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Assessment of a Futile Cycle Involving Reconversion of Fructose 6-Phosphate to Fructose 1,6-Bisphosphate During Gluconeogenic Growth of *Escherichia coli*

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In gluconeogenesis, fructose 6-phosphate is formed from fructose 1,6-bisphosphate, and if fructose 1,6-bisphosphate were reformed by the phosphofructokinase reaction there would be a "gluconeogenic futile cycle." We assessed the extent of this cycling in *Escherichia coli* growing on glycerol 3-phosphate, using a medium containing $^{32}P_i$. Fructose 1,6-bisphosphate coming from glycerol 3-phosphate should be unlabeled, but any coming from fructose 6-phosphate should contain label from the γ-position of ATP. The amount of labeling of the 1-position of fructose 1,6-bisphosphate was only 2 to 10% of that of the γ-position of ATP in a series of isogenic strains differing in phosphofructokinases (Pfk-1, Pfk-2, or Pfk-2*). In control experiments with glucose 6-phosphate instead of glycerol 3-phosphate, the two positions were equally labeled. Thus, although the presence of Pfk-2* causes gluconeogenic impairment (Daldal et al., Eur. J. Biochem., **126**:373–379, 1982), gluconeogenic futile cycling cannot be the reason.

The *pfkB* gene of *Escherichia coli* specifies a minor phosphofructokinase, Pfk-2, in wild-type strains. The *pfkB1* mutation increases the amount of Pfk-2, allowing Pfk-2 to substitute for the major phosphofructokinase, Pfk-1, in *pfkA* mutants. Strains which also carry the closely linked mutation *pfkB10* grow slightly faster on sugars in the *pfkA* mutant background and contain a mutant form of Pfk-2 called Pfk-2*. However, strains with a high level of Pfk-2* are impaired in growth on substances such as glycerol and malate (i.e., in gluconeogenic growth), whether or not they contain Pfk-1 (5a, 6).

It is not known how an alteration in phosphofructokinase would impair growth on substances for whose metabolism phosphofructokinase is unnecessary. (Mutants completely lacking either phosphofructokinase grow normally gluconeogenically [6].) Under conditions of impaired growth, the cells contain abnormally low amounts of hexose monophosphates and elevated amounts of fructose 1,6-bisphosphate (fructose 1,6-P_2) (5a). Therefore, one possible explanation of the impairment might be that the altered phosphofructokinase, Pfk-2*, converts fructose 6-phosphate (fructose 6-P), formed from fructose 1,6-P_2 by the fructose bisphosphatase reaction, so effectively back to fructose 1,6-P_2 as to cause a deficiency of fructose 6-P and its essential products. In this paper, we report an assessment of this "gluconeogenic futile cycle."

The method is illustrated in Figure 1. *E. coli* uses glycerol 3-phosphate (glycerol 3-P) intact (9), so in growth on unlabeled glycerol 3-P, fructose 1,6-P_2 derived from glycerol 3-P should also be unlabeled. If the medium also contains $^{32}P_i$, the γ-position of ATP should be at least partially labeled from P_i via the glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase reactions and by oxidative phosphorylation. In gluconeogenic growth, fructose 6-P is formed by the action of fructose bisphosphatase on fructose 1,6-P_2. Therefore, if there is reformation of fructose 1,6-P_2 from the fructose 6-P by the action of phosphofructokinase, radioactivity should be introduced into the 1-position of fructose 1,6-P_2. Hence, comparison of the amount of label in ATP γ-position with the amount of label in the fructose 1,6-P_2 1-position should indicate the relative rates of the fructose bisphosphatase and phosphofructokinase reactions.

MATERIALS AND METHODS

Bacterial strains. Strains used are listed in Table 1.

Growth and media. Growth conditions were described elsewhere (5a). Experiments 1 through 4 in Table 2 used the same high-phosphate (0.1 M) medium, M63 (5), as before (5a). All other experiments used a medium with 10 mM phosphate (10) and the following: 100 mM Tris-hydrochloride (pH 7.5), 10 mM KCl, 10 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, and 2 μM $Fe_2(SO_4)_3$. Carbon sources were, as

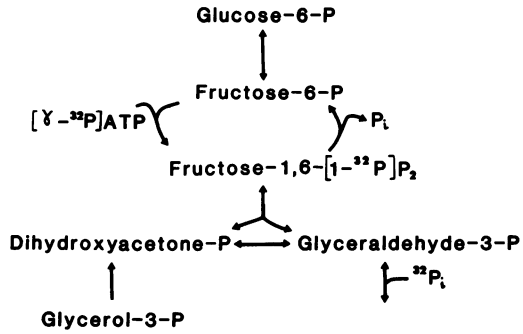

FIG. 1. ^{32}P incorporation into fructose 1,6-P_2 during growth on glycerol 3-P or glucose 6-P in a medium containing $^{32}P_i$.

indicated, glycerol (43 mM), pyruvate (45 mM), glycerol 3-P (10 mM), or glucose 6-phosphate (glucose 6-P; 10 mM). The amount of $^{32}P_i$ (see Table 3) was between 0.5 and 2.5 mCi/50 ml of culture, giving specific activities of 0.4×10^4 to 2×10^4 cpm/nmol.

