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PROPERTIES AND REGULATION OF SYNTHESIS OF THE GLYCEROL DEHYDROGENASE PRESENT IN *KLEBSIELLA AEROGENES* NCTC 418, GROWING IN CHEMOSTAT CULTURE

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1. Introduction

Klebsiella aerogenes, a member of the Enterobacteriaceae, is able to use glycerol as the sole carbon and energy source when growing either aerobically or anaerobically, and it has been shown to possess two glycerol assimilatory pathways [1,2]. In the first pathway, glycerol is converted to glycerol 3-phosphate (by glycerol kinase) which then is oxidized to dihydroxyacetone phosphate by a specific flavoprotein-linked dehydrogenase. The second pathway consists of an NAD-linked glycerol dehydrogenase, which oxidizes glycerol to dihydroxyacetone, and this product is then thought to be phosphorylated to dihydroxyacetone phosphate. Neijssel et al. [2] further showed that modulation between the alternative pathways of glycerol metabolism was not dependent solely upon the degree of aeration of the culture, as was suggested by Lin et al. [1], but also that it was influenced by the level of exogenous glycerol present in the growing culture. Thus, glycerol kinase was only detected in aerobically growing organisms when the carbon and energy source was present in growth-limiting concentrations. In contrast, glycerol dehydrogenase was found in cells growing both aerobically and anaerobically whenever glycerol-sufficient conditions prevailed. This finding conflicted with the conclusions of Lin et al. [1] who suggested that glycerol dehydrogenase was inactivated in the presence of oxygen and therefore was not functionally relevant to aerobic growth.

Although the evidence presented by Neijssel et al. [2] seemed convincing, it could be criticised on the grounds that the activities of glycerol dehydrogenase measured in cell-free extracts of aerobically grown organisms never exceeded 20 nmoles/min. mg protein, a value that was far below that needed to account for the rate of glycerol metabolism expressed by the growing culture (and by washed suspensions). Moreover, their findings did not exclude the possibility of there being a third pathway of glycerol assimilation present in these organisms. Consequently, if it is assumed that the glycerol dehydrogenase activities reported by these workers are functionally insignificant, then the regulation mechanism proposed by Lin [3] still could be valid.

The present communication shows firstly that the glycerol dehydrogenase activities found previously in cells growing aerobically were, in fact, underestimated, and are indeed functionally significant; and secondly, that the influence that the concentration of extracellular glycerol exerts on the activity of glycerol kinase and glycerol dehydrogenase in growing cultures fully accords with the proposal made previously [2] that these two pathways represent alternative high- and low-affinity glycerol uptake systems for the aerobic growth of *Klebsiella aerogenes* NCTC 418.

2. Material and Methods

2.1. Organism

K. aerogenes NCTC 418 was maintained by monthly subculture on tryptic meat-digest agar slopes.

2.2. Growth conditions

Organisms were grown in mineral salts media [4] in 500 ml Porton-type chemostats [5]. The media

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contained 1 mM KCl as the potassium source and concentrations of glycerol varying from 65 mM (glycerol-limitation) to 390 mM (potassium-limitation). The culture pH value was maintained automatically at 6.8 ± 0.1, and the temperature at 35°C. The dilution rate was set at 0.30 ± 0.02 h^{-1}.

2.3. Enzyme analyses

Cell-free extracts were prepared, and enzyme activities measured, as described previously [2].

2.4. Chemical analyses

The concentration of glycerol in the culture extracellular fluids was determined enzymically [6] as was acetate [7]. Protein was estimated by the biuret method.

3. Results

It was reported previously [2] that glycerol dehydrogenase was present in ammonia- and suphate-limited *K. aerogenes* organisms that were growing aerobically in the presence of excess glycerol, and that these organisms seemingly were devoid of glycerol kinase activity. It was concluded, therefore, that glycerol was assimilated via the glycerol dehydrogenase reaction. However, the activities found for the latter enzyme were far too low to account for the glycerol uptake rate exhibited by the growing organisms, suggesting either that partial inactivation of the enzyme had occurred during the isolation procedure or else that sub-optimal test conditions had been employed. Inactivation was thought improbable since extracts of anaerobically grown organisms possessed a very considerable glycerol dehydrogenase activity. It seemed reasonable, therefore, to suspect that the assay conditions used were the cause of this discrepancy. In this connection, the test conditions used differed chemically from the growth environment in several important respects. In particular, the former lacked trace metals (Ca^{2+}, MoO_4^{2-}, Cu^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}) as well as magnesium and the chelating agent (citrate). Therefore the influence of these ions on the activity in vitro of glycerol dehydrogenase was studied.

