Glucose Metabolism in *Escherichia coli* and the Effect of Increased Amount of Aldolase†

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ABSTRACT: We present a comparative study of *Escherichia coli* with normal and increased amounts of fructose-1,6-bisphosphate aldolase. Most experiments employed a resting cell system involving a high cell density (so as to obtain the soluble pool by direct extraction) and anaerobic incubation in the presence of chloramphenicol. Glucose use is linear with time with a rate ca. half of that in growth, fermentation is almost quantitative, and metabolite concentrations reach a quasi steady state. Increased amount of aldolase had little effect on glucose flux; fructose-1,6-P_2 concentration decreased by ca. one-third, and the extent of equilibration of its two halves, measured by a dismutation procedure on samples taken during metabolism of [6-^{14}C]glucose, increased from 0.33 [(cpm in C1–3)/(cpm in C1–6)] to 0.43. Using the simplest model, that increased amount of aldolase does not perturb net flux or later metabolites, together with the steady-state rate equations for aldolase and triose-P isomerase, we show that the results with resting cells fit with the extra enzyme being fully active, and do not necessitate special assumptions concerning a glycolytic complex, metabolite compartmentation, or secondary mechanisms assuring high metabolite concentration. However, the fit does require that the measured V_{max} values substantially underestimate the actual ones. Calculation also shows that the forms of the predicted curves—and hence the fit with experimental data—of fructose-1,6-P_2 concentration and labeling as a function of the amount of aldolase are highly dependent on glyceraldehyde-3-P concentration but independent of the kinetic parameters of aldolase.

The present work derives from genetic studies of the glycolytic pathway in microbes. Strains with increased amounts of glycolytic enzymes have usually been obtainable, as if their growth and metabolism are not much perturbed. This paper is a quantitative study of one such case. We introduce a convenient methodology for assessing glucose metabolism in resting cells of *Escherichia coli*. We apply it to a study of the effects of increased amount of fructose-1,6-bisphosphate aldolase on glucose flux, fructose-1,6-P_2 concentration, and the equilibration of its two halves. We also describe a way of calculating the expected effects.

MATERIALS AND METHODS

Strains, Media, and Growth. The wild-type *E. coli* strain employed in this laboratory, DF1001, is a prototrophic K12 HfrC strain cured of λ prophage (Sedivy et al., 1984). Most experiments employed a derivative, strain DF716, which carries a *pcnB* (plasmid copy number) mutation (see Results) introduced by its close linkage with the tetracycline transposon *zad*::Tn*10* (Lopilato et al., 1986). A few experiments used a different *E. coli* strain, MC1061 (Casadaban & Cohen, 1980), likewise transduced to *pcnB* (i.e., strain DF711). The aldolase plasmid, here referred to as p*fba*$^+$, is pDF11 (Babul & Fraenkel, 1988), a 1.5 kbp *Eco*RI/*Eco*RV fragment in vector pUC18 deriving from the original aldolase plasmid pLC33-5 (Thomson et al., 1979); this fragment is bp 4130–5665 of the sequence reported by Alefounder and Perham (1989), and includes the aldolase structural gene *fba*$^+$ and flanking sequences of ca. 0.3 kbp 5′ and 0.2 kbp 3′. Some experiments employed a plasmid, p*fba*$^-$, where transposon Tn5 (kanamycin resistance) is located about midway in the gene, inactivating it (this laboratory, unpublished experiments). Cells were grown as specified, usually in minimal medium M63 (Miller, 1972); in a few experiments, we used L-broth (Miller, 1972) or M63 supplemented with 0.02% casamino acids. Except for strain DF1001, growth media also contained ampicillin (200 μg/mL). Growth was in flasks filled to one-tenth or less of capacity and rapidly agitated on a gyrotory shaker, and was monitered at A_{600}; for resting cell experiments or assays, the cultures were harvested at an A_{600} value of 1 or less. All rates are referred to A_{600} units [1 unit is 1 mL of culture at an A_{600} of 1, and is ca. 0.33 mg dry weight and 0.83 μL of cell water (Lowry et al., 1971); the latter value was used to convert the concentration of intracellular metabolites in extracts to their concentration in the cells]. For the rate of glucose use (v_{glu}) during growth, glucose assayed in samples of culture supernatant fluid was plotted vs A_{600}, and the linear slope was multiplied by the growth rate, μ (h^{-1}).

"Resting Cell" Incubations. Cells were obtained from the growth condition specified, washed once in M63 salts medium, and resuspended to a final A_{600} of 10–50 in M63 containing chloramphenicol (20 μg/mL) to prevent new protein synthesis, and ca. 2 μL of Antifoam B (Sigma). One to five milliliters of such a suspension was incubated with continuous mixing by bubbling of N_2. After 30-min preincubation, glucose was added (usually to 10 mM), and samples were obtained over the succeeding 20 min.

Glucose utilization was linear with time (see Figure 1), and linearity (but different rates) was observed over a temperature range of 4–40 °C; in the present report, with the exception noted, all the incubations were done at 30 °C. When radioactive glucose was employed in such incubations, almost all radioactivity remained soluble after perchloric acid extraction, comprising unused glucose, fermentation products, and soluble metabolites (see Figure 2A). Perchloric acid

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insoluble material (i.e., assimilation) was 2% or less of the glucose used. An assessment of fermentation products in such an incubation with wild-type strain DF1001 showed for use of 5.9 μmol/mL glucose, from 16 mM initial concentration, formation of 8.9 μmol/mL lactate, 3.3 μmol/mL formate, 1.7 μmol/mL acetate, and 2.0 μmol/mL ethanol, i.e., a typical mixed-acid fermentation. These latter assays used ion-exclusion chromatography [as in Margolis et al. (1988)].

Glucose was determined after brief centrifugation or in the same extracts as employed for intracellular metabolites, for which 0.5-mL samples were added to tubes containing 0.028 mL of 11.7 M perchloric acid containing 20 mM Na_2EDTA, vigorously mixed 60 s, and left in an ice bath up to 2 h before neutralization with 0.078 mL of 3 M $KHCO_3$ and centrifugation to remove salts and cell debris. In some of the early experiments, the cell samples were rapidly frozen in liquid nitrogen before perchloric acid extraction [i.e., as in the method employed for cells collected by filtration from growing cultures (Lowry et al., 1971)], but control experiments showed this step to be unnecessary for the metabolites of interest. Direct acid treatment of cultures has often been employed to obtain metabolites [e.g., see Betz and Chance (1964) and Zwaig et al. (1970)].