Metabolite determination. Metabolites were measured in the acid-soluble pool, as described previously (12).

Measurement of label in 1-position of fructose 1,6-P_2 and γ-position of ATP. Inocula were from stationary-phase cultures in 0.1% glucose or, for *pfkA* mutants, 0.1% gluconate and were diluted into the radioactive medium to give an initial absorbance at 600 nm of 0.05 to 0.1. At an absorbance at 600 nm of 0.3 to 0.5, approximately 50 ml was rapidly filtered, and the acid-soluble fraction (ca. 2 ml) was obtained as done previously (5a), except that the filter was extracted twice with 1 ml of 0.3 N $HClO_4$, and glucose 6-P, fructose 6-P, fructose 1,6-P_2, and ATP were measured. Nucleotides were absorbed from 1.5 ml of the acid-soluble fraction by the addition of 25 mg of activated charcoal (2). (The charcoal was prepared by washing with 6 N HCl and water and then drying at 150°C. It was then preabsorbed by suspension of 1 g in 10 ml of 18.5 mM adenine and centrifuged, and the pellet was washed once with water and three times with a mixture of 95% ethanol–14.5 N NH_4OH–water, 65:0.5:35. The charcoal was then washed with water, filtered, and dried at 37°C. The preabsorbtion step was necessary for efficient elution [60 to 70%] of ATP later.) After the charcoal was washed with water, nucleotides were extracted by two successive 2.5-ml washes with the ethanol-ammonia-water mixture, evaporated to dryness (in a Rotary Evapo Mix, Buchler Instruments Div., Nuclear-Chicago Corp.), and the residue was dissolved in 0.1 ml of water. ATP was assayed (10 to 20 μl containing 1 to 2 nmol) in the usual 1-ml assay system containing glucose, hexokinase, glucose 6-P dehydrogenase, and $NADP^+$ (12); a control incubation lacked hexokinase. Then, we added 10 μmol of glucose 6-P and 10 μmol of 6-phosphogluconate to the same tubes, and nucleotides were reabsorbed by the addition of 10 mg of activated charcoal. After centrifugation and one wash with water, radioactivity was determined by scintillation counting in Aquasol (New England Nuclear Corp.). The difference in counts per minute between the two final supernatants, one from the complete incubation and the other from the incubation without hexokinase, was taken as counts in the γ-position phosphate of ATP, and specific activity was calculated according to the amount of ATP assayed in the same pair of incubations.

The original acidic extracts (ca. 1.2 ml), after the charcoal treatment, were treated with 0.1 ml of 0.08 M $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ at room temperature until a yellow color was seen, indicating complex formation

TABLE 1. *E. coli* strains used[a]

Strain	Genotype	Origin
DF903	Δ(*rha-pfkA*)*15 pfkB1 zdh-1*::Tn*10*[b]	5a
DF905	Δ(*rha-pfkA*)*15 pfkB1 pfkB10 zdh-1*::Tn*10*[b]	5a
DF929	*glpK* Δ(*phoA*) Δ(*rha-pfkA*)*15 zig-1*::Tn*10*[c]	P1/DF877 (6) × no. 4 Lin (11), Tet^r Rha$^-$ Mtl$^-$
DF930	*glpK* Δ(*phoA*) *zdh-1*::Tn*10*[c]	P1/DF868 (6) × no. 4 Lin (11), Tet^r α-glyP$^+$, normal Pfk-2 activity
DF931	*glpK* Δ(*phoA*) *pfkB1 pfkB10 zdh-1*::Tn*10*[c]	P1/DF868 (6) × no. 4 Lin (11), Tet^r α-glyP$^+$, high Pfk-2* activity
DF932	*glpK* Δ(*phoA*) *pfkB1 zdh-1*::Tn*10*[c]	P1/DF865 (6) × no. 4 Lin (11), Tet^r, high Pfk-2 activity
DF955	*pgi-2 zwf-2 glpK zig-1*::Tn*10*[b]	P1/DF929 × DF2000 (7), Tet^r Gly$^-$

