Arch Microbiol (1989) 151:257–260

Archives of
Microbiology

The separate roles of PQQ and apo-enzyme syntheses in the regulation of glucose dehydrogenase activity in *Klebsiella pneumoniae* NCTC 418

R. W. J. Hommes[1,*], P. T. D. Herman[1], P. W. Postma[2], D. W. Tempest[3], and O. M. Neijssel[1]

[1] Department of Microbiology, Biotechnology Centre, University of Amsterdam, P.O. Box 20245, 1000 HE Amsterdam, The Netherlands
[2] Department of Biochemistry, Biotechnology Centre, University of Amsterdam, Amsterdam, The Netherlands
[3] Department of Microbiology, University of Sheffield, Sheffield S10 2TN, UK

Abstract. No holoenzyme pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase and only very low apo-enzyme levels could be detected in cells of *Klebsiella pneumoniae*, growing anaerobically, or carrying out a fumarate or nitrate respiration. Low glucose dehydrogenase activity in some aerobic glucose-excess cultures of *K. pneumoniae* (ammonia or sulphate limitation) was increased significantly by addition of PQQ, whereas in cells already possessing a high glucose dehydrogenase activity (phosphate or potassium limitation) extra PQQ had almost no effect. These observations indicate that the glucose dehydrogenase activity in *K. pneumoniae* is modulated by both PQQ synthesis and synthesis of the glucose dehydrogenase apo-enzyme.

Key words: *Klebsiella pneumoniae* – Glucose dehydrogenase – Pyrroloquinoline quinone – Enzyme regulation – Anaerobiosis – Chemostat culture

A variety of bacteria possess an NAD(P)-independent membrane-bound glucose dehydrogenase (E.C. 1.1.99.17), that oxidizes glucose to glucono-1,4-lactone (for a review, see: Duine et al. 1986) which in its turn is hydrolyzed non-enzymatically or by means of a glucono-1,4-lactonase to gluconic acid (Jermyn 1960; Hucho and Wallenfels 1972). The cofactor of glucose dehydrogenase is pyrroloquinoline quinone [PQQ; systematic name 2,7,9-tricarboxy-1H-pyrrolo-(2,3-*f*)-quinoline-4,5-dione]. In some bacteria glucose dehydrogenase is synthesized constitutively (de Bont et al. 1984; Berka et al. 1984), whereas in others the enzyme is induced or derepressed depending upon the growth conditions (Dawes 1981; Hommes et al. 1985). Clearly, glucose dehydrogenase activity can be influenced by the synthesis of either the apo-enzyme or the cofactor PQQ. Apo-enzyme synthesis without PQQ production was first detected in *Acinetobacter lwoffi* (van Schie et al. 1984) and in *Escherichia coli* (Hommes et al. 1984). Furthermore, only apo-enzyme is produced by *Pseudomonas aeruginosa* when growing anaerobically and carrying out a nitrate respiration, while aerobically both components are synthesized (van Schie et al. 1984). Similar results have been obtained with anaerobically cultured *Rhodopseudomonas sphaeroides* (Niederpruem and Doudoroff 1965). On the other hand, it has been reported that some organisms exhibited an overproduction of PQQ (Ameyama et al. 1984; Duine et al. 1985). From these observations it is clear that the syntheses of apo-glucose dehydrogenase and PQQ are not always coordinately regulated.

Klebsiella pneumoniae (formerly known as *K. aerogenes*) also possesses a PQQ-linked glucose dehydrogenase (Neijssel et al. 1983), which, together with an FAD-linked gluconate dehydrogenase (Matsushita et al. 1982), forms a direct glucose oxidative pathway. Aerobically, the level of glucose dehydrogenase has been shown to be strongly influenced by the culture conditions, being high under those circumstances where the energetic demand for growth and/or maintenance functions is high (Hommes et al. 1985). However, no discrimination was made as to whether this was caused by an enhanced apo-enzyme synthesis or an increased production of PQQ or both. Furthermore, when cultured anaerobically under similar growth-limiting conditions or in batch cultures where nitrate or fumarate was present as an electron acceptor, no indirect evidence for glucose dehydrogenase activity, such as gluconate production, was found (Teixeira de Mattos and Tempest 1983; Forget and Pichinoty 1964). Nevertheless, there might have been apo-glucose dehydrogenase and/or PQQ synthesized. Therefore we examined more closely the role of PQQ and glucose dehydrogenase apo-enzyme syntheses in the regulation of the glucose dehydrogenase activity in *K. pneumoniae*.