Assays. Metabolites and glucose were assayed spectrophotometrically: glucose with hexokinase, ATP, glucose-6-P dehydrogenase, and NADP; dihydroxyacetone-P with glycerol-3-P dehydrogenase and NADH; and fructose-1,6-P_2 likewise with addition of aldolase and triose-P isomerase. For assay of aldolase activity, cells in the specified growth condition were obtained by centrifugation, washed once with cold M63, resuspended (20–50 A_{600}/mL) in 50 mM potassium phosphate, pH 7.6, and 0.1 mM Na_2EDTA, and vigorously shaken 30 min at 37 °C after addition of one drop of toluene per milliliter. The aldolase assay, 30 °C, contained cell sample, 80 mM Tris-HCl and 100 mM KCl, pH 7.6, 2 mM fructose-1,6-P_2, 0.3 mM NADH, and 2 μL of glycerol-P dehydrogenase/triose-P isomerase (Boehringer 127787, 10 mg/mL), and activities are expressed in the same units as glucose utilization, i.e., micromoles of fructose-1,6-P_2 converted per A_{600} of cells per hour. Comparison of this assay system and others, and of permeabilized cells vs conventional extracts prepared by French press or ultrasonic treatment, showed it to give the highest rates, although the differences between various assay systems were generally less than 2-fold. As known (Doelle & Manderson, 1971), high concentrations of fructose-1,6-P_2 were inhibitory at pH 7.5 (ca. 80% inhibition with 20 mM fructose-1,6-P_2). The only variation which substantially increased activity over that reported here was to employ a higher pH (Doelle & Manderson, 1971; Szwergold, 1986), pH 9 giving values 2–4 times that at pH 7.6.

Triose-P isomerase K_m values were obtained using a crude extract of wild-type *E. coli* strain HB101 carrying the aldolase plasmid pDF11. The assay employed 5 mM triethanolamine and 10 mM $MgCl_2$, pH 7.4, plus glyceraldehyde-3-P, 0.3 mM NADH, and 2 μL of glycerol-3-P dehydrogenase (Boehringer 127124, 2 mg/mL), or plus dihydroxyacetone-P, 1 mM NAD, 10 mM Na_2HAsO_4, 7 mM 2-mercaptoethanol, and 2 μL of glyceraldehyde-3-P dehydrogenase (Boehringer 125684, 10 mg/mL).

Dismutation of Fructose-1,6-P_2. These experiments used [6-^{14}C]glucose during the resting cell incubation, usually of sufficient volume for a single sample: e.g., 0.55 mL with 10 mM glucose, 2.4 × 10^3 cpm/nmol. The acid-soluble metabolites were fractionated by anion exchange (Beckman Ultrasil AX, 10-μL pore, 4.5 × 25 cm main column and 4.5 × 3.2 cm precolumn) with two 4.5 × 3.2 cm protective precolumns containing HPLC sorbent (Sigma H7256 silica gel) and Waters HPLC equipment. Sample application was followed by 4 mL of 5 mM potassium phosphate, pH 6.7, and a 26-mL linear gradient of 5 mM–0.4 M NaCl in the same buffer; fractions of 1 and 0.5 mL were collected. As shown in Figure 2A, most radioactive material in such an extract, comprising unused glucose and fermentation products, is not retained by the column, and the few percent which is retained is mostly found in the peaks labeled II and IV, which include hexose monophosphates and dihydroxyacetone-P, and fructose-1,6-P_2, respectively. Pooled peak IV fractions were subjected to a dismutation procedure: 1-mL final volumes contained 0.3 mL of sample, 2 mM NAD, and 50 mM sodium arsenate, and addition of 5 μL of glyceraldehyde-3-P dehydrogenase (Boehringer 10586, 10 mg/mL) and 5 μL of aldolase (Boehringer 102652, 10 mg/mL) gave rise to glycerate-3-P and dihydroxyacetone-P, as observed by reduction of the NAD at A_{340}. After completion of the reaction, 2 μL of glycerol-3-P dehydrogenase (Boehringer 127124, 2 mg/mL) was added and the NADH reoxidized, with conversion of the dihydroxyacetone-P to glycerol-3-P. After being heated 5 min at 100 °C, 40-μL portions or less were applied to the same chromatographic regime as the original extract. The glycerol-3-P, coming from the top three carbons of fructose-1,6-P_2, appears in fractions II and, depending on sample size, I, and the glycerate-3-P in fraction III (see Figure 2B).

For cells labeled in growth, for experiment 10 of Table II, 20 mL of a growing culture at an A_{600} of 1 was collected by filtration with perchloric acid extraction of the filtered cell mass (Lowry et al., 1971; Sedivy et al., 1986). For experiment 11, the process of extract preparation was exactly as in the resting cell experiments, but used 1 mL of a growing culture; the radioactive cells were extracted in the presence of a 20-fold excess of unlabeled cells obtained from the same growth condition.

Calculation. As discussed below, the application of Scheme I to obtain fructose-1,6-P_2 concentration and labeling could be done "by hand", i.e., in the order eq 3, 1, 4, 2, 5–8. However, the curves in Figure 3 were generated using a spreadsheet model, "Fit" (Kretschmer, 1992), a program for nonlinear regression and forcasting, implemented in EXCEL. The spreadsheet model was also used with eq 9–14 to obtain a value of glyceraldehyde-3-P concentration which gave equal rates of triose-P isomerase flux for the two cases of normal and 15-fold increased amount of aldolase.

RESULTS

Glucose Flux and Amount of Aldolase. In growth, the rate of glucose metabolism, v_{glu}, is the product of the growth rate constant, μ (h^{-1}), and the reciprocal of the yield (micromoles of glucose used per A_{600} increment of growth). For the wild-type strain DF1001 in growth in minimal medium M63 at 30 °C with glucose as the sole carbon source, the value of v_{glu} was ca. 2 μmol A_{600}^{-1} h^{-1} (Table I, line 1).

