approximately 80% of the consumed glucose was converted into gluconate, 2-ketogluconate, biomass and CO_2. Minor products were acetate, pyruvate, 2,3-butanediol and sometimes lactate (D = 0.3; pH = 6.0; t = 35°C)

[$CaCl_2$] in medium μM	Glucose dehydrogenase activity			q_{ss} mmol · gdw^{-1} · h^{-1}			
	In vitro nmol WB · min^{-1} · mg $protein^{-1}$		In vivo mmol · gdw^{-1} · h^{-1}	gla	kgla	CO_2	O_2
	Holoenzyme	Holo- + apoenzyme					
0	6	122	0	0	0	14.5	14.5
0 + 200 nM PQQ	26	275	0	0	0	14.2	14.7
20	29	336	4.5	1.5	3	13.2	15.9
100	42	625	12	3.8	8.2	12.3	20.9
200	48	264	18	5.7	12.3	10.3	23.4
1000	71	280	22	7.5	14.5	10.3	27.1

of these cations on the activity of the PQQ synthesizing enzymes and/or the GDH holoenzyme itself cannot be excluded yet.

We conclude that the low GDH activities that were originally observed in magnesium-limited cultures of *K. pneumoniae*, were merely due to the low concentration of *both* magnesium and calcium ions. Although an absolute growth requirement for calcium by this strain of *K. pneumoniae* has not been demonstrated (i.e. it is typically a trace element, see Table 2), one can conclude from the observation that magnesium-limited growth conditions with elevated Ca^{2+} concentrations resulted in high GDH activities, that Ca^{2+} ions are functionally involved in the assembly of the GDH holoenzyme. It is, of course, difficult to establish which ion, Mg^{2+} or Ca^{2+}, is the "preferred" ionic species for the binding of PQQ, since potassium-limited cultures (20 μM $CaCl_2$ and 1.25 mM $MgCl_2$) were fully competent to synthesize large amounts of GDH holoenzyme. One must conclude therefore that in this respect calcium and magnesium ions are interchangeable, and that one should pay attention to the concentration of calcium ions in magnesium-limited cultures.

Hommes et al. (1985) proposed that in *K. pneumoniae* GDH, together with gluconate dehydrogenase (GaDH), acts as an auxiliary energy generating system. Indeed, a stimulation of the GDH activity (by the addition of Ca^{2+} to the medium) resulted in an increased specific oxygen consumption rate. However, this was accompanied by a decrease in the oxidation rate of the NADH formed during glycolysis and TCA cycle activity as reflected by the decrease in qCO_2 (Table 2). This suggests that the ATP requirement is fulfilled to a greater extent by the GDH and that ATP synthesis via glycolysis and TCA cycle activity must have been diminished. Hommes et al. (1989b) have obtained evidence that in this organism oxidation of $PQQH_2$ by the respiratory chain impedes NADH oxidation. The data reported here would support this proposal. Since the oxidation of NADH would generate more ATP than the oxidation of $PQQH_2$, it is not certain whether the increased oxygen consumption rates, that were observed in cultures grown in the presence of higher calcium concentration, are indicative of increased energy (ATP) production rates. On the other hand, previously high GDH activities in vivo have been observed only whenever leakage occurred of protons, potassium or phosphate ions (Hommes et al. 1985). Thus, an increase in the specific energy production rate would decrease the residual concentration of the growth-limiting ion in the culture fluids. Therefore it seems justified to assume that high GDH activities resulted indeed in a net increase of the specific energy production rate. For this reason, we propose that the high GDH activity in magnesium-limited cultures growing in the presence of 1 mM $CaCl_2$ is caused again by the energetic demands of the maintenance of a high transmembrane ion (in this case: Mg^{2+}) gradient.

Acknowledgement. JLB was the recipient of a training grant from the EEC [no. CII-0220-NL (SP)].

