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pfkA Locus of *Escherichia coli*

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pfkA was known, on the basis of three mutants, as the likely locus of phosphofructokinase in *Escherichia coli*, and the unlinked *pfkB1* mutation suppressed these mutations by restoring some enzyme activity (Morrissey and Fraenkel, 1972). We now report a new search for the complete inactivation of *pfkA* (e.g., by deletion or amber mutation), done to assess whether the *pfkB1* suppression is by an independent enzyme, phosphofructokinase activity 2 (Fraenkel, Kotlarz, and Buc, 1973). Ten new phosphofructokinase mutants all were at *pfkA*, rather than at *pfkB* or *pfkC*. One of them (*pfkA9*) gave temperature-sensitive reverants with heat-labile enzyme. Another (*pfkA11*) proved genetically to be a nonsense mutation, but showed no restored activity when suppressed by *supF*. However, even unsuppressed it was found to contain an enzyme related to phosphofructokinase activity 1 kinetically (more allosteric), physically (almost identical subunit), and antigenically. All the *pfkA* mutants apparently contained cross-reacting material to activity 1. All (including *pfkA11*) were suppressed by the *pfkB1* mutation. Several results support the idea that *pfkA* is the structural gene for the main phosphofructokinase of *E. coli* (activity 1), but that there is some restriction to its complete inactivation.

Mutant analysis of *Escherichia coli* phosphofructokinase has shown several deficiency mutants to be altered at a locus called *pfkA*, and that phosphofructokinase activity is restored to these mutants by a suppressor lying elsewhere, *pfkB1* (14-16). A strain wild type at *pfkA* and also carrying the suppressor (i.e., *pfkA*$^+$ *pfkB1*) was found to contain two phosphofructokinase activities in similar amounts; the first, activity 1, was like the major enzyme in wild-type strains, and the second, activity 2, was kinetically and physically different from activity 1, resembling a component present in low amount in wild-type strains (9).

In an accompanying paper we have described a new type of mutation, *pfkB*$^-$, which reduces to zero the residual activity in *pfkA*$^-$ mutants; we also found a mutation of similar effect, *pfkC*$^-$, which lay elsewhere (22).

In this paper we return to *pfkA*. We wanted deletions or early amber mutants to assess whether *pfkB1* indeed controls an independent enzyme. No certain mutations of this type at *pfkA* were found, for even a nonsense mutant proved to make a related protein of similar size. Of 11 new independent phosphofructokinase mutants, all were at *pfkA* and were suppressed by *pfkB1*. Many lines of evidence suggest that *pfkA* is the structural gene for activity 1.

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MATERIALS AND METHODS

Media. Minimal medium 63 was used (6), supplemented (per milliliter) with 1 μg of thiamine hydrochloride, 25 μg of amino acids or uracil, as required, and 0.4% carbon source. Minimal plates also contained 2% agar (Difco). Media for propagation and assay of phage P1 were those of Lennox (13), and for phages ϕ80 and T4 were those of Gottesman and Beckwith (10).

Genetic procedures. Most genetic procedures were described earlier (21). Strains were tested for carrying a nonsense suppressor according to ability to grow a phage T4 coat protein amber mutant, B17 (8); broth cultures of strains to be tested, and of known *sup*$^+$ and *sup*$^-$ controls, were inoculated as drops on H plates (10) and allowed to dry. Portions (1 μl) of T4 amber mutant or wild type (10^7/ml) were applied to the center of the inoculated area, and the plates were incubated. Suppression of phenotypes by *supF*, or sensitivity to this suppressor, was scored by drawing a toothpick with the bacterial strain through a line of ϕ80*psupF* (ca. 10^9 phage) on the selective plate and observing stregthened or weakened growth below the line. Wild-type ϕ80 and ϕ80*psupF*$^+$ and known *sup*$^+$ and *sup*$^-$ bacterial strains served as controls.

Bacterial strains. Strains are listed in Table 1 with genotypes and derivations. Map positions are shown in Fig. 1 of the accompanying paper (22).

