ISOLATION AND CHARACTERIZATION OF ESCHERICHIA COLI MUTANTS DEFECTIVE IN ENZYMES OF GLYCOLYSIS

Meher Irani and P. K. Maitra
Molecular Biology Unit
Tata Institute of Fundamental Research
Bombay 400005, India

Received October 30, 1973

SUMMARY

A selection procedure has been developed that has yielded Escherichia coli mutants lacking the enzymes glyceraldehyde 3-P dehydrogenase (GAD), glycerate 3-P kinase (PGK) and enolase (ENO). The mutants fail to grow on sugar alone or on glycerol alone or on any oxidative substrate alone. They grow on synthetic media when glycerol and an oxidative substrate are present together; the addition of glucose, gluconate and many other sugars inhibits their growth. Reversion studies performed with these mutants suggest that the enzyme losses are due to single gene lesions. Preliminary mapping studies indicate the gene order as argG pgk lysA thyA eno gad tyrA.

INTRODUCTION

The genetic investigation of the Embden - Meyerhof pathway has been rather fragmentary and consists to date only in the determination of a number of loci governing the synthesis of a few glycolytic enzymes. One of the serious limitations has been the nonavailability of mutants blocked in each of the steps. The multifunctional nature of the pathway and the general impermeability of phosphorylated intermediates make a rationale for selecting glycolytic mutants inherently difficult. So far, mutations affecting only four of the enzymes interconverting glucose 6-P and the triose phosphates have been reported (1,2,3,4). Based on the logic that mutants blocked in a physiologically bidirectional sequence will require compounds at both ends for growth, we have been able to devise a selection procedure that yields mutants in enzymes involved in the further metabolism of glyceraldehyde 3-P to pyruvic acid*. Here we report the isolation and

* Reported in the Symposium on "Control Mechanisms in Cellular Processes" held at the Bhabha Atomic Research Centre, Bombay 400085 in February, 1973.

some characteristic properties of mutants in the enzymes GAD, PGK and ENO.

MATERIALS AND METHODS

The parent strain used was A, a *lac*$^+$, *gal*$^+$ derivative of strain AN16 (F$^-$ *trp leu proA his argE ilvC tyrA lac gal str*-S) obtained from Dr F. Gibson (5). The Hfr strains KL16 (O-*lysA*, *his*, *trp*) and JC12 (O-*argG*, *str*, *ampA*) were obtained from Dr K. G. Eriksson-Grennberg (6). The basal mineral salts medium consisted of 0.58% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl and 0.01% $MgSO_4$; pH was 6.7. It was supplemented with 1 µg/ml thiamine hydrochloride and a carbon source as required and 20 µg/ml of the necessary L-amino acids. Glucose was added at a concentration of 10 mM, glycerol at 25 mM and sodium succinate at 40 mM unless stated otherwise. Medium X was used for growing and maintaining the mutants. It consisted of the basal medium supplemented with 1 µg/ml thiamine hydrochloride, 0.5% tryptone, 40 mM sodium succinate and 4 mM glycerol.

Mutagenesis was induced by N-methyl-N'-nitro-N-nitrosoguanidine according to the procedure of Adelberg (7). The mutants were allowed to express in medium X. A penicillin enrichment step in glucose was used prior to plating. All conjugation experiments were performed aerobically with gentle shaking at 37°C with logarithmic-phase cultures of donor and recipient cells in medium X appropriately supplemented. Transductions using phage P1kc were carried out by a modification of the Lennox method (8).

Cells were harvested in the stationary phase for preparing the crude extracts. Enzyme assays were made on 20,000 x g supernatants from sonicated extracts prepared in 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM 2-mercaptoethanol and 2 mM EDTA. Glycolytic enzymes were assayed fluorimetrically (9). For the enolase mutant the extract was prepared in 50 mM Tris-buffer, pH 7.4 containing 2 mM 2-mercaptoethanol and 10 mM $MgCl_2$. The assay was performed by incubating the lyzate in a mixture containing 2 mM glycerate 3-P and 1 unit of glycero-P mutase for suitable periods of time. The reaction was stopped by acidification with $HClO_4$ and the P-enol pyruvate formed was assayed fluorimetrically in the neutralized supernatant. Protein content of the extract was measured by the Folin-Ciocalteu method (10) using bovine serum albumin as standard.