* *Present address:* Gist-brocades NV, P.O. Box 1, 2600 MA Delft, The Netherlands

Offprint requests to: O. M. Neijssel

Abbreviations: PQQ, 2,7,9-tricarboxy-1H-pyrrolo-(2,3-f)quinoline-4,5-dione; WB, Wurster's Blue (1,4-bis-(dimethylamino)-benzene perchlorate)

Materials and methods

Organism

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

Growth conditions

Organisms were cultured either in a Porton-type chemostat (Herbert et al. 1965) or in a Modular Fermenter 700 Series III (LH Engineering Co. Ltd., Stoke Poges, Bucks, England). In order to obtain glucose-, ammonia-, sulphate-, phosphate- or potassium-limited growth conditions, simple salts media were used as specified by Evans et al. (1970) with glucose added as the carbon source. Carbon-limited media contained 5 g/l glucose, whereas all other media contained 30–40 g/l glucose to ensure carbon excess under potassium (0.5 mM KCl input), sulphate (0.15 mM sulphate input), ammonia (11.25 mM ammonia input), or phosphate limitation (0.5 mM phosphate input). Medium for anaerobic cultures carrying out a nitrate respiration was adjusted by adding 10 mM $NaNO_3$ and 50 mg/l $NaMoO_4 \cdot 2\,H_2O$, while the NH_4Cl concentration was lowered to 50 mM. Medium for anaerobic cultures carrying out a fumarate respiration was adjusted by adding 40 mM fumaric acid and replacing the NH_4Cl by NH_4OH. The dilution rate was set at $0.30 \pm 0.02\ h^{-1}$. The pH value of the culture was maintained automatically at pH 6.0 ± 0.1 for the aerobic cultures and at pH 6.5 ± 0.1 for the anaerobic cultures using sterile 2N NaOH as titrant, and the temperature was maintained at 35° C. Fully aerobic conditions were maintained throughout by injecting air, at a rate of 600–700 ml/min, into the region of the impeller which was rotating at about 1700 rpm. Anaerobiosis was maintained by the method described previously by Teixeira de Mattos and Tempest (1983).

Preparation of cell-free extracts

Cell-free extracts were prepared as reported previously (Hommes et al. 1985). The buffer used throughout the preparation was a 10 mM phosphate buffer (pH 6.0) containing 5 mM $MgCl_2$.

Enzyme assay

The spectrophotometric assay of glucose dehydrogenase using Wurster's Blue (WB) as the electron acceptor was carried out at 35° C with freshly-prepared cell-free extracts using a Beckman DU40 spectrophotometer (Beckman Instruments Inc., Irvine, USA) according to Hommes et al. (1985). To determine maximal glucose dehydrogenase activity (as a measure for the apo-enzyme level), the cell-free extracts were incubated for 15 min with 100 μM PQQ at 35° C prior to the assay.

PQQ-pulse procedure

In order to determine maximal glucose dehydrogenase activity in situ, 2 ml of a sterilized solution of PQQ was injected into the culture by means of a syringe, providing a final concentration of 0.2 μM, while all other culture conditions remained unchanged. Samples were taken at regular intervals, the cells were sedimented in an Eppendorf centrifuge and the supernatants were frozen to be analyzed later.

Table 1. Glucose dehydrogenase activities in cell-free extracts of *Klebsiella pneumoniae*, grown anaerobically in chemostat cultures ($D = 0.3\ h^{-1}$; pH 6.5; 35° C). When extra PQQ was added, the mixture was preincubated with 100 μM PQQ for 15 min at 35° C prior to glucose addition

Culture conditions	Glucose dehydrogenase activity nmol WB reduced · min^{-1} · mg protein^{-1}	
	−PQQ	+PQQ
Glucose limitation	<1	4
Potassium limitation	<1	12
Potassium limitation + fumarate	<1	11
Potassium limitation + nitrate	<1	15

Analyses

Glucose, gluconate, and 2-ketogluconate were determined by HPLC (LKB, Bromma, Sweden) with an Aminex A28 column (Biorad, Richmond, USA) using a 2142 Refractive Index Detector (LKB, Bromma, Sweden), an SP 4270 Integrator (Spectra Physics, San Jose, USA) and 0.3 M formic acid adjusted to pH 5.5 with ammonia as eluent at 55° C. Protein was assayed by the biuret method using bovine serum albumin as standard (Gornall et al. 1949). Oxygen consumed, and carbon dioxide produced by the cultures were determined by passing the effluent gas from the fermenter through an oxygen analyzer (Taylor Servomex Type OA 272, Crowborough, Sussex, England) and a carbon dioxide analyzer (Servomex IR Gas Analyzer PA404, Crowborough, Sussex, England).