In nongrowing ("resting") cells, glucose metabolism can also be rapid (Figure 1), and is obtained as the dividend of the linear rate of glucose use (micromoles of glucose per hour) by the amount of cells (A_{600}). The value for the same wild-type strain, DF1001, was 1.3 (Table I, line 1). Since glucose utilization was proportional to the amount of cells over a wide range (up to 100 A_{580}/mL, data not shown), and metabolites could be obtained by direct extraction, resting cells were used for most of the experiments which follow.

Table I: Effect of Increasing Aldolase on Glucose Flux and Metabolites

			growth				resting cells[e]		
line	strain[a]	aldolase act.[b]	med[c]	temp (°C)	μ[d]	v_{glu}[b]	v_{glu}[b]	[FbP][f]	[DHAP][f]
1	DF1001	1.2	Glu	30	0.52	2.0	1.3	10.5	3.2
2a	DF716/pUC18	1.0	Glu	30	0.40	ND	1.0	9.4	3.2
2b	DF716/pUC18	1.4	Glu	37	0.62	ND	1.1	8.4	2.6
3a	DF716/p*fba*$^+$	1.4	Glu	30	0.33	1.9	1.2	8.5	2.9
3b	DF716/p*fba*$^+$	3.1	Glu	34	0.44	2.1	1.3	7.5	2.6
3c	DF716/p*fba*$^+$	20	Glu	37	0.53	3.0	1.3	6.4	2.3
4	DF1001/p*fba*$^+$	51	Glu	30	0.22	1.0	0.6	5.8	3.7
5a	DF1001	0.8	LB	30	ND[g]	NA[h]	0.7	5.2	2.3
5b	DF1001/p*fba*$^+$	34	LB	30	ND	NA	0.6	4.8	2.9
6a	DF711/p*fba*$^-$	1.7	LB	37	ND	NA	1.0	4.9	5.0
6b	DF711/p*fba*$^+$	8.7	LB	37	ND	NA	1.0	3.8	5.5
7a	DF1001	0.8	GAA	30	ND	ND	0.7	14.5	3.9
7b	DF1001/p*fba*$^+$	36	GAA	30	ND	ND	0.7	12.8	6.7
8a	DF1001	ND	GAA	30	ND	ND	0.06[e]	11.7	2.7
8b	DF1001/p*fba*$^+$	ND	GAA	30	ND	ND	0.06	11.0	2.6

[a] All strains are congenic with strain DF1001, except for DF711, which is a derivative of MC1061. All strains without plasmids carry the normal chromosomal allele for aldolase, *fbaA*$^+$. Plasmids are pUC18 (the vector alone), p*fba*$^+$ (pUC18 carrying *fbaA*$^+$), and p*fba*$^-$ (which is p*fbaA*$^+$ with the aldolase gene inactivated by insertion of Tn5). To reduce plasmid copy number, strains DF711 and DF716 also have the chromosomal *pcnB* mutation. [b] Aldolase activities and glucose flux (v_{glu}) values are expressed as micromoles per hour per A_{600} unit of cells. [c] med, medium employed for growth: Glu, minimal medium with glucose as the sole carbon source; LB, L-broth; GAA, minimal medium with casamino acids (0.02%) and glucose. [d] First-order growth rate constant, h^{-1}. [e] Resting cell incubations were all 30 °C except for lines 8a,b which was 5 °C. [f] Concentrations are millimolar, intracellular. FbP, fructose-1,6-P_2; DHAP, dihydroxyacetone-P. [g] Not determined. [h] Not applicable.

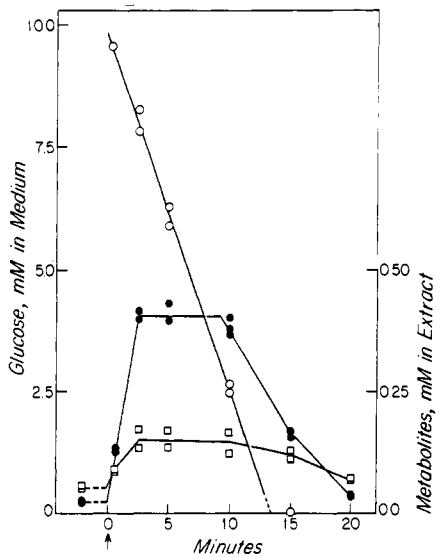


FIGURE 1: Glucose utilization and metabolite concentration in resting cells at 30 °C. Glucose was added at 0 min. Strain DF1001 was grown in minimal medium with glucose at 30 °C. Glucose, open circles; fructose-1,6-P_2, closed circles; dihydroxyacetone-P, open squares.

For increased aldolase levels, we used strains with the aldolase structural gene (*fba*$^+$) carried on a plasmid (i.e., p*fba*$^+$) and hence in multiple copy. To control and reduce copy number, most experiments employed strains (e.g., DF716 and DF711) also carrying the chromosomal mutation pcn*B80* (plasmid copy number). Plasmid replication in *pcnB80* strains is apparently cold-sensitive, so the amount of aldolase in a 30 °C culture of DF716/p*fba*$^+$ was near normal, but it was increased by growth at higher temperatures (Table I, lines 3a–c). The temperature effect was not observed in the control strain carrying just the vector, whose aldolase is being normally expressed from its single chromosomal locus (Table I, lines 2a,b). As shown for cells from minimal medium, the v_{glu} values in resting cells were unchanged by increasing amount of aldolase. The only clear exception was the strain with the highest amount of aldolase, strain DF1001 (*pcn*$^+$) carrying p*fba*$^+$, with v_{glu} values reduced by about half both in resting and in growing cells (line 4). This strain contains aldolase as ca. 20% of its soluble protein (Babul & Fraenkel, 1988), and is also somewhat impaired in its growth and genetically unstable.

