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Control of Glycolysis by Glyceraldehyde-3-Phosphate Dehydrogenase in *Streptococcus cremoris* and *Streptococcus lactis*

BERT POOLMAN, BOUKJE BOSMAN, JAN KIERS, AND WIL N. KONINGS*

Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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The decreased response of the energy metabolism of lactose-starved *Streptococcus cremoris* upon readdition of lactose is caused by a decrease of the glycolytic activity (B. Poolman, E. J. Smid, and W. N. Konings, J. Bacteriol. 169:1460-1468, 1987). The decrease in glycolysis is accompanied by a decrease in the activities of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase. The steady-state levels of pathway intermediates upon refeeding with lactose after various periods of starvation indicate that the decreased glycolysis is primarily due to diminished glyceraldehyde-3-phosphate dehydrogenase activity. Furthermore, quantification of the control strength exerted by glyceraldehyde-3-phosphate dehydrogenase on the overall activity of the glycolytic pathway shows that this enzyme can be significantly rate limiting in nongrowing cells.

In a previous communication, we have presented evidence that the survival of *Streptococcus cremoris* upon lactose starvation in chemostat cultures is related to the glycolytic capacity maintained (12). A rapid decrease of glycolytic activity was observed for lactose-starved cells in growth medium (anaerobically) and in phosphate-lactate buffer (aerobically) (12). Initially, the decrease of glycolysis did not result in a lower steady-state level of the proton motive force (Δp), but the rate of proton motive force generation was severely affected. However, when glycolysis decreased further over time, the high proton motive force could not be maintained and the activity of proton motive force-driven processes, like amino acid transport, decreased (12). In this paper, the cause of the drop in glycolytic activity upon (lactose) starvation has been analyzed.

Enzymatic activities. The decrease in glycolysis during starvation as described previously (12) was also observed for *S. cremoris* Wg2 (and *S. lactis* ML3) cells grown on complex broth (4) in batch cultures (Fig. 1). Cells were grown to an optical density at 660 nm of about 0.8, washed twice, and suspended in sterile phosphate-lactate buffer (pH 6.4) as described elsewhere (12). At the times indicated, samples were taken and the acidification of the medium was monitored (11). To study the effect of starvation on the activities of glycolytic enzymes, cell-free extracts were prepared (10) after three different periods of starvation, as indicated by the arrows (Fig. 1). Enzymatic activities were determined by standard procedures involving NAD, NADH-, or NADP-coupled assays (3). The effects of starvation on the activities of glyceraldehyde-3-phosphate (G3P) dehydrogenase (EC 1.2.1.12) and phosphoglycerate mutase (EC 5.4.2.1) are shown in Table 1. Both activities decreased concomitant with the decrease in glycolysis. The activities of all other glycolytic enzymes were not significantly affected by the starvation regime (data not shown). The activities of these enzymes varied between 1 and 6 U/mg of protein, except for phosphoglycerate kinase (EC 2.7.2.3), which had an activity of about 0.4 U/mg of protein, independent of the starvation period.

Glycolytic intermediates. To analyze whether G3P dehydrogenase or phosphoglycerate mutase was primarily responsible for the decrease in glycolytic activity, the steady-

state levels of glycolytic intermediates were analyzed after various periods of starvation (Fig. 1) upon refeeding with lactose. When the flux through a metabolic pathway is decreased, specifically by a decrease in activity of a single enzyme, the steady-state concentrations of substrate(s) and product(s) of that enzyme can be expected to increase and decrease, respectively. At times indicated (Fig. 1), lactose was added to parallel suspensions of starved *S. cremoris* cells. Samples (1.0 ml) were withdrawn (after 0 to 60 min of glycolysis), and glycolytic intermediates were extracted and assayed by fluorescence spectrophotometry with NADH- or NADP-coupled indicator systems, as described by Maitra and Estabrook (9) and modified by Poolman et al. (12). The steady-state concentrations (taken from time curves) of G3P plus dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3-PG), 2-phosphoglycerate (2-PG), and phosphoenolpyruvate upon renewed addition of lactose after various periods of starvation are shown in Table 2. Time courses of intracellular concentrations of glycolytic intermediates indicated that steady-state concentrations of 3-PG, 2-PG, and phosphoenolpyruvate were reached within 10 min of glycolysis, independent of starvation time, whereas the time to reach steady-state concentrations of DHAP plus G3P increased with the starvation period (data not shown). Upon addition of lactose during starvation, the steady-state levels of DHAP plus G3P increased, whereas the concentrations of intermediates following G3P dehydrogenase decreased (Table 2). The concentrations of 1,3-diphosphoglycerate, a product of the reaction catalyzed by G3P dehydrogenase, were too low to estimate accurately. The increase in DHAP plus G3P concentration most probably reflects the decreased activity of G3P dehydrogenase, since triose-phosphate isomerase (EC 5.3.1.1) was not affected by starvation and operates fast and close to equilibrium. The comparable effects of starvation on the steady-state concentrations of 3-PG (substrate of phosphoglycerate mutase) and 2-PG (product) indicate that the immediate decrease in glycolysis upon starvation (Fig. 1) is not likely to be caused by a decrease in the activity of phosphoglycerate mutase. The decrease of glycolytic activity upon lactose starvation is thus most probably due to a decrease in G3P dehydrogenase activity.