Chemicals

All reagents used were of the best analytical grade commercially available. Wurster's Blue was prepared according to Michaelis and Granick (1943). PQQ was kindly provided by Prof. J. A. Duine (Dept. of Microbiology, Delft University of Technology, The Netherlands).

Results and discussion

When growing aerobically, *Klebsiella pneumoniae* is able to produce gluconate and 2-ketogluconate (Neijssel and Tempest 1975) by means of a direct oxidative pathway, in which the activity of the key enzyme, glucose dehydrogenase, is strongly dependent on the growth conditions (Hommes et al. 1985). Not surprisingly, however, gluconate or 2-ketogluconate were never detected as fermentation products in anaerobic cultures of *K. pneumoniae* (Teixeira de Mattos and Tempest 1983). Nevertheless, this does not necessarily imply that anaerobically grown cells were devoid of glucose dehydrogenase activity or at least of the apo-enzyme or the cofactor PQQ. Hence we undertook a search for glucose dehydrogenase activity in cells of *K. pneumoniae* that had been cultured under different anaerobic conditions.

As is shown in Table 1 no holo-glucose dehydrogenase could be detected, not even when the cells were provided with an electron acceptor which allowed anaerobic respiration to take place. That both fumarate and nitrate could support anaerobic respiration was clearly evident from the increased

Table 2. Glucose dehydrogenase activities in cell-free extracts of *Klebsiella pneumoniae*, grown aerobically in chemostat cultures (D = 0.3 h^{-1}; pH 6.0; 35°C), and the increase (%) in glucose dehydrogenase activity after preincubation with 100 µM PQQ for 15 min at 35°C prior to the glucose addition

Limitation	Glucose dehydrogenase activity nmol WB reduced · min^{-1} · mg $protein^{-1}$		
	−PQQ	+PQQ	Increase (%)
Carbon	25	66	160
Sulphate	26	240	820
Ammonia	13	170	1200
Potassium	150	200	33
Phosphate	120	200	67

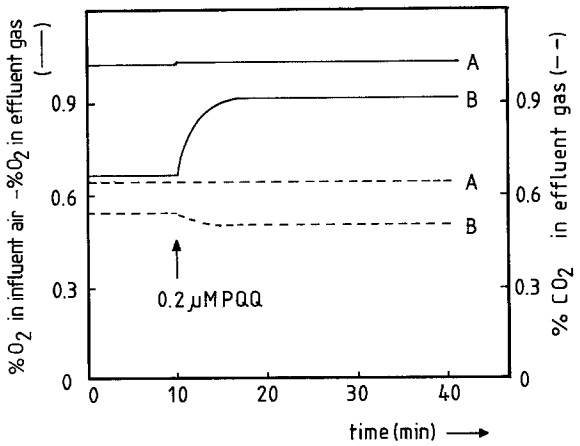

Fig. 1. Recorder tracings of oxygen consumption and carbon dioxide production of chemostat cultures of *Klebsiella pneumoniae* (D = 0.3 h^{-1}; pH 6.0; 35°C), grown under potassium-limited conditions (*A*), and under ammonia-limited conditions (*B*). At t = 10 min a sterile solution of PQQ was added to the steady state cultures to obtain a final concentration of 0.2 µM PQQ

production of acetate by these cultures of *K. pneumoniae*, implicating a corresponding increase in NADH generation (data not shown). When PQQ was added to the cell-free extracts only very low apo-glucose dehydrogenase activities were observed. These results indicate that under anaerobic conditions the synthesis of both the apo-enzyme and PQQ were repressed. This form of regulation of apo-enzyme synthesis is clearly different from that found with *Pseudomonas aeruginosa* PAO1, where an undiminished level of apo-glucose dehydrogenase was detected, when organisms were grown anaerobically on glucose in the presence of nitrate (van Schie et al. 1984), or, again, in anaerobically grown *Rhodopseudomonas sphaeroides* (Niederpruem and Doudoroff 1965), although the identity and structure of the cofactor was unknown at that time.