Table I also includes data for cells obtained from growth in other media. Cells from L-broth, which contains yeast extract and tryptone but no added glucose, were also competent for rapid anaerobic glucose metabolism in the resting state, and similar v_{glu} values were obtained for strains with normal or high amounts of aldolase (lines 5a,b). Likewise, in a quite different strain background, DF711, the same glucose flux values were seen with cells containing normal or 5-fold increased amount of aldolase (lines 6a,b). Similarly, the aldolase plasmid made no difference to glucose flux in cells grown in glucose minimal medium supplemented with casamino acids (lines 7a,b), and there was also no difference between the same pair of strains incubated at 5 °C although the v_{glu} values were much reduced (lines 8a,b).

Thus, apart from the extreme case of strain DF1001 (*pcn*$^+$) with the aldolase plasmid and the highest aldolase level, substantial increases in the amount of aldolase did not affect the rate of glucose metabolism.

Concentrations of Metabolites. If flux is unaltered, an increase in activity of an enzyme catalyzing a reversible reaction would change forward and backward rates by the same amount. The effect on substrate, fructose-1,6-P_2, in the present case would depend on how much the V_{max} of aldolase was already in excess to glucose flux: the greater the excess normally, the less the effect of having more enzyme (as calculated below). According to the in vitro assay (Table I), the aldolase V_{max} was about equal to glucose flux in the wild-type strain, which would predict large changes in fructose-1,6-P_2 concentration in the strains having more enzyme.

Figure 1 shows how in a typical resting cell incubation fructose-1,6-P_2 and dihydroxyacetone-P levels rapidly rise after glucose addition to quasi-steady-state levels where they remain until glucose is exhausted. The two metabolite concentrations are given in the right-hand columns of Table I. In general, only small differences were observed between the normal and high-level aldolase strains. Considering all strains with 20-fold or greater increases in amount of aldolase (i.e., even including the strains with the highest levels of aldolase which are impaired in their growth), the lowering of fructose-1,6-P_2

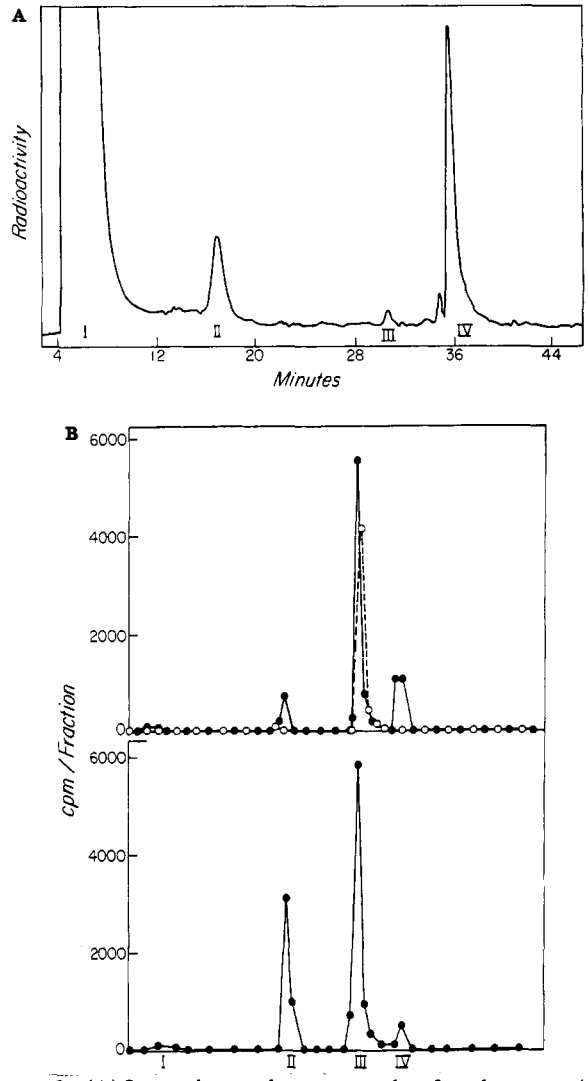


FIGURE 2: (A) Ion-exchange chromatography of crude extract (see Materials and Methods). Strain DF1001, resting cells from growth in minimal medium with glucose, 5 min after addition of [6-^{14}C]-glucose, total 0.5-mL sample, 13 × 10^6 cpm. Radioactive elution profile, full scale 3 × 10^5 cpm. The regions indicated as I (flow-through), II, III, and IV contained, by integration, 93.7, 1.7, 0.24, and 4.0% of the total cpm, respectively. Fructose-1,6-P_2 is in peak IV, and dihydroxyacetone-P and hexose monophosphates are in peak II. (B) Upper panel: Dismutation of fructose-1,6-P_2 from strain NP315 (*fbaA*^{ts}). The strain was grown in minimal medium with glucose and casamino acids at 30 °C. Resting cells were incubated with [6-^{14}C]glucose at 40 (open circles) and 30 °C (closed circles). Lower panel: Dismutation of fructose-1,6-P_2 from peak IV of the experiment shown in (A).

concentration averaged to 27% and the increase in dihydroxyacetone-P concentration to 33%.

Equilibration in Vivo. Another way to assess aldolase activity in vivo was to measure the distribution of label in the two halves of fructose-1,6-P_2 during metabolism of [6-^{14}C]-glucose. Label arriving in fructose-1,6-P_2 from glucose will be in its 6-position, but to the degree that there is equilibration of the triose-P's, then the higher the activity of aldolase, the greater the labeling expected for the 1-position of fructose-1,6-P_2 (also calculated below).

We measured the relative labeling of the two halves of fructose-1,6-P_2 by a dismutation (see Materials and Methods and Figure 2). Fructose-1,6-P_2, obtained by ion-exchange chromatography of the extracted metabolites (e.g., peak IV of Figure 2A), was treated to yield glycerate-3-P from carbons 4–6 and glycerol-3-P from carbons 1–3, which were separated by a second chromatographic step.