RESULTS

In *E. coli* glucose is metabolized by the glycolytic pathway and the hexose monophosphate shunt (1). In the absence of any other route, the enzymes GAD, PGK, glycero-P mutase (GPM) and ENO are essential for growth on glucose and glycerol. The methylglyoxal bypass discovered recently (11) does not seem to be capable of supporting growth (11, 12) probably due to limiting amounts of glyoxalase present in the cell (12). Thus a mutation leading to the loss of any of the enzymes between triose-P isomerase and pyruvate kinase should not be able to grow on glucose or glycerol. Since the same enzymesare generally believed to be functioning during gluconeogenesi

(13), these mutants should also fail to grow on oxidative substrates such as pyruvate, lactate or any tricarboxylic acid cycle intermediate. However, supplementation with small amounts of sugar or a precursor of sugar such as glycerol should elicit growth on respiratory substrates. Glycerol seemed to be the most suited for this purpose. Its metabolism is kept slow under the feed-back control of fructose 1, 6-diP (14), thus preventing accumulation of high levels of glycolytic intermediates which could be toxic for growth.

A number of mutants were isolated which were unable to grow on any one of glucose, glycerol or succinate as a single carbon source. They could grow only when glycerol and an oxidative substrate such as succinate were present together. Enzyme analysis of this class of mutants revealed many of them to have lost either GAD or ENO activities. One such isolate was found to be devoid of PGK activity. Results shown in Fig. 1 indicate that these mutants have lost over 98% of the respective mutated enzyme activities. However, they have normal levels of the rest of the glycolytic enzymes. Appropriate control experiments indicated that the absence of a particular enzyme activity in an extract was not due to the presence of any inhibitor therein.

Spontaneous revertants appeared at a frequency of 10^{-9} - 10^{-8} when the mutants were plated heavily on media containing each of glucose, glycerol or succinate as the single carbon source. The revertants had regained the lost glycolytic enzymes and the ability to grow on all the three carbon sources (Fig. 2 and Table I). The pleiotropic phenotype was therefore caused by a lesion in the gene leading to the respective defects.

The mutants in the glycolytic enzymes GAD, PGK and ENO isolated by the above selection procedure fail to grow on all sugars so far tested, which are metabolized by the Embden-Meyerhof pathway and the hexose monophosphate shunt as also on gluconate which is primarily metabolized via the Entner-Doudoroff pathway (1). In the absence of glycerol, none of the

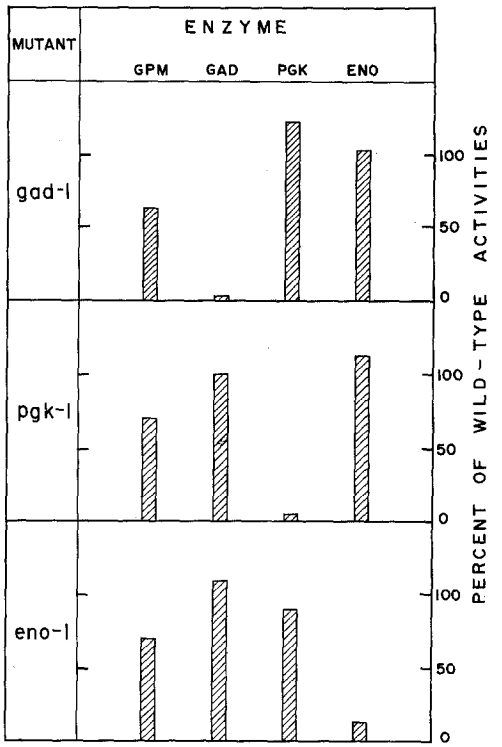

Figure 1. Enzyme activities of the glycolytic mutants. The values for the mutants are expressed in terms of the specific activities of the corresponding wild-type enzymes. The mutated enzyme activities are shown on a ten-fold magnified scale.

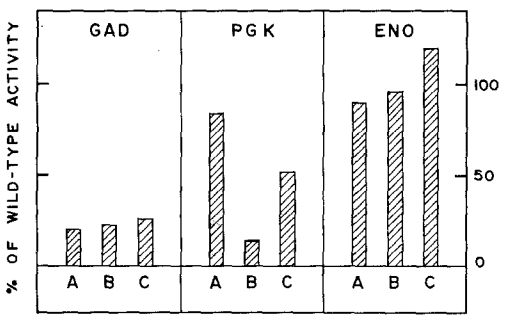

Figure 2. Enzyme activities of revertants from GAD, PGK and ENO mutants. Results shown under columns GAD, PGK and ENO refer to their respective revertants obtained on glucose (A), succinate (B) and glycerol (C) respectively. For the enolase assays the extracts were prepared in 50 mM Tris-buffer, pH 7.4 containing 2 mM 2-mercapto-ethanol and 10 mM $MgCl_2$.