References

Ameyama M, Nonobe M, Hayashi M, Shinagawa E, Matsushita K, Adachi O (1985) Mode of binding of pyrroloquinoline quinone to apoglucose dehydrogenase. Agr Biol Chem 49:1227–1231

Beardmore-Gray M, Anthony C (1986) The oxidation of glucose by *Acinetobacter calcoaceticus*: interaction of the quinoprotein glucose dehydrogenase with the electron transport chain. J Gen Microbiol 132:1257–1268

Evans CGT, Herbert D, Tempest DW (1970) The continuous cultivation of micro-organisms. II. Construction of a chemostat. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 2. Academic Press, London New York, pp 277–327

Gornall AG, Bardawill CJ, David MA (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751–766

Groen BW, van Kleef MAG, Duine JA (1986) Quinohaemoprotein alcohol dehydrogenase apoenzyme from *Pseudomonas testosteroni*. Biochem J 234:611–615

Herbert D, Phipps PJ, Strange RE (1971) Chemical analysis of microbial cells. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 5B. Academic Press, London New York, pp 277–327

Hommes RWJ, van Hell B, Postma PW, Neijssel OM, Tempest DW (1985) The functional significance of glucose dehydrogenase in *Klebsiella aerogenes*. Arch Microbiol 143:163–168

Hommes RWJ, Herman PTD, Postma PW, Tempest DW, Neijssel OM (1989a) The separate roles of PQQ and apo-enzyme syntheses in the regulation of glucose dehydrogenase activity in *Klebsiella pneumoniae* NCTC 418. Arch Microbiol 151:257–260

Hommes RWJ, Postma PW, Tempest DW, Neijssel OM (1989b) The influence of the culture pH value on the direct glucose oxidative pathway in *Klebsiella pneumoniae* NCTC 418. Arch Microbiol 151:261–267

Hucho F, Wallenfels K (1972) Glucono-δ-lactonase from *Escherichia coli*. Biochim Biophys Acta 276:176–179

Jermyn MA (1960) Studies on the glucono-δ-lactonase of *Pseudomonas fluorescens*. Biochim Biophys Acta 37:78–92

Matsushita K, Nonobe M, Shinagawa E, Adachi O, Ameyama M (1987) Reconstitution of a pyrroloquinoline quinone-dependent D-glucose oxidase respiratory chain of *Escherichia coli* with cytochrome *o* oxidase. J Bacteriol 169:205–209

Matsushita K, Shinagawa E, Ameyama M (1982) D-Gluconate dehydrogenase from bacteria, 2-keto-D-gluconate-yielding, membrane bound. Methods Enzymol 89:187–193

Mulder MM, Teixeira de Mattos MJ, Postma PW, van Dam K (1986) Energetic consequences of multiple K^+ uptake systems in *Escherichia coli*. Biochim Biophys Acta 851:223–228

Neijssel OM, Tempest DW (1975) Production of gluconic acid and 2-ketogluconic acid by *Klebsiella aerogenes*. Arch Microbiol 105:183–185

Neijssel OM, Tempest DW, Postma PW, Duine JA, Frank Jzn J (1983) Glucose metabolism by K^+-limited *Klebsiella aerogenes*: evidence for the involvement of a quinoprotein glucose dehydrogenase. FEMS Microbiol Lett 20:35–39

Nelson DL, Kennedy EP (1972) Transport of magnesium by a repressible and a nonrepressible system in *Escherichia coli*. Proc Natl Acad Sci USA 69 (5):1091–1093

Schie BJ van, Mooy OH de, Linton JD, Dijken JP van, Kuenen JG (1987) PQQ-dependent production of gluconic acid by *Acinetobacter*, *Agrobacterium* and *Rhizobium* species. J Gen Microbiol 133:867–875

Tempest DW, Hunter JR (1965) Magnesium-limited growth of *Aerobacter aerogenes* in a chemostat. J Gen Microbiol 39:355–366