Table II: Dismutation of Fructose-1,6-P_2

entry[a]	strain	aldolase act. [μmol h^{-1} (A_{600} unit of cells)$^{-1}$]	growth temp (°C)	fraction of label in C1–3 of fructose-1,6-P_2
1	DF1001	1.2	30	0.33
2	DF1001	1.1	37	0.35
3	DF1001/p*fba*$^+$	28	30	0.46
4	DF716/pUC18	1.4	37	0.33
5	DF716/p*fba*$^+$	1.4	30	0.34
6	DF716/p*fba*$^+$	3.1	34	0.36
7	DF716/p*fba*$^+$	20	37	0.43
8	DF711/p*fba*$^-$	1.7	37	0.39
9	DF711/p*fba*$^+$	8.7	37	0.42
10	DF1001	ND[b]	30	0.38
11	DF1001	ND	30	0.36

[a] Entries 1–9 are dismutations of fructose-1,6-P_2 obtained from resting cell incubations with [6-^{14}C]glucose at 30 °C. Growth, for entries 1–7, was in minimal medium with glucose and for entries 8 and 9 in L-broth. Entries 10 and 11 are for samples obtained during growth in minimal medium with [6-^{14}C]glucose. [b] Not determined.

Figure 2B (lower panel) shows a typical result for the wild-type strain, substantial label in the C1–3 positions. Control experiments used aldolase mutant strain NP315, which has a temperature-sensitive aldolase (strain h8; Bock & Neidhardt, 1966; Singer et al., 1991), as confirmed by an experiment on resting cells: v_{glu} values were 0.83 μmol h^{-1} A_{600}^{-1} at 30 °C but only 0.08 μmol h^{-1} A_{600}^{-1} at 40 °C. With [6-^{14}C]glucose, the fructose-1,6-P_2 labeling was near normal at 30 °C and, as expected, limited to its lower half at 40 °C (Figure 2B, top).

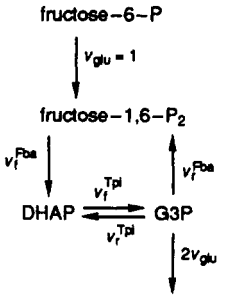
Collected results for the experimental strains are in Table II; fractional labeling is expressed as (cpm in glycerol-3-P)/(cpm in glycerol-3-P plus glycerate-3-P). For the strains with a normal level of aldolase, the average fraction of label in the C1–3 portion of fructose-1,6-P_2 was 0.33, indicative of a considerable back-reaction of aldolase. The trend in the strains with higher amount of aldolase was to increase the ratio.

Expectations had been different, for we had reported low labeling of the 1-position of fructose-1,6-P_2 from 6-labeled glucose, during growth (Chambost & Fraenkel, 1980); a similar finding by NMR has also been cited (Shulman et al., 1979). In view of the present results, we also performed the dismutation procedure on samples labeled during growth (see Materials and Methods). Both experiments gave the same high value found in resting cells (Table II, entries 10 and 11).

Calculations. Since fermentation was almost quantitative and the pentose-P cycle is not used in anaerobic conditions (Fraenkel & Vinopal, 1973), the net flux in each reaction, including aldolase and triose-P isomerase, is equal to the glucose flux (which in the following calculations is normalized to a value of 1). These net fluxes are unchanged in the clone. The clone carries only the structural gene for aldolase and is only known, and expected, the change the amount of aldolase. If the excess aldolase has normal activity, in order for net flux to remain 1 both reverse and forward reactions will increase, equally. This increase should be reflected in increased relative labeling of C1–3 of fructose-1,6-P_2 and a lower concentration of this substrate, and should be in accord with the steady-state rate equations for triose-P isomerase (Scheme I,eq 1) and aldolase [Scheme I, eq 2 (Segel, 1975) for an ordered uni-bi reaction (Rose et al., 1965; Hill et al., 1976)], i.e., depend on the amounts of these enzymes, their kinetic parameters, and the net flux.

If the various enzyme parameters and the concentration of glyceraldehyde-3-P were known, one could predict the expected fructose-1,6-P_2 concentration and labeling in wild-type and clone, as well as the actual values of forward and reverse flux

Scheme I[a]



$$v^{Tpi} = v_f^{Tpi} - v_r^{Tpi} = (V_f^{Tpi}[DHAP]/K_{DHAP}^{Tpi})/D1 - (V_r^{Tpi}[G3P]/K_{G3P}^{Tpi})/D1 \quad (1)$$

$$D1 = 1 + [DHAP]/K_{DHAP}^{Tpi} + [G3P]/K_{G3P}^{Tpi}$$

$$v^{Fba} = v_f^{Fba} - v_r^{Fba} = (V_f^{Fba}V_r^{Fba}[FbP])/D2 - (V_f^{Fba}V_r^{Fba}[DHAP][G3P]/K_{eq}^{Fba})/D2 \quad (2)$$

$$D2 = K_{FbP}^{Fba}V_r^{Fba} + [FbP]V_r^{Fba} + K_{DHAP}^{Fba}[G3P]V_f^{Fba}/K_{eq}^{Fba} + K_{G3P}^{Fba}[DHAP]V_f^{Fba}/K_{eq}^{Fba} + [G3P][DHAP]V_f^{Fba}/K_{eq}^{Fba} + [FbP][G3P]V_r^{Fba}/K_{iG3P}^{Fba}$$

$$[DHAP] = K_{DHAP}^{Tpi}[1 + ([G3P]/K_{G3P}^{Tpi})(1 + V_r^{Tpi})]/(V_f^{Tpi} - 1) \quad (3)$$

$$[FbP] = (K_{eq}^{Fba})^{-1}\{V_f^{Fba}[V_r^{Fba}[G3P][DHAP] + [G3P]K_{DHAP}^{Fba} + [DHAP]K_{G3P}^{Fba} + [DHAP][G3P]] + V_r^{Fba}K_{FbP}^{Fba}K_{eq}^{Fba}\}\{V_r^{Fba}[V_f^{Fba} - 1 - [G3P]/K_{iG3P}^{Fba}]\}^{-1} \quad (4)$$