Phosphofructokinase. The assay was described earlier (9) and contained (in 1 ml) 0.1 M tris(hydroxy-

TABLE 1. *E. coli K-12 strains used*[a]

Strain	Sex	Genotype	Derivation, source, or reference
113	F^-	*gal glpK lac mal str sup*[b] *thi*	E.C.C. Lin
161	F^-	*argECBH glpK his ilv metB mtl str sup*[b]	E.C.C. Lin
AM1	HfrC	*pfkA1 tonA22 lambda*	(14)
DF(85, 86, 87)	F^-	*edd-1 galK his pfkA(1,2,3) pps-1 pyrD str*	(16)
DF89	F^-	*edd-1 galK his pfkB1 pfkB2 pyrB str*	(22)
DF1651	F^-	*edd-1, galK his pyrD pps-1 str tyrA*	(16)
K10	HfrC	*tonA22 lambda*	(2)
K10-15-16	HfrC	*pfkA21*[c] *rel-1 tonA22* T_2^r *zwf*	(12; CGSC)
PA3	Hfr(P4X)	*kdgA2*[d] *kdgP3 metB thi*	(17)
RT(14,26)	F^-	*pfkA(10,11)* in strain 113	—[e]
RT(59, 60, 61, 62, 64, 65)	HfrC	*pfkA(4,5,6,7,8,9)* in strain K10	K10, EMS, pcs for Mtl^- [f]
RT(116, 117, 126)	HfrC	*pfkA9 pfkA (22,23,24)* in strain K10	—[g]
RT(136, 137, 139)	F^-	*pfkA(12,13,14)* in DF89	DF89, EMS, pcs for Man^-
RT142	F^-	*edd-1 galK his metB pps-1 pyrD str sup*[b]	(21)
RT143	F^-	*metB* trp_{amber} *pps-3 str*	X7187, $PyrF^+$(trp_{amber}) trans.
RT151	F^-	*pfkA11* trp_{amber} *pps-3 str*	RT143, Met^+(Mtl^-) trans. (RT26)
RT229	F^-	*pfkA11* in RT142	RT142, Met^+(Man^-) trans. (RT26)
RT235	F^-	*pfkA9 pfkA23* in RT142	RT142, Met^+(Man^-) trans. (RT117)
RT247	F^-	*edd-1 galK his pfkA11 pyrD str sup*[b]	RT229, $Lact^+$($pfkB^+$) trans. DF1651B1 (16)
RT534	F^-	*pfkA9 pyrF trp pps-3 str*	X7187, Met^+(Mtl^-) trans. (RT65)
RT559	F^-	*pgi* in RT534	RT534, Mtl^+ revt. (see text)
RT686	HfrC	$lacZ_{amber}his_{amber}supF$	R. T. Vinopal
RT849	F^-	*pfkA11 pps-3 str supF*	RT151, Trp^+(sup^+) trans. (RT686)
RT850	F^-	*pfkA11 pps-3 str* sup^+	RT151, Trp^+(sup^-) trans. (RT686)
RT851	F^-	*pfkA11 pfkB1 pfkB2 pps-3 str supF*	RT849, $Lact^+$($pfkB^-$) trans. (DF89)[h]
RT853	F^-	*pfkA11 pfkB1 pfkB2 pps-3 str* sup^+	RT850, $Lact^+$($pfkB^-$) trans. (DF89)[h]
RT862	F^-	*pfkA10 pps-3 str supF*	—[i]
X7187	F^-	*metB pps-3 pyrF str trp*	(21)

[a] Gene designations are according to Taylor and Trotter (20) and all known markers are included. Special abbreviations and other conventions were described elsewhere (21); e.g., Man, mannose utilization; Mtl, mannitol utilization, Lact, lactate utilization; revt., revertant; trans., transduction with phage P1 (followed by donor strain in parenthesis): i.e., derivation of RT534 is "transduction of X7187 to methionine independence by phage P1 grown in strain RT65, with co-inheritance of *pfkA9* as scored on mannitol." CGSC, strain obtained through the courtesy of B. Bachmann, Coli Genetic Stock Center, Yale University.

[b] Sup^+ without gene designation means suppression of T4 amber mutant B(22), with genetic locus unknown.

[c] *pfkA21* is our allele designation.

[d] *kdgA* is called *eda* in ref. 20.

[e] Derived by nitrosoguanidine-induced comutation (11) from strain 113, with selection for $glpK^+$ and scoring for $pfkA^-$ (see text).

[f] Selections were done at 22 C, but these mutants are not phenotypically temperature sensitive or cold sensitive (see text).

[g] Spontaneous temperature-sensitive revertants of RT65, selected on mannitol (see text).

[h] Presence of "$pfkB^-$" (*pfkB1 pfkB2*) was ascertained by transduction as in reference 22.