[a] Gene designations are in Bachmann and Low (1), and positions of Tn*10* insertions are according to the notation of Chumley et al. (4). Abbreviations: Tet, tetracycline; Rha, rhamnose; Mtl, mannitol; Gly, glycerol, α-glyP, L-α-glycerophosphate; Pfk, phosphofructokinase. All strains come by P1 transduction (as, for example, DF929: transduction of no. 4 Lin with phage P1 grown on DF877, selection on rich medium containing 15 μg of tetracycline per ml, and scoring for lack of growth on rhamnose and mannitol). Pfk-2 and Pfk-2* activities were determined by assay of crude extracts (5a, 6) treated with a serum containing antibodies against Pfk-1.

[b] Derivative of DF1000 which is strain K10, HfrC *relA1 spoT pit-10 tonA22* T2^r (markers according to B. Bachmann, Coli Genetic Stock Center, Yale University).

[c] Derivative of K10 known as strain no. 4 Lin (11).

with P_i; 0.1 ml of 0.2 M triethylamine hydrochloride was then added, and after 10 min at 0°C, the precipitated phosphate complex was removed by centrifugation (14). After neutralization with 0.1 volume of 3 M $KHCO_3$ and centrifugation, the supernatant was used for determination of the amount of label at the 1-position of fructose 1,6-P_2. A sample of 0.1 to 0.2 ml containing 0.5 to 3 nmol of fructose 1,6-P_2 was assayed in the usual way for glucose 6-P, fructose 6-P, and fructose 1,6-P_2 (by successive additions of $NADP^+$ and glucose 6-P dehydrogenase, phosphoglucose isomerase, and fructose bisphosphatase [12]). Then, 5 μmol of glucose 6-P, 5 μmol of fructose 6-P, 5 μmol of fructose 1,6-P_2, and 5 μmol of 6-phosphogluconate were added, followed by 0.1 ml of 12 M $HClO_4$ and 0.1 ml of 10 mM KH_2PO_4, and P_i was precipitated, as above. The precipitated phosphate complex was washed with 1 ml of water, dissolved in 0.1 ml of 14.5 M NH_4OH, and diluted by 0.5 ml of water, and its radioactivity was determined in Aquasol. The radioactivity difference between the two original incubations, one complete and the other omitting fructose bisphosphatase, was taken as counts in the 1-position of fructose 1,6-P_2, and specific activities were obtained according to the amount of fructose 1,6-P_2 assayed in the same pair of incubations.

In one case (strain DF903 grown on glucose 6-P), the protocol for obtaining the amount of label in the 1-position of fructose 1,6-P_2 was modified because the extract contained high concentrations of hexose monophosphates, as compared with the concentration of fructose 1,6-P_2. After assay of the hexose monophosphates, 2.5 μmol of oxidized glutathione and 50 μg of glutathione reductase (Boehringer Mannheim Corp.) were added to reoxidize the accumulated NADPH. After the latter incubation, the mixture was boiled for 1 min to inactivate enzymes, and a portion, usually 0.2 ml, was assayed for fructose 1,6-P_2 as usual. The extra incubation did not cause detectable hydrolysis of fructose 1,6-P_2, as no hexose monophosphate was observed in the final incubation without the addition of fructose bisphosphatase.

In all cases, radioactivity and overall recovery were measured in all samples to follow the course of fractionation and to indicate any marked deviations in yield. Specific activity of the P_i used in the medium was determined at the same time as the other measurements of radioactivity.

Chemicals. Carrier-free $^{32}P_i$ was from New England Nuclear Corp. and was used after being boiled for 10 min. All of the enzymes were from Boehringer Mannheim Corp., with the exception of fructose bisphosphatase, which was from Sigma Chemical Co. Activated charcoal was from Darco Atlas Chemical Ind., and triethylamine was from Eastman Kodak Co.

RESULTS AND DISCUSSION

Since the method for determining gluconeogenic futile cycling involved growth on glycerol 3-P in a medium containing $^{32}P_i$ (Fig. 1), it was first necessary to establish whether growth on glycerol 3-P was impaired with the typical metabolite pattern shown for glycerol and malate (5a) and whether the effect also occurred in a medium with a lower phosphate concentration better suited to radioactive experiments. In Table 2, the first two lines repeat data from Daldal et al. (5a), comparing the *pfkB1* and *pfkB1 pfkB10* strains for growth and metabolite levels on glycerol as the carbon source in M63 medium (0.1 M P_i). Lines 3 and 4 show data obtained for growth on glycerol 3-P in the same medium; both growth impairment and deficiency of hexose monophosphate were observed.