$$sa(DHAP) = sa(G3P)v_r^{Tpi}/v_f^{Tpi} \quad (5)$$

$$sa(1\text{–}3)FbP = sa(DHAP)v_r^{Fba}/v_f^{Fba} \quad (6)$$

$$sa(4\text{–}6)FbP = sa(F6P)v_{glu} + sa(G3P)v_r^{Fba}/v_f^{Fba} \quad (7)$$

$$[cpm(1\text{–}3)FbP]/[cpm(1\text{–}6)FbP] = B = [sa(1\text{–}3)FbP]/[sa(1\text{–}3)FbP + sa(4\text{–}6)FbP] \quad (8)$$

$$K_{eq}^{Tpi} = V_f^{Tpi}K_{G3P}^{Tpi}/V_r^{Tpi}K_{DHAP}^{Tpi} \quad (9a)$$

$$K_{eq}^{Fba} = V_f^{Fba}K_{iDHAP}^{Fba}K_{G3P}^{Fba}/V_r^{Fba}K_{FbP}^{Fba} \quad (9b)$$

$$[DHAP] = [G3P]v_f^{Tpi}/K_{eq}^{Tpi}v_r^{Tpi} \quad (10)$$

$$[FbP] = [G3P][DHAP]v_f^{Fba}/K_{eq}^{Fba}v_r^{Fba} \quad (11)$$

$$[FbP]K_{eq}^{Fba}K_{eq}^{Tpi}/[G3P]^2 = [(1 + v_r^{Tpi})(1 + v_r^{Fba})]/v_r^{Tpi}v_r^{Fba} = A \quad (12)$$

$$v_r^{Tpi} = (1 + v_r^{Fba})/(Av_r^{Fba} - v_r^{Fba} - 1) \quad (13)$$

$$v_r^{Fba} = (1 - 2AB - B)/(AB + B - 1) \text{ (see eq 8 for "}B\text{")} \quad (14)$$

[a] Equations 1–8 describe the calculation of fructose-1,6-P_2 concentration and labeling from knowledge of enzyme parameters and amount and concentration of glyceraldehyde-3-P. Equations 9–14 may be used to calculate rates of aldolase and triose-P isomerase reactions, forward and reverse, from two equilibrium constants, fructose-1,6-P_2 concentration and labeling, and the glyceraldehyde-3-P concentration. See text. Abbreviations: F6P, fructose-6-P; FbP, fructose-1,6-P_2; G3P, glyceraldehyde-3-P; DHAP, dihydroxyacetone-P; Fba, fructose-1,6-P_2 aldolase; Tpi, triose-P isomerase; capitalized V is used for V_{max} and lower case v for actual rate, both normalized to v_{glu}; f, forward; r, reverse; sa, specific activity. K values (K_{Fbp} etc.) are K_m or K_d's; K_i's are inhibition constants.

for each reaction, as follows. [V_{max} values are represented with capital V's and actual rates with lower case v's either net (without subscript) or forward (f subscript) or reverse (r subscript)]. Dihydroxyacetone-P concentration comes from eq 3, and setting (net) v^{Tpi} to 1, the forward and reverse rates for triose-P isomerase would then be obtainable from eq 1. Fructose-1,6-P_2 concentration (eq 4) would come analogously from eq 4, and setting v^{Fba} to 1, and hence the forward and reverse rates being obtainable from eq 2. Specific activities of dihydroxyacetone-P and the 1–3- and 4–6-positions of fructose-1,6-P_2, eq 5–7, are derived readily from conservation equations, cpm entering = cpm leaving, and depend only on the specific activity of glyceraldehyde-3-P and the various one-way rates. Thus, for input [6-^{14}C]glucose of normalized specific activity 1, and total metabolism being glycolytic, the specific activity of glyceraldehyde-3-P should be 0.5, and the fractional labeling (C1–3/C1–6) of fructose-1,6-P_2 (i.e., the values in Table II) is given by eq 8. We will refer to such calculation as "forward", i.e., proceeding from in vitro enzyme parameters to effects on metabolism.

A somewhat arbitrary example uses available data on the enzymes, together with reasonable guesses, as follows. Triose-P isomerase is, by assay, in considerable excess to glucose flux. We use a value of 20 (normalized to v_{glu}) and K_m's of 0.30 and 2.8 mM, glyceraldehyde-3-P and dihydroxyacetone-P, respectively (Materials and Methods), which, for a K_{eq} of 0.04 (Newsholme & Start, 1975) and Haldane eq 9a gives a V_f^{Tpi} of 7.4. For aldolase, a K_m for fructose-1,6-P_2 of 0.133 mM was obtained in the present work, and a K_i value of 0.6 mM for glyceraldehyde-3-P was reported (Szwergold, 1986). Assuming a V_{max} ratio, reverse reaction/forward reaction, of 2, which is reasonable in view of the known enzymes (Callens et al., 1991; Richards & Rutter, 1961; Penhoet et al., 1969), and using its Haldane equation (eq 9b) and the K_i value for dihydroxyacetone-P of 0.3 mM (Szwergold, 1986) give a K_m for glyceraldehyde-3-P of 0.088 mM; we use the same K_m value for dihydroxyacetone-P [as in Richards and Rutter (1961) and Berthiaume et al. (1991)]. For the concentration of glyceraldehyde-3-P, which was not reported in the most extensive survey of *E. coli* (Lowry et al., 1971), we would have observed—but did not—an amount 10% that of dihydroxyacetone-P (i.e., 0.25 mM). For values of glyceraldehyde-3-P below ca. 0.05 mM, it may be calculated that the C1–3/C1–6 labeling of fructose-1,6-P_2 would not reach 0.4 (unless there were more triose-P isomerase). Also, 0.1 mM would fit with equilibrium of triose-P isomerase, and so that was the value used, which together with the other parameters gave the fructose-1,6-P_2 concentration (from eq 4) and fractional label in C1–3 (from eq 8), plotted in Figure 3A as a function of the amount of aldolase, i.e., its V_{max} in the forward reaction. As expected, both values reach a limit with increasing aldolase concentration.

Placed on Figure 3A also are the measured values of V_{max}, fructose-1,6-P_2 concentration, and labeling (from Tables I and II) for entries 3a–c of Table I (cells from growth in minimal medium and for aldolase in normal, twice normal, and 15-fold normal amounts). None of the points fall on the predicted curves (unless one assumes that V_{max} values are low by a factor of ca. 10, in which case at least the three values of labeling would fit). Indeed, considering that so many enzyme parameters are needed for the forward calculation and various cumulative errors, one might suppose it to be of little use for in vivo modeling anyway.