Crude cell-free extracts were prepared from glycerol-grown aerobic potassium-limited cultures and assayed for glycerol dehydrogenase activity. A range of concentrations of MoO_4^{2-} and the various cations were then added to the test solutions and the effects noted. Table 1 shows the activities of glycerol dehydrogenase at the optimal concentration of each element as a percentage of the activity found under the "standard" test conditions [2]. Clearly, of the cations tested, only manganese exerted a profound effect. Thus, in the presence of 80 μM Mn^{2+} the V_{max} of the enzyme reaction was increased 5-fold whilst the apparent K_m value was unchanged (Fig. 1). The glycerol dehydrogenase activity so measured now could account for about 30% of the rate of glycerol metabolism expressed by the growing organisms, which is sufficient to allow of the conclusion that it actually functions in glycerol assimilation by aerobically growing glycerol-sufficient cultures. In subsequent experiments the glycerol dehydrogenase activity was routinely assayed in the presence of 80 μM Mn^{2+}.

It was important at this stage to establish how the activities of glycerol dehydrogenase and glycerol kinase varied with the concentration of glycerol in the culture extracellular fluids. Therefore an experiment was performed in which the extracellular glycerol concentration was varied whilst all the other

TABLE 1

Influence of metal ions on the activity of glycerol dehydrogenase present in crude extracts of potassium-limited *Klebsiella aerogenes*, grown in the presence of excess glycerol

Additions	Concentration [a] (mM)	Activity [b] (%)
None	–	100
Mg^{2+}	3.8	50
MoO_4^{2-}	0.3	90
Cu^{2+}	0.008	190
Fe^{2+}	0.15	190
Co^{2+}	0.015	190
Mn^{2+}	0.08	500
$Mn^{2+} + Co^{2+}$	0.08; 0.015	490
$Mn^{2+} + Co^{2+} + Cu^{2+}$	0.08; 0.015; 0.008	250

[a] Concentrations effecting maximum stimulation of activity.
[b] Activity is given as a percentage of that expressed under "standard" test conditions [2].

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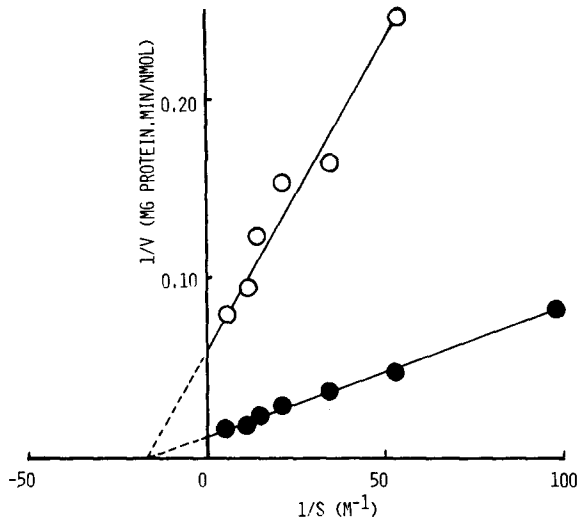

Fig. 1. Lineweaver-Burke plot of the glycerol dehydrogenase activity expressed by crude extracts of glycerol-grown potassium-limited *Klebsiella aerogenes*. Activities in the absence (○) and presence (●) of 80 μM $MnCl_2$.

growth conditions were held constant. This was achieved by growing *K. aerogenes* aerobically in a chemostat under conditions such that glycerol availability was limiting the growth rate with all other nutrients in a large excess, except for potassium which was present in an amount only slightly greater than that needed to meet the organisms' needs. Thus, by increasing the glycerol concentration in the input medium the growth of the culture could be changed to being potassium-limited, leaving an excess of glycerol in the extracellular fluid in a graded concentration. After each step-wise increase in the medium glycerol concentration, steady state conditions could be established and the organisms then tested for their glycerol dehydrogenase and glycerol kinase activities. The growth rate was kept constant (at 0.3 h^{-1}) throughout.