Lines 5 through 8 show growth of several strains on glycerol 3-P in a medium containing only 10 mM P_i. The same correlations were observed among *pfkB1 pfkB10*, impaired growth, and metabolite concentrations as were observed for the high-phosphate medium. The strains used for these experiments (DF929, DF930, DF931, and DF932) differed from the strains in earlier experiments in also carrying a mutation, *glpK*, causing lack of glycerol kinase; the latter mutation was included to avoid rephosphorylation of any glycerol which might be formed from glycerol 3-P.

Lines 9 and 10 of Table 2 show experiments with glucose 6-P as the sole carbon source. Glucose 6-P is also used intact by *E. coli* (8) and was employed as a control in the radioactive experiments to see whether, as expected, the amount of label in the 1-position of fructose 1,6-P_2 would then equal the amount of label in the γ-position of ATP. Both strains tested (DF903 [*pfkB1*, Pfk-2] and DF905 [*pfkB1 pfkB10*, Pfk-2*]) grew well on glucose 6-P and contained high concentrations of hexose monophosphates.

Results of labeling experiments are in Table 3. The protocol (see above for details) was as follows. The acid-soluble fraction was obtained from cultures growing on glycerol 3-P (or glucose 6-P) in a medium containing $^{32}P_i$. Nucleotides were removed. Then, fructose 1,6-P_2 and sugar monophosphates were assayed enzymatically with fructose bisphosphatase, phosphoglucose isomerase, and glucose 6-P dehydrogenase. Radioactivity from the 1-position of fructose 1,6-P_2 was obtained by precipitation of the P_i produced in the same incubation. In the nucleotide fraction, ATP was measured enzymatically by treatment with glucose and hexokinase, and its γ-position label was obtained from the same incubation, as radioactivity not reabsorbed by charcoal.

The first line of Table 3 shows a control experiment from growth on a non-phosphorylated carbon source, pyruvate, in the $^{32}P_i$ medium. The amount of label in the γ-position of ATP and the amount of label in the 1-position of fructose 1,6-P_2 equaled the amount of label of the P_i confirming the validity of the experimental techniques.

Lines 2 and 3 are controls with glucose 6-P as

TABLE 2. Bacterial growth and metabolite concentrations[a]

Strain	Relevant genotype	Carbon source	P_i concn in medium (mM)	Doubling time (min)	Metabolite concn (mM)		
					Glucose 6-P	Fructose 6-P	Fructose 1,6-P_2
DF903[b]	*pfkA pfkB1*	Glycerol	100	95	1.0	0.2	3.4
DF905[b]	*pfkA pfkB1 pfkB10*	Glycerol	100	350	0.05	ND[c]	5.8
DF903	*pfkA pfkB1*	Glycerol 3-P	100	150	1.22	0.33	2.25
DF905	*pfkA pfkB1 pfkB10*	Glycerol 3-P	100	290	ND	ND	9.5
DF930	*pfkA*$^+$ *pfkB*$^+$	Glycerol 3-P	10	104	0.58	0.03	2.44
DF932	*pfkA*$^+$ *pfkB1*	Glycerol 3-P	10	96	0.38	0.27	2.49
DF931	*pfkA*$^+$ *pfkB1 pfkB10*	Glycerol 3-P	10	410	ND	ND	7.41
DF929	*pfkA pfkB*$^+$	Glycerol 3-P	10	120	1.0	0.2	2.98
DF903	*pfkA pfkB1*	Glucose 6-P	10	70	58.3	9.9	3.9
DF905	*pfkA pfkB1 pfkB10*	Glucose 6-P	10	66	52.1	5.8	12.8

[a] Cultures were grown as indicated, and metabolite concentrations were determined from the acid-soluble fraction (see text). Each determination was done at least twice with less than 15% variation.
[b] Data from Table 3 or 4 of Daldal et al. (5a).
[c] ND, Not detectable (<0.05 mM).

the sole carbon source. In both strains (DF903 [Pfk-2] and DF905 [Pfk-2*]) the amount of label in the 1-position of fructose 1,6-P_2 was about equal to the amount of label in the γ-position of ATP. These results confirm that the 1-position of fructose 1,6-P_2 indeed derives from the γ-position of ATP when growth depends on Pfk-2 or Pfk-2*, as expected if ATP is the phosphate donor. Admittedly, the results do not prove that the transfer is direct, but they lend no support to speculation that the true donor might not be ATP.