However, for the model presented by eq 1–8 of Scheme I, over a large range the actual values of the kinetic parameters

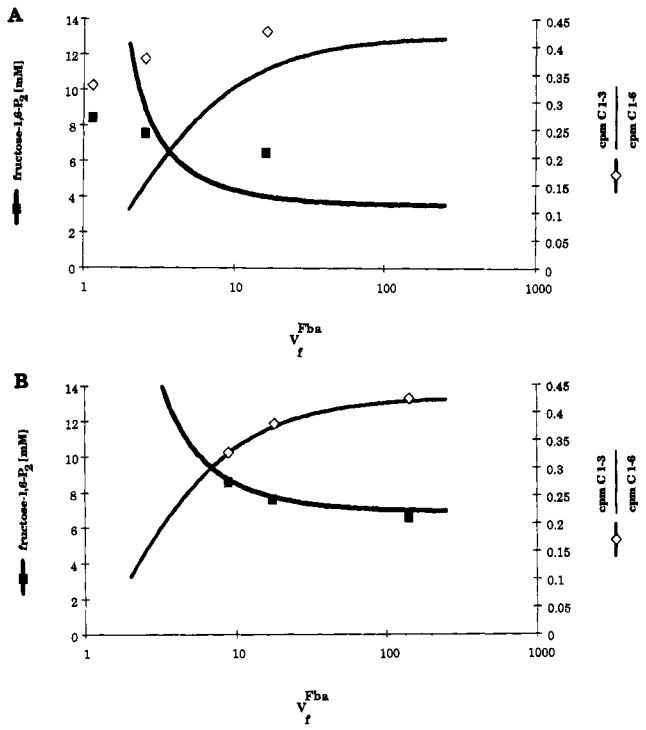


FIGURE 3: Fructose-1,6-P_2 concentration (left-hand axis) and its fraction of radioactivity in C1–3 during metabolism of [6-^{14}C]glucose (right-hand axis) are calculated as a function of the amount of aldolase ($V_{max\text{-}forward}$) from eq 1–8 of Scheme I. The parameters employed (see text) are as follows: for triose-P isomerase, K_{eq} = 0.04, K_{G3P} = 0.3 mM, K_{DHAP} = 2.8 mM, $V_{max\text{-}forward}$ = 20, and $V_{max\text{-}reverse}$ = 7.4; for aldolase, K_{eq} = 0.1 mM, K_{FbP} = 0.133 mM, K_{G3P} = K_{DHAP} = 0.088 mM, K_{iG3P} = 0.6 mM, and a ratio of reverse to forward V_{max} of 2. For (A), a value of 0.1 mM was employed for G3P concentration, and for (B), a value of 0.143 mM was obtained through application of eq 9–14 of Scheme I (see text). For (A), the measured values of fructose-1,6-P_2 concentration and labeling (lines 3a–c from Table I and entries 5–7 from Table II, respectively, i.e., from the minimal medium grown cells with aldolase in normal, twice normal, and 15-fold normal amounts) are placed at their assayed positions (Table I), normalized to v_{glu}, on the abscissa. For (B), to show the fit, the three pairs of values are shifted on the abscissa by a factor of 7.5.

are not critical (examples are given below). Rather, the values of fructose-1,6-P_2 concentration and its relative C1–3 labeling together, merely, with the equilibrium constants of aldolase and triose-P isomerase and a value for glyceraldehyde-3-P concentration, are sufficient to determine the one-way fluxes in aldolase, which, after all, is what one wants to know about the effect of increasing amount of aldolase. Thus, from the forward and reverse rates of triose-P isomerase (the individual terms of eq 1) and its Haldane equation (eq 9a), one obtains an expression for dihydroxyacetone-P concentration (eq 10) and, from the two rates of aldolase (the individual terms of eq 2), an expression for fructose-1,6-P_2 concentration (eq 11). Combining the latter two equations and rearranging give an expression for the rates in question in terms of the two equilibrium constants and fructose-1,6-P_2 and glyceraldehyde-3-P concentrations (eq 12). Then, using the expression (eq 8) for the specific activity of fructose-1,6-P_2 in terms of the same two rates, one may solve for the actual rates of triose-P isomerase (eq 13) and of aldolase (eq 14) in terms of the above quantities. Use of these equations will be referred to as a "back" calculation, i.e., from the measured values of fructose-1,6-P_2 concentration and labeling to in vivo rates.

Such back-calculation still requires a value for glyceraldehyde-3-P concentration. However, aside from the previous considerations for an estimate of 0.1 mM, it turns out through use of eq 9–14 that values outside of a most narrow range predict unrealistic reaction rates for one or the other enzyme. For example, for the wild-type case, a value of glyceraldehyde-3-P concentration of 0.13 mM predicts a reverse rate for aldolase of 108, while a value of 0.15 mM gives a triose-P isomerase reverse rate (normalized to v_{glu}) of 123, and a similar calculation with the data from cells with 15-fold increased amount of aldolase places glyceraldehyde-3-P concentration between 0.14 and 0.15 mM. Calculation within those ranges shows that there is a single value of glyceraldehyde-3-P concentration, 0.143 mM, where, for both the normal and high aldolase situations, the one-way fluxes in triose-P isomerase are unchanged: a back-reaction rate of triose-P isomerase of ca. 3.52 and hence a forward rate of 4.52. For these two cases, the aldolase rate in the reverse direction is 3.43 (forward rate 4.43) in the wild-type strain and 62 (forward rate 63) in the high-level strain. Thus, without considering the enzyme parameters, the back-calculation seems to accord fairly well with the model, increased aldolase concentration causing substantial increase in the reaction in vivo.