Table I

Colony size of glycolytic mutants and their revertants

Strain	Colony diameter (mm)			
	Glucose	Glycerol	Succinate	Glycerol* + succinate
A-(wild-type)	1.0	0.9	0.8	1.0
gad-1	0.0	0.0	0.0	0.8
gad-1 R_{suc}	1.0	0.6	0.8	1.0
pgk-1	0.0	0.0	0.0	0.8
pgk-1 R_{glu}	0.7	0.3	0.3	0.3
eno-1	0.0	0.0	0.0	0.6
eno-1 R_{gly}	1.0	0.8	0.8	0.8

Cultures were spread on agar plates so as to give about 200 colonies per plate, and average colony size was measured after 60 hrs. of incubation. The subscripts suc, glu and gly refer to the revertants isolated on succinate, glucose and glycerol respectively.

* Glycerol was present at a concentration of 4 mM.

gluconeogenic substrates tested could support growth. The addition of glucose, gluconate and many other sugars to cultures of the mutants growing on medium X causes inhibition of growth. No growth could be observed on agar plates supplemented with glycerol and succinate as carbon sources in the presence of sugars.

A preliminary attempt was made at mapping the gad, pgk and eno mutations. Mapping by conjugation indicated pgk-1 to be a late marker (later than 50') for Hfr K10 (O-purE, leu, ilv) and a terminal marker for Hfr's JC12 and KL16. The gad-1 and eno-1 mutations could be placed between tyrA and the origin of Hfr KL16. eno-1 was found to be contransducible with lysA and thyA, the order being lysA thyA eno. We propose the

provisional gene order as argG pgk lysA thyA eno gad tyrA.

DISCUSSION

The rationale behind the selection of mutants of the enzymes of the Embden-Meyerhof pathway intervening between triose-P isomerase and pyruvate kinase was based on the assumption that these enzymes are essential for both glycolysis and gluconeogenesis. The successful isolation of GAD, PGK and ENO mutants strongly supports this. A number of aldolase and triose-P isomerase mutants have also been obtained by minor modifications of this method. So far we have not found any GPM mutant, possibly because we have not screened a large enough number of isolates. On the other hand, there may exist more than one GPM or there may be an alternative route operating from either glycerate 3-P or glycerate 2-P.

As pointed out earlier, the use of glycerol as a biosynthetic supplement rather than of glucose circumvents the inhibition of growth by sugars. It has been observed that glycerol can be substituted by L-arabinose, for example. The condition necessary for the growth of these mutants appears to be the provision of a gluconeogenic compound below the block and a sugar or sugar precursor above. In order that rapid metabolism does not flood the intracellular pool, the sugar or the sugar precursor must have a slow or controlled flux. Glycolytic mutants blocked in the metabolism of glyceraldehyde 3-P should in principle be able to grow slowly on gluconate which is primarily metabolized by the inducible Entner-Doudoroff pathway. Their inability to grow on gluconate could be due to the accumulation of toxic compounds produced from the non-metabolizable part of the carbon.

Our preliminary mapping results indicate that GAD, PGK and ENO mutants lie between the 50' and 60' region of the E. coli chromosome. However, it seems unlikely that these loci are tightly linked. Further

genetic and biochemical characterization is in progress.

ACKNOWLEDGMENTS

We thank F. Gibson and K. G. Eriksson-Grennberg for supplying us with some of the bacterial strains and S. L. Chakrabarti for stimulating discussions.

REFERENCES

1. Fraenkel, D. G. and Levisohn, S. R., J. Bacteriol. 93, 1571 (1967).
2. Morrissey, A. T. and Fraenkel, D. G., Biochem. Biophys. Res. Commun. 32, 467 (1968).
3. Böck, A. and Neidhardt, F. C., J. Bacteriol. 92, 470 (1966).
4. Anderson, A. and Cooper, R. A., FEBS Letters 4, 19 (1969).
5. Huang, M. and Gibson, F., J. Bacteriol. 102, 767 (1970).
6. Eriksson-Grennberg, K. G., Nordström, K. and Englund, P., J. Bacteriol. 108, 1210 (1971).
7. Adelberg, E., Mandel, M. and Chein Ching Chem, G., Biochem. Biophys. Res. Commun. 18, 788 (1965).
8. Lennox, E. S., Virology, 1, 190 (1955).
9. Maitra, P. K. and Lobo, Z., J. Biol. Chem. 246, 475 (1971).
10. Layne, E., in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. III, Academic Press, Inc., New York, 1953, p. 448.
11. Cooper, R. A. and Anderson, A., FEBS Letters 11, 273 (1970).
12. Freedberg, W. B., Kistler, W. S. and E. C. C. Lin, J. Bacteriol. 108, 137 (1971).
13. Krebs, H. A. Advan. Enzyme Regul. 1 385 (1963).
14. Zwaig, N., Kistler, W. S. and E. C. C. Lin, J. Bacteriol. 102, 753 (1970).