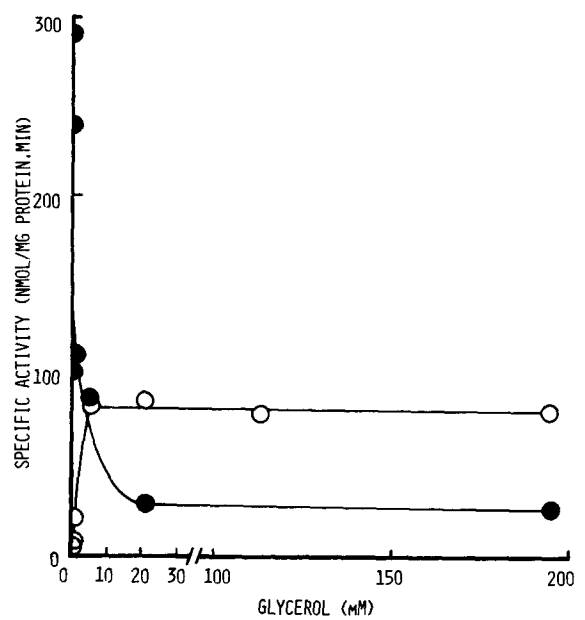

Fig. 2. Activities of glycerol kinase (●) and glycerol dehydrogenase (○) present in crude extracts of *Klebsiella aerogenes* organisms that were cultured in the presence of varying extracellular concentrations of glycerol.

The results obtained (Fig. 2) show unequivocally that the extracellular glycerol concentration exerts a profound influence on the regulation of glycerol metabolism; as its concentration is increased, so the activity of glycerol dehydrogenase sharply increased to a level of 80 nmoles glycerol oxidized/min. mg protein. This activity was about 30% of that necessary to account fully for the rate of glycerol utilization extant in the growing culture. In contrast, glycerol kinase activity decreased sharply, with increasing glycerol concentration, attaining a residual activity of only 7% of that expressed under glycerol-limiting conditons (where the extracellular glycerol concentration is vanishingly small).

Finally, we determined the effect of a sudden increase in the extracellular glycerol concentration on the glycerol kinase and glycerol dehydrogenase activites of *K. aerogenes* growing aerobically under glycerol-limiting conditions. The organisms were cultured at a dilution rate of 0.3 h^{-1} with glycerol as the sole carbon and energy source, and after a study state had been attained the extracellular concentration of glycerol was suddenly increased to 180 mM. Figs. 3a and 3b show the measured activities of glycerol kinase and glycerol dehydrogenase, and the changes in the extracellular concentration of glycerol, as functions of time following the pulse. Clearly, glycerol kinase synthesis was severely repressed when exogenous glycerol was present in concentrations above 30 mM; below this concentration the organisms regained their glycerol kinase activity. Concomitant with the change in glycerol kinase activity, glycerol dehydrogenase initially increased, but there was no further increase once the glycerol had been largely consumed.

Regarding the changes observed in the glycerol

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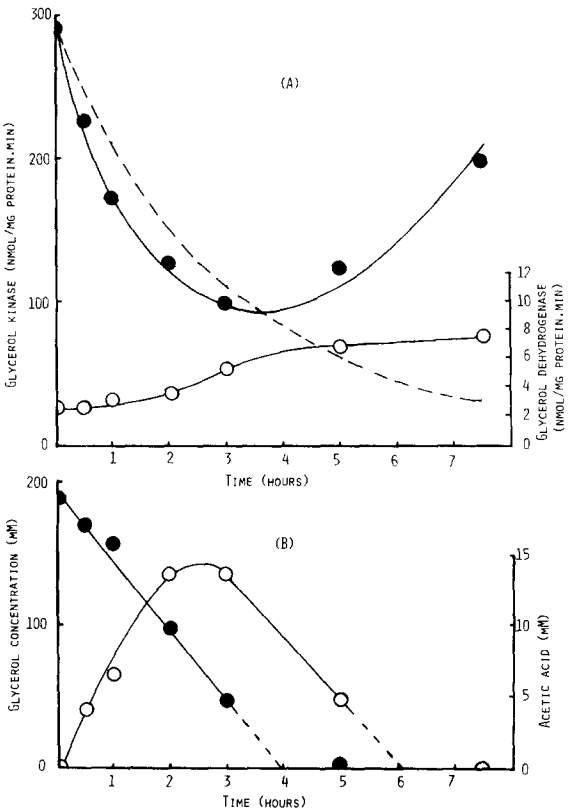