[i] Like RT849, but from a *pfkA10* derivative of RT143.

methyl)aminomethane-hydrochloride, pH 8.2, 0.2 mM nicotinamide adenine dinucleotide, reduced form, 1 mM sodium fructose 6-phosphate, 1 mM sodium adenosine 5'-triphosphate (ATP), 10 mM $MgCl_2$, 2 mM NH_4Cl, 120 μg of fructose-1,6-P_2 aldolase, 3 μg of triose phosphate isomerase, and 30 μg of α-glycerophosphate dehydrogenase (auxiliary enzymes were from Boehringer). The control mixture lacked ATP. Absorbancy at 340 nm was followed at 25 C with a Gilford spectrophotometer. Specific activities are units (micromoles of fructose-1,6-P_2 formed per minute) per milligram of protein. For activity 1* (see Results) the assay was supplemented with 0.5 mM sodium adenosine 5'-diphosphate (ADP).

Enzyme purification. Starting with about 180 g of frozen cells, preparation of activity 1 used the initial steps described earlier. The final purification step was a Cibacronblau F_3GA column, as mentioned (9); we used Sepharose 4B instead of Sephadex G200, but with the same coupling technique (19). Typically, we used an approximately 60-ml column (2.4 by 13 cm) washed with buffer C [0.1 M tris(hydroxymethyl)-aminomethane, pH 7.6, 0.01 M $MgCl_2$, and 14 mM 2-mercaptoethanol]. About 1,000 U (ca. 5 mg) of enzyme from the heat step (65 C, 5 min) was washed in, followed by 50 ml of buffer 3 and 75 ml of buffer 3 containing 10% $(NH_4)_2SO_4$. The enzyme could be eluted with 75 ml of the latter mixture containing also 1 mM ATP. The enzyme was concentrated by addition of solid $(NH_4)_2SO_4$ to 80% and centrifugation after 18 h at 4 C, and such final preparations were pure according to gels (see Results). For activity 1* the protocol was slightly modified. Heat treatment was at 48 C for 3 min (all activity was lost at 65 C). The column step was the same as with activity 1, but, since we had variable results with ammonium sulfate concentration of the final fraction, we used concentration versus buffer A containing also 0.2 mM ADP (Sartorius Membranefilter, Collodion bags SM13200). The enzyme preparations shown in Fig. 3 were:

activity 1 from strain K10 (*pfkA*$^+$ *pfkB*$^+$), glucose minimal medium; activity 1*, from strain RT247 (*pfkA11 pfkB*$^+$), fructose-supplemented broth. Gel electrophoresis, as in Davis (7), was in 7.5% gels, and was according to Weber and Osborn (24) for sodium dodecyl sulfate gels. Staining was with Coomassie brilliant blue and destaining was with 7.5% acetic acid in 5% methanol.

Antiserum to phosphofructokinase. Pure activity 1 from strain K10 (gels shown in Fig. 3), approximately 400 U (2 mg), was mixed with an equal volume (0.75 ml) of complete Freund adjuvant (Difco) and injected into the hind feet and nuchal regions of a male rabbit; 1 month later another 1 mg (without adjuvant) was given by the same route. Bleeding was 1 week later, and the antiserum was used without further fractionation. Antibody titrations were done in a series of incubations containing approximately 0.04 U of enzyme (pure or crude) and increasing amounts of antiserum (0 to 2 μl) in a total volume of 0.25 ml of 0.1 M sodium phosphate, pH 7.5, containing 20 mg of bovine plasma albumin per ml; after 1 h in ice the mixtures were centrifuged and the supernatants were assayed. The slopes, activity remaining versus antiserum, were converted to units precipitated (or inactivated)/milliliter of antiserum. For cross-reacting material (CRM) the incubations contained the usual amount of pure enzyme and also a volume of mutant crude extract which, if wild type, would have contained about the same activity. Then, if for a particular amount of antiserum, s_1 units were lost from the enzyme alone and s_2 units from the mixture containing CRM, then the relative amount of CRM was $(s_1 - s_2)/s_2$, and this value was converted to enzyme equivalent units per milligram of crude extract.

For double diffusion we used Immunoplates (pattern C, no. 085-073; Hyland Laboratories, Costa Mesa, Calif.).

Chemicals. The sources were described previously (21). 2-Keto-3-deoxygluconate was a kind gift of J. Pouysségur (17).