Control strength of G3P dehydrogenase. The experiments presented thus far indicate that the overall glycolytic activity

* Corresponding author.

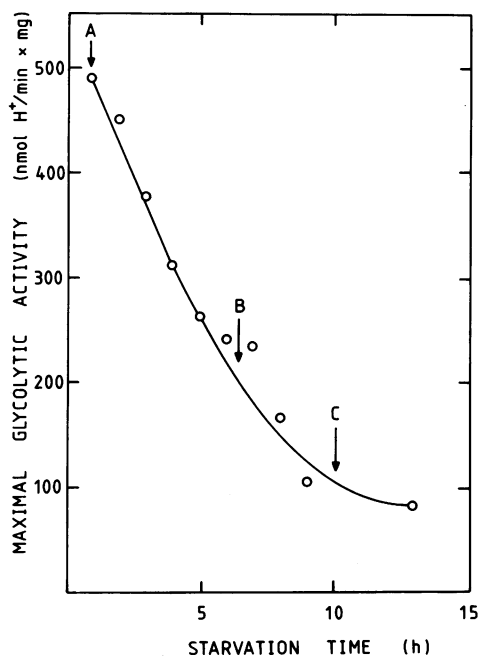


FIG. 1. Effect of starvation on the maximal glycolytic activity of *S. cremoris* Wg2. Batch-grown cells were starved (at 30°C) in 50 mM potassium phosphate (pH 6.4)–50 mM sodium lactate–1 mM MgSO₄ at a final protein concentration of 1.5 mg/ml. At times indicated, the cells were diluted 10-fold in 5 mM K-piperazine-*N,N'*-bis(2-ethanesulfonic acid) (K-PIPES) (pH 7.0)–50 mM KCl–5 mM MgSO₄. After equilibration at 30°C, lactose was added to a final concentration of 10 mM. pH changes were converted into nanomoles of H⁺/min × mg of protein, as described previously (11). The sample identifications (A, B, and C) were used to denote the starvation times at which samples were withdrawn for measurement of glycolytic enzymes (Table 1) and the steady-state levels of glycolytic intermediates after readdition of lactose (Table 2).

of *S. cremoris* cells is determined to a large extent by the activity of G3P dehydrogenase. To quantify the control strength exerted by G3P dehydrogenase on the flux through the glycolytic pathway, the principles of the metabolic control theory were applied (5, 7). The control strength ($C_{E_i}^J$) of a step in a metabolic pathway is defined as the fractional change in flux through the pathway induced by a fractional change in the enzyme concerned, or in mathematical terms

$$C_{E_i}^J = \frac{(dJ/J)}{(d[e]/[e])_{ss}} \quad (1)$$

where J is the steady state pathway flux, $[e]$ is the enzyme concentration, and ss refers to steady state conditions. Under conditions in which the initial concentrations of substrate(s) and product(s) are kept constant, it can be shown that the sum of all control strengths in the pathway is equal to 1 (5, 7). Instead of varying the concentration of a particular enzyme, which obviously has practical limitations, the concept of the metabolic control theory can also be applied to enzymes for which a specific irreversible inhibitor is available. The following equation can now be derived for $C_{E_i}^J$ (16):

$$C_{E_i}^J = \frac{\{(dJ/J)/(dI/I)\}_{ss}}{\{(dv_i/v_i)/(dI/I)\}_{S,P_i}} \quad (2)$$

TABLE 1. Effect of starvation on activities of G3P dehydrogenase and phosphoglycerate mutase in *S. cremoris* Wg2

Sample	Glycolytic activity (%) ^a	G3P dehydrogenase (U/mg of protein) ^b	Phosphoglycerate mutase (U/mg of protein) ^b
A	100	0.69	3.90
B	40	0.27	0.86
C	21	0.14	0.26

^a Relative glycolytic activities were taken from Fig. 1.