In order to investigate the possible role of PQQ synthesis in the modulation of glucose dehydrogenase activity, *K. pneumoniae* was also grown under aerobic conditions in chemostat cultures. Since at low pH values a high glucose dehydrogenase activity was manifest in variously-limited chemostat cultures of this organism (Hommes 1988), and in vitro a high affinity of the apo-glucose dehydrogenase of *Acinetobacter calcoaceticus* for PQQ was observed (van Schie et al. 1987), the organisms were cultured aerobically at pH 6.0 under different nutrient limitations. As expected from previous findings (Hommes et al. 1985), the highest holo-glucose dehydrogenase activities were observed in cell-free extracts of *K. pneumoniae* grown under either phosphate- or potassium-limitation (Table 2). Just as was found with organisms growing anaerobically, glucose dehydrogenase activity was regulated in part by apo-enzyme synthesis, the cell-free extract of carbon-limited cells having lower glucose dehydrogenase activity after preincubation with saturating amounts of PQQ as compared with the other cell-free extracts from glucose-sufficient cultures. However, the apo-glucose dehydrogenase levels in these latter cell-free extracts showed no marked differences (Table 2), suggesting that the glucose dehydrogenase activities, as manifest in vivo, are regulated by the synthesis of the cofactor PQQ (cf. activities with and without added PQQ). On the other hand the glucose dehydrogenase activities expressed in vivo under glucose-excess conditions might be regulated in some other (e.g. kinetic) way, or the differences in holo-enzyme activity might be due simply to the loss of varying amounts of PQQ from the enzyme during the preparation of the cell-free extracts. Therefore to test the hypothesis that, beside the apo-glucose dehydrogenase synthesis, PQQ synthesis is also an important factor in the regulation of the glucose dehydrogenase enzyme activity, we studied the effect of addition of extra PQQ to chemostat cultures of *K. pneumoniae*.

If the glucose dehydrogenase activity, as expressed in the culture, is directly proportional to the amount of PQQ available, an increased concentration of this cofactor should be followed by a stimulated enzyme activity. Hence, once steady state conditions prevailed, 2 ml of a sterilized solution of PQQ was injected into the culture, providing an end concentration of 0.2 µM, while all other culture conditions remained constant. An increased glucose dehydrogenase activity would give rise to a stimulated rate of gluconate production with a concomitant increase in oxygen consumption. Therefore, to obtain an indication of the glucose dehydrogenase activity, the oxygen consumption and the carbon dioxide production of the cultures were monitored simultaneously. Whereas the addition of extra PQQ to a potassium-limited culture seemingly had no effect, the oxygen consumption of an ammonia-limited culture of *K. pneumoniae* increased instantaneously (Fig. 1). This increase, of almost 40%, represented an extra consumption rate of oxygen of 5.8 mmol $O_2 \cdot h^{-1} \cdot$ (g dry wt cells)$^{-1}$. Moreover, not only the oxygen consumption rate increased, but also the carbon dioxide production showed a small but reproducible decrease. At first sight, this seems difficult to explain, but bearing in mind the fact that glucose dehydrogenase feeds electrons to the respiratory chain (probably) at the level of ubiquinone (Beardmore-Gray and Anthony 1986; Matsushita et al. 1987), and thus will compete with, and might impede, respiratory NADH oxidation, the lowering of the CO_2 production rate well could be a consequence of a "back pressure" exerted on the tricarboxylic acid cycle enzymes due to a diminished availability of NAD^+. Similar differential results were obtained using a phosphate-limited culture (no effect after addition of PQQ) and a sulphate-limited culture (large effect after addition of PQQ). These results show that, whereas the amounts of apo-enzyme that were detected in cell-free extracts of potassium- or phosphate-limited cells could have been caused by loss of PQQ from the enzyme during the preparation procedure, in

sulphate- or ammonia-limited cells the major part of the enzyme molecules were present in vivo in the apo-form.

The combined rate of gluconate and 2-ketogluconate production in a chemostat culture is an indication of the glucose dehydrogenaes activity expressed by the actively growing cells. Whereas the sum of the rates of production of gluconate [1.2 mmol · h^{-1} · (g dry wt cells)$^{-1}$] and 2-ketogluconate [1.3 mmol · h^{-1} · (g dry wt cells)$^{-1}$] by *K. pneumoniae* growing in a sulphate-limited chemostat culture, indicates an in vivo activity of the glucose dehydrogenase of 2.5 mmol · h^{-1} · (g dry wt cells)$^{-1}$), the rate of gluconate production immediately rose to 12 mmol · h^{-1} · (g dry wt cells)$^{-1}$) after the addition of extra PQQ to the culture. This shows that the increased oxygen consumption rate was indeed due to a higher glucose dehydrogenase activity in situ and these observations emphasize the important role of PQQ in the regulation of the glucose dehydrogenase activity. Even the production of 2-ketogluconate increased slightly as a result of the extra gluconate formed by the glucose dehydrogenase [in agreement with an observed overcapacity of the gluconate dehydrogenase in situ (RWJ Hommes, unpublished results)]. It has to be noted that the increase in the rate of gluconate production after addition of PQQ to the sulphate-limited chemostat culture was smaller than the increase in glucose dehydrogenase activity as measured in vitro (Table 2). Although it is likely that some loss of PQQ during the preparation of the cell-free extracts occurred (cf. potassium- or phosphate-limited cells), it is important to realize that electron acceptor in the in vitro assay (Wurster's Blue) is clearly different from the electron acceptor in vivo and this will have a profound effect on the activity of the enzyme. In addition, it has been shown that higher concentration of PQQ in the chemostat cultures did not lead to any further effect, indicating that 0.2 μM PQQ fully saturated the apo-enzyme with the cofactor (unpublished results).