RESULTS

New pfkA mutants. We wanted a new set of phosphofructokinase mutants, which hopefully would include interesting ones (temperature sensitive, cold sensitive, nonsense, deletion, etc.) or serve for selection of useful phenotypes later by reversion. Several methods were used.

(i) A selection was done with strain K10, using ethyl methane sulfonate mutagenesis and penicillin counterselection in mannitol—the type of protocol used for the selection of *pfkA1* (14), but modified in that 22 C was the temperature used throughout (Table 1, RT [59-65]). We hoped that this procedure might yield cold-sensitive strains, or even temperature-sensitive lethals, but the only phosphofructokinase mutants obtained (*pfkA* alleles 4-9) had the usual phenotype at 22, 32, and 42 C. Likewise, no interesting temperature-dependent phenotypes (which proved to be phosphofructokinase mutants) were found in similar selections done at 30 C or with modifications which required growth at some permissive temperature (e.g., counterselection at 22 C but growth on mannitol at 37 C).

(ii) Another type of selection was directed mutagenesis. Since nitrosoguanidine often gives multiple mutations in closely linked genes (11) and *glpK* is close to *pfkA* (15), we used this mutagen on strain 113 (*glpK*$^-$ *pfkA*$^+$), selected reversion to growth on glycerol, and screened the revertants for impaired growth on mannitol and the phosphofructokinase mutant phenotype. This protocol yielded two more *pfkA* mutants (alleles 10 and 11).

(iii) Early attempts to obtain deletions of *pfkA* using positive selection for loss of the nearby markers *glpK* or *rha* had failed (A.T.E. Morrissey, Ph.D. thesis, Harvard University, Cambridge, Mass., 1971). Since then, other close loci have been identified. One of them, *tpi* (triose phosphate isomerase) is linked 82% by transduction to *pfkA* (1), counterclockwise. A deletion lacking both would not have appeared in the earlier selections; such a strain would be expected to grow, however, on a medium containing both succinate and glycerol. Another nearby marker, *kdgP,T*, specifies the permease for 2-keto-3-deoxygluconate (KDG), also counterclockwise to *pfkA* and linked 75% by transduction (17). Loss of this marker would not have interfered in the earlier selections. It offered the advantage of a positive selection for loss of function: *kdgP* mutation confers resistance to KDG in a strain such as PA3 which lacks KDGP aldolase and is constitutive for KDG permease. Accordingly, we selected for KDG resistance of strain PA3 on plates containing 0.4% succinate and 0.015% glycerol, i.e., a positive selection for loss of *kdgP,T* in conditions which should allow recovery of *pkfA-tpi* deletions. Of 80 such independent derivatives of strain PA3, most must have indeed been mutant at *kdgP,T*, since all were still gluconate negative (and hence still mutant at KDGP aldolase) and only one-fifth were resistant to galacturonate (likely mutants in KDG kinase, which maps elsewhere [18]). None of these strains gave evidence of having lost *tpi* or *pfkA*, since all grew normally on mannose.

(iv) In another selection we used strain DF89 (*pfkA*$^+$ *pfkB*$^-$). (*pfkB*$^-$ is discussed in an accompanying paper [22]. It reduces to zero the usual low phosphofructokinase activity found in *pfkA* mutants, and, correspondingly, makes the growth phenotype less "leaky." A strain of genotype *pfkA*$^+$ *pfkB*$^-$ contains activity 1 and

has a generally normal growth phenotype on most sugars.) After ethyl methane sulfonate mutagenesis, penicillin counterselection was done in mannose minimal medium and survivors were screened for the typical *pfkA⁻ pfkB⁻* phenotype. The three mutants obtained also proved eventually to be *pfkA* (alleles 12–14) and had no unusual properties.

(v) We did one selection for mutants of the usual phenotype starting with a *pfkA⁺ pfkB1* strain and found none.

In all these selections, although we screened primarily for the known phosphofructokinase mutant phenotypes, many isolates of different phenotype were found. Some looked like well-known mutant phenotypes (e.g., specific mannitol negatives or phosphotransferase mutants) and others were novel. Many were assayed anyway, since it seemed likely that phosphofructokinase mutants of unsuspected phenotype might exist; none was obviously altered in phosphofructokinase, and these strains will not be discussed further.

In contrast, all the new mutant strains of expected phenotype were apparently *pfkA* mutants. The position of the mutation was first screened by co-transduction with *metB*, and then most of the mutants were mapped by three-point transduction with respect to *metB* and *glpK* and were found, as with the