^b Enzymatic activities were determined in 100 mM triethanolamine-KOH (pH 7.6)–20 mM KCl–10 mM MgCl₂ containing 5 to 10 μl of cell-free extract (about 10 mg of protein per ml) and the appropriate concentrations of substrates and coupling enzymes. For G3P dehydrogenase activity, the following additions (final concentrations) were made: G3P (2.0 mM), sodium arsenate (15 mM), Na-EDTA (1.0 mM), cysteine (5.0 mM), and NAD⁺ (1.0 mM). For phosphoglycerate mutase activity, 3-PG (2.0 mM), 2,3-diphosphoglycerate (0.2 mM), ADP (5.0 mM), NADH (0.2 mM), enolase (0.5 U/ml), pyruvate kinase (2.3 U/ml), and lactate dehydrogenase (3.2 U/ml) were added. Reaction rates were estimated at 30°C from the increase or decrease at 340 nm in a double-beam spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme which utilized 1 μmol of substrate per minute under the assay conditions described.

where J and ss have a similar meaning as indicated above, I refers to the inhibitor concentration, v_i refers to the activity of the enzyme (modulated by the inhibitor) when isolated from the pathway and in the presence of constant concentrations of substrate(s) (S_i) and product(s) (P_i). An estimate of the value for $C_{E_i}^J$ derived in equation 2 can be obtained directly from a plot in which the normalized enzymatic activity and the normalized pathway flux are plotted as a function of the inhibitor concentration and by extrapolating both activities to zero inhibitor concentration (8).

Since G3P dehydrogenase can be inhibited specifically (and irreversibly) by iodoacetate (IAA), the $C_{E_i}^J$ of this enzyme in glycolysis could be determined. To achieve this determination, freshly harvested cells of *S. lactis* (or *S. cremoris*) were washed twice and suspended in buffer as described in the legend to Fig. 2. Subsequently, these resting-cell suspensions were incubated with various concentrations of IAA (Fig. 2). After 15 min of incubation at 30°C, the cells were pelleted by centrifugation, washed twice again, and suspended in buffer. At this moment, the age of the cell suspension corresponded to that of sample A in Fig. 1. Half of each of these cell suspensions was used to measure the glycolytic activity, whereas the other half of each was used to prepare cell-free extracts in which the activity of G3P dehydrogenase could be determined. Control experiments verified that other enzymes, like phosphoglycerate mutase, pyruvate kinase (EC 2.7.1.40), and L-lactate dehydrogenase (EC 1.1.1.27), which are inhibited by IAA only at millimolar concentrations (14), were not affected by

TABLE 2. Effect of starvation on steady-state levels of glycolytic intermediates in glycolyzing cells of *S. cremoris* Wg2^a

Sample	DHAP + G3P (mM)	3-PG (mM)	2-PG (mM)	PEP (mM)
A	1.8	1.7	0.2	0.5
B	4.1	0.5	0.1	0.3
C	5.8	0.2	<0.1	0.1

^a After various periods of starvation, lactose (30 mM) was added to cell suspensions at times indicated in Fig. 1 (samples A, B, and C). Portions (1.0 ml) were withdrawn at regular time intervals and mixed with ice-cold perchloric acid (7% [wt/vol] final concentration). After pH neutralization, the concentrations of glycolytic intermediates were determined as described previously (12). Abbreviation: PEP, phosphoenolpyruvate.