Doing the forward calculation with the same set of enzyme parameters as for Figure 3A but now using the value of glyceraldehyde-3-P just obtained gives the curves of Figure 3B. In this case, placing of values of fructose-1,6-P_2 concentration and labeling at their measured V_{max}'s again would show no fit; however, a shift of V_{max} by a factor of 7.5 would give fair fit to both sets of values (concentration and labeling), as shown. As predicted, this fit is independent of aldolase parameters, although the position of the fit on the V_{max} axis does depend on them. Thus, the kinetic parameters of the muscle enzyme, which are in the 0.01–0.1 mM range (Callens et al., 1991), would place the points for wild-type strain at a V_{max} of 16 (instead of the value of 9 in Figure 3B), while the parameters for the yeast (class II) enzyme, which are in the millimolar range (Richards & Rutter, 1961), would place the points for the wild-type strain at a V_{max} of 30. As an extreme case, using the *E. coli* values changed only so as to simulate an almost irreversible reaction—a ratio of reverse to forward V_{max} of 0.02 instead of 2—gives equally good fit but requires a V_{max} for the wild type of ca. 350. Similar calculations for triose-P isomerase, however, demonstrate that the fit worsens, regardless of the V_{max}, for K_m values of glyceraldehyde-3-P above the (measured) 0.3 mM value employed so far while the fit would improve for lower K_m values.

DISCUSSION

As shown in the calculations, increasing the amount of aldolase, an enzyme catalyzing a reversible and presumably non-rate-limiting reaction in a pathway, should have consequences which are relatively independent of its kinetics. In that context, the main conclusion from the present work is that *E. coli* aldolase indeed does not contribute in a major way to flux control and that the modest changes in fructose-1,6-P_2 concentration and labeling in strains with more aldolase may be in accord with what is known of the kinetics of the enzyme. No special hypotheses are needed about altered kinetics of the cloned enzyme, of a glycolytic enzyme complex to which the cloned enzyme might not belong, of the inappropriateness of the measured metabolite pools, or for separate mechanisms to maintain them. This is not to say that such interesting possibilities [e.g., for an *E. coli* glycolytic complex (Gorringe & Moses, 1980) or for channeling of fructose-1,6-P_2/triose-P interconversion as in erythrocytes (Magetto et al., 1992)] are disproven but only that conventional assumptions do not demand them to explain the present data.

This conclusion, however, requires that absolute V_{max}'s of aldolase, measured in vitro, be low by the substantial factor of ca. 8. In part, this factor comes from using a pH of 7.6 for assay of aldolase instead of the optimal pH of 9 where values are higher by a factor of ca. 2–4 (Doelle & Manderson, 1971; Szwergold, 1986); the lower pH was employed because it is close to the known internal pH of *E. coli* (Maloney, 1987). It may be that items such as ionic strength, pH, or unappreciated activators account for the difference. Of course, the factor could be smaller or larger if certain kinetic constants differ very considerably in vivo from those in vitro. A similar uncertainty about the aldolase V_{max} in mammalian tissues has also been noted (Newsholme & Start, 1975). Regarding the kinetics of aldolase, there is surely much to be learned. Adenylates inhibit some class I aldolases (Palczweski & Kachman, 1987; Callens et al., 1991), but not the *E. coli* class II enzyme (Szwergold, 1986). *E. coli* aldolase contains phosphate (Babul & Fraenkel, 1988) but in low amount (unpublished data) and of unknown function. Also, there is relatively little information on the condensation reaction for any aldolase.

Another limitation to a model such as Scheme I is the assumption that later metabolites in the pathway are unaffected by alteration of the enzyme in question. Although this assumption is reasonable for a first calculation, it may be incorrect. Perhaps the modest increase in dihydroxyacetone-P concentration in the strains with a very high amount of aldolase (data not employed for the present calculations) is related to lower fructose-1,6-P_2 concentration and hence decreased activation of a pyruvate kinase (Malcovati & Valentini, 1982). Also, since the concentration of glyceraldehyde-3-P is not known, it still might have a value which simply would not even fit the data for the wild-type strain (e.g., as in Figure 3A), let alone be unchanged in the high-level aldolase cases. The present modeling is extremely sensitive to glyceraldehyde-3-P concentration.

Both of these qualifications point to the general difficulties of in vivo/in vitro comparison of metabolism; data of this type, particularly for microbes, are scarce.

Two other matters related to *E. coli* class II aldolase have been touched upon in this paper. First, the finding that in a known temperature-sensitive aldolase mutant 1–3-labeling of fructose-1,6-P_2 was normal at 30 °C but absent at 40 °C (Figure 2B) fits with the expected function of this enzyme in glucose catabolism. This result was not a foregone conclusion, since in at least one *E. coli* strain a class I fructose-1,6-P_2 aldolase can be present in a substantial amount (Baldwin & Perham, 1978), and it has been detected also in a K12 strain (Scamuffa & Caprioli, 1980).

Second, as mentioned, experiments using other methods had shown low 1-position labeling of fructose-1,6-P_2 from 6-labeled glucose. Indeed, those findings were one stimulus for the present work with high-level strains. Although our earlier findings (Chambost & Fraenkel, 1980) were for growing cells, the dismutation method employed here gave no indication of low labeling in growing cells either (Table II, entries 10 and 11). The difference from the earlier results is thus not clearly related to growth and may be methodological; the present technique, being more direct, is preferred.

Other Comments. Studies of microbes have tended to emphasize growth, but it has long been known that sugar catabolism in nongrowing microbes can be rapid [e.g., see Gunsalus and Shuster (1961)]. The regime employed for most of the present work, of anaerobic incubation and inhibition of protein synthesis, is experimentally convenient and gives linear glucose use at half or more of the rate in growth, marginal assimilation, and quantitative fermentation. The use of high cell densities allows rapid extraction of metabolites without filtration or freezing. The current application concerns the in vivo effect of altering the amount of an enzyme; the system should also be useful in studies of strains with altered enzyme properties.

Finally, the present report belongs to a small but growing number of studies of metabolic effects of increased enzyme level (Fraenkel, 1992). In a particularly detailed one, Brindle (1987) described the effect of increased phosphoglycerate kinase levels in *Saccharomyces cerevisiae*. This caused an approximate doubling of P_i/ATP exchange but marginal effects on glucose flux and amounts of metabolites. In that case, the measured enzyme activity in the wild-type strain was in ca. 20-fold excess to glucose flux.

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