Two additional comments should be made about the data from the glucose 6-P experiments. First, the equality of label in the γ-position of ATP and the 1-position of fructose 1,6-P_2 fits reports that the 6- and 1-positions of fructose 1,6-P_2 are not in equilibrium (3, 13); otherwise the 1-position of fructose 1,6-P_2 should have one-half the specific activity of the γ-position of ATP.

Second, the amount of label in the γ-position of ATP was only about 10% of the specific activity of the P_i of the medium, so 90% derived from glucose 6-P itself. The latter phosphate does go to ATP directly by the pyruvate kinase reaction, but this could not account for more than 50% of the ATP, since an equivalent amount must come from P_i via the glyceraldehyde 3-P dehydrogenase reaction, and more comes by oxidative phosphorylation. Therefore, most of the P_i used for ATP synthesis must come

TABLE 3. ^{32}P-labeling of γ-position of ATP and 1-position of fructose 1,6-P_2[a]

Strain	Relevant genotype	Carbon source	Ratio of sp act[b]		B/A[c]
			(A) γ-position of ATP/P_i	(B) 1-position of fructose 1,6-P_2/P_i	
DF931	*pfkA*$^+$ *pfkB1 pfkB10*	Pyruvate	0.970	0.979	1.01
DF903	*pfkA pfkB1*	Glucose 6-P	0.104	0.118	1.14
DF905	*pfkA pfkB1 pfkB10*	Glucose 6-P	0.071	0.069	0.98
DF930	*pfkA*$^+$ *pfkB*$^+$	Glycerol 3-P	0.311	0.030	0.097
DF932	*pfkA*$^+$ *pfkB1*	Glycerol 3-P	0.128	0.0045	0.035
DF931	*pfkA*$^+$ *pfkB1 pfkB10*	Glycerol 3-P	0.228	0.005	0.024
DF929	*pfkA pfkB*$^+$	Glycerol 3-P	0.196	0.006	0.033
DF955[d]	*pfkA*$^+$ *pfkB*$^+$	Glycerol 3-P	0.317	0.025	0.080

[a] Cultures were grown on the (unlabeled) carbon sources in a medium containing $^{32}P_i$. The amount of label in the positions specified was determined (see text). Measurements were performed at least twice and agreed within 15%.
[b] These columns give the ratios of the counts per minute per nanomole determined for the γ-position of ATP or the 1-position of fructose 1,6-P_2 with respect to the specific activity of the P_i in the growth medium (0.4×10^4 to 2×10^4 cpm/nmol in the various experiments).
[c] Ratio of the values in the two preceding columns.
[d] DF955 also carries a phosphoglucose isomerase (*pgi*) mutation.

ultimately from glucose 6-P rather than from the radioactive P_i supplied. (Presumably, the pathways to P_i are via ATP hydrolysis and the phosphoenolpyruvate carboxylase reaction.) The mechanism of relative exclusion of exogenous P_i is unknown. The phenomenon was also observed with glycerol 3-P, but was less pronounced (Table 3, lines 4 through 8).

For the experiments with glycerol 3-P (Table 3, lines 4 through 8), the results for five different strains show a range of relative labeling of the 1-position of fructose 1,6-P_2 and the γ-position of ATP of only 2 to 10%. These values represent the relative rates of the phosphofructokinase and fructose bisphosphatase reactions. For the single strain for which the physiological results suggested that there might be futile cycling (strain DF931 [Pfk-1, Pfk-2*]), the value was the lowest of the series (2%), indicating that futile cycling cannot account for the gluconeogenic impairment. The low values for the other strains suggest that there is little if any gluconeogenic futile cycling regardless of which phosphofructokinase is present (DF932, Pfk-1 and Pfk-2; DF929, only low-level Pfk-2; DF930, wild type). If anything, it was in the wild-type strain (DF930) that there was slight indication of 1-position labeling of the fructose 1,6-P_2 (ca. 10%), but the value was marginal. The final entry in the table is for strain DF955, which has wild-type phosphofructokinases but lacks phosphoglucose isomerase. This strain was used because of the slight indication of cycling in DF930, and we wondered whether or not the amount might increase substantially if use of fructose 6-P was impaired. The labeling experiment (Table 3, line 8) gave no indication of increased cycling.

We conclude, therefore, that there is little, if any, gluconeogenic futile cycling in *E. coli*. This finding complements the earlier one that futile cycling in growth on glucose is also marginal (3). Furthermore, the results also seem to rule out gluconeogenic futile cycling as an explanation for the gluconeogenic impairment of strains carrying Pfk-2*.

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