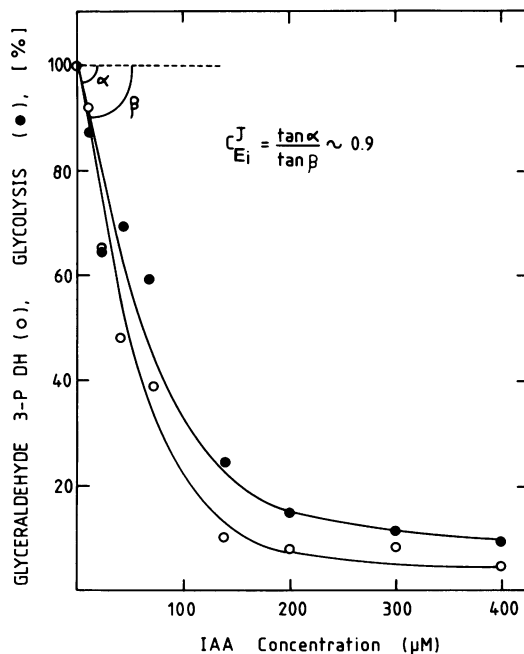


FIG. 2. Effect of IAA on glycolysis and on the activity of G3P dehydrogenase of *S. lactis* ML3. Batch-grown cells were suspended in 5 mM K-PIPES (pH 7.0)–50 mM KCl–5 mM MgSO₄ at a final protein concentration of 1.3 mg/ml. Portions (20 ml) were incubated with varying concentrations of IAA for 15 min at 30°C. The reaction was stopped upon addition of ice-cold buffer, and rapid centrifugation was done as described in the text. The glycolytic activity was measured as described in the legend to Fig. 1. G3P dehydrogenase was assayed as described in footnote *b* of Table 1. All measurements were performed at 30°C.

IAA under the conditions used (data not shown). The effects of increasing concentrations of IAA on the glycolytic flux and on the activity of G3P dehydrogenase are shown in Fig. 2. The control strength on glycolysis of G3P dehydrogenase was estimated from the ratio of the tangents of the angles between the titration curves and the horizontal line at zero IAA concentration (Fig. 2). The $C_{E_i}^J$ was determined in two independent experiments and was found to be about 0.9. Similar results were obtained for *S. cremoris* Wg2 (data not shown). Surprisingly, when cells were starved for approximately 10 h (corresponding with sample C in Fig. 1) before incubation with IAA, the remaining activity of the glycolytic pathway and G3P dehydrogenase was not significantly affected by IAA up to a 1 mM concentration of IAA. The insensitivity towards IAA could be due to oxidation of the reactive SH group in G3P dehydrogenase during starvation.

The observation that the $C_{E_i}^J$ of G3P dehydrogenase is close to 1 does not necessarily mean that glycolysis is rate limited by this enzyme only (it should be noted that other enzymes may have negative flux control coefficients [15, 16]). Furthermore, flux-control coefficients are not constants but are likely to vary with experimental (environmental) conditions, e.g., in growing cells, the value of 0.9 could be expected to be lower (8, 16). The experiment, however, confirms the suggestion that inactivation of G3P dehydrogenase during starvation immediately influences the capacity of the glycolytic pathway. Interestingly, the increased glycolytic activity of continuously cultured *S. sanguis* upon shifting from glucose-excess to glucose-limited conditions has been attributed to an increase in the synthesis

of the glucose phosphotransferase system and G3P dehydrogenase (6), indicating that these enzyme systems largely control the pathway flux in this organism.

At present, the $C_{E_i}^J$ of glycolytic enzymes other than G3P dehydrogenase cannot easily be measured, since no specific irreversible inhibitors are available for these enzymes. Alternatively, $C_{E_i}^J$ values can be determined by varying enzyme levels by genetic means. Upon cloning of the gene for phospho- β -galactosidase in *S. lactis* ML3, the expression of this enzyme has been increased from 21 to 54% without having a significant effect on the rate of acid production in milk (1). This finding indicates that the $C_{E_i}^J$ for phospho- β -galactosidase in the glycolytic pathway was zero under the conditions employed.

Nature of G3P dehydrogenase inactivation. To determine whether G3P dehydrogenase was specifically degraded during starvation, the relative amounts of the enzyme were determined by crossed immunoelectrophoresis (13). G3P dehydrogenase was identified in a single precipitation line by zymogram staining. The relative amounts of the enzyme per milligram of protein were not affected during starvation. Taking advantage of the same zymogram staining technique, the *pI* of G3P dehydrogenase was estimated by isoelectric focusing on agarose (2% [wt/vol]) slab gels (2). A single band, with a *pI* of about 8.7, was observed independent of the starvation time preceding the preparation of cell-free extracts, indicating that G3P dehydrogenase was not inactivated by chemical modification resulting in a change of the overall charge of the protein. The mechanism of inactivation of G3P dehydrogenase during starvation is currently being investigated further.

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