Fig. 3. (a) Glycerol kinase (•) and glycerol dehydrogenase (○) activities following the pulse addition of 180 mM glycerol to a glycerol-limited culture of *Klebsiella aerogenes*. The broken line shows the theoretical washout rate for glycerol kinase assuming complete repression of its synthesis following addition of the excess glycerol. (b) Concentrations of (○) acetate and (•) glycerol as a function of time following the pulse-addition of glycerol.

kinase activity, it should be noted that a complete inhibition of glycerol kinase synthesis following the pulse would have led to a washout of that activity present initially in the growing cells at a rate that was a function of the culture dilution rate. The broken line (Fig. 3a) represents this theoretical washout rate for a culture growing at a $D = 0.3\ h^{-1}$; and comparison of this with the observed decrease clearly reveals that there must have occurred an immediate and total cessation of glycerol kinase synthesis following addition of the excess glycerol to the culture.

The initial glycerol consumption rate on the growing culture, calculated from the decrease in extracellular glycerol concentration 1 h following the pulse, was equal to that estimated from the glycerol kinase activity (assuming a cellular protein content of 70%, w/w). The glycerol consumption rate expressed by the steady state culture prior to the pulse was, however, only half this value. Clearly, the glycerol oxidizing potential of glycerol-limited cells was substantially greater than could be expressed under carbon-limiting conditions, a conclusion that is further supported by the finding of a rapid excretion of acetate following the glycerol pulse (Fig. 3b).

4. Discussion

The results presented here reinforce the conclusion drawn previously [2] that *aerobic* cultures of *K. aerogenes* NCTC 418 possess the ability to modulate their glycerol assimilatory mechanisms in response to changes in the extracellular glycerol concentration. Excess glycerol triggers both repression of glycerol kinase synthesis and induction of glycerol dehydrogenase synthesis. However, glycerol per se may not be the inducer/repressor molecule, rather it may follow from other changes associated with the saturation of the organisms with glycerol. One might speculate that organisms possess this type of regulatory mechanism in order to prevent traumatic effects exerted by an unrestricted uptake of glycerol and intracellular accumulation of phosphorylated derivatives. In this connection, results obtained with *Escherichia coli* K12 are relevant since it was suggested [8,9] that accumulation in these cells of phosphorylated intermediates provoked the synthesis of methylglyoxal, a growth inhibitory compound. Therefore, a rapid switch from glycerol kinase, with a low apparent K_m for glycerol ($\sim 10^{-6}$ M), to glycerol dehydrogenase, with a high apparent K_m (20 mM), at elevated glycerol concentrations, safeguards the organisms against similar traumatic effects.

Below an extracellular glycerol concentration of 20 mM, glycerol kinase still is present in potassium-limited cells in measurable amounts (Fig. 2), which may be necessary to compensate for glycerol dehydrogenase functioning far below its maximum activity at low glycerol concentrations. Further increase of the glycerol concentration promotes the synthesis of glycerol dehydrogenase and the rate of glycerol assimilation now can be almost fully regulated by the redox

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state extant within the cell since this reaction cannot proceed in the absence of NAD^+. Hence a high NADH/NAD^+ ratio will impede glycerol oxidation and circumvent accumulation of phosphorylated intermediates. On the other hand, induction of glycerol kinase under carbon-limiting conditions guarantees a rapid and efficient uptake of glycerol.

With regards to quantitative aspects of the modulation mechanism, one might argue that the glycerol kinase activity as well as the glycerol dehydrogenase activity present in organisms growing at steady state extracellular glycerol concentrations between 5 and 20 mM, together are too low to account for the glycerol uptake rate expressed by the growing organisms (about 12 mmoles glycerol/g dry weight.h). However, it is not unreasonable to suppose that determinations in vitro of enzyme activities often may provide underestimates of the actual activities expressed in vivo. Therefore, considering the substantial activities obtained, there seems to be no good reason to suspect the presence of *K. aerogenes* of yet a third glycerol assimilating enzyme system.

The presence of glycerol dehydrogenase, as the first enzyme of the low-affinity glycerol assimilatory system requires the participation of an enzyme phosphorylating dihydroxyacetone. Although such an enzyme has yet to be detected in glycerol-sufficient organisms, its presence is clearly suggested by the fact that washed suspensions of *K. aerogenes* NCTC 418, obtained from glycerol-grown ammonia- or sulphate-limited cultures, rapidly oxidized dihydroxyacetone at a rate equal to that of glycerol (Hueting, unpublished result). Glycerol kinase, which also is able to phosphorylate dihydroxyacetone, was not detectable under these conditions [2].

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