In conclusion, it will be clear from the results presented herein that the activity of the glucose dehydrogenase in *K. pneumoniae* is regulated by both the glucose dehydrogenase apo-enzyme synthesis and by the synthesis of the cofactor PQQ. This mode of regulation is clearly different from that found in other organisms; that is, that regulation of glucose dehydrogenase activity occurs primarily by the way of a modulation of PQQ synthesis (van Schie et al. 1984).

Acknowledgements. This research was supported by the Foundation for Fundamental Biological Research (BION), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

References

Ameyama M, Hayashi M, Matsushita K, Shinagawa E, Adachi O (1984) Microbial production of pyrroloquinoline quinone. Agr Biol Chem 48:561–565

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

Berka RT, Allenza P, Lessie TG (1984) Hyperinduction of enzymes of the phosphorylative pathway of glucose dissimilation in *Pseudomonas cepacia.* Curr Microbiol 11:43–148

Bont JAM de, Dokter P, Schie BJ van, Dijken JP van, Frank Jzn J, Duine JA, Kuenen JG (1984) Role of quinoprotein glucose dehydrogenase in gluconic acid production by *Acinetobacter calcoaceticus.* Antonie van Leeuwenhoek 50:76–77

Dawes EA (1981) Carbon metabolism. In: Calcott PH (ed) Continuous cultures of cells, vol II. CRC Press, Boca Raton, Florida, USA, pp 1–38

Duine JA, Frank Jzn J, Jongejan JA (1985) The coenzyme PQQ and the quinoproteins, a novel class of oxidoreductase enzymes. In: Ovchinikov Y (ed) Proceedings of the 16th FEBS Congress, Part A, VNU Science Press, Utrecht, pp 79–88

Duine JA, Frank Jzn J, Jongejan JA (1986) PQQ and quinoprotein enzymes in microbial oxidations. FEMS Microbiol Rev 32:165–178

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. Academic Press, London New York, pp 277–327

Forget P, Pichinoty F (1964) Influence de la respiration anaérobie du nitrate et du fumarate sur le métabolisme fermentaire d'*Aerobacter aerogenes.* Biochim Biophys Acta 82:441–444

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

Herbert D, Phipps PJ, Tempest DW (1965) The chemostat: Design and instrumentation. Lab Pract 14:1150–1161

Hommes RWJ (1988) The role of the PQQ-linked glucose dehydrogenase in the physiology of *Klebsiella aerogenes* and *Escherichia coli.* PhD thesis, University of Amsterdam

Hommes RWJ, Postma PW, Neijssel OM, Tempest DW, Dokter P, Duine JA (1984) Evidence of a quinoprotein glucose dehydrogenase apo-enzyme in several strains of *Escherichia coli.* FEMS Microbiol Lett 24:329–333

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

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

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

Michaelis L, Granick S (1943) The polymerization of the free radicals of the Wurster Dye type: the dimeric resonance bound. J Am Chem Soc 65:1747–1755

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

Niederpruem DJ, Doudoroff M (1965) Cofactor-dependent aldose dehydrogenase from *Rhodopseudomonas sphaeroides.* J Bacteriol 89:697–705

Schie BJ van, Dijken JP van, Kuenen JG (1984) Non-coordinated synthesis of glucose dehydrogenase and its prosthetic group PQQ in *Acinetobacter* and *Pseudomonas* species. FEMS Microbiol Lett 24:133–138

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

Teixeira de Mattos MJ, Tempest DW (1983) Metabolic and energetic aspects of growth of *Klebsiella aerogenes* NCTC 418 on glucose in anaerobic chemostat cultures. Arch Microbiol 134:80–85

Received July 26, 1988/Accepted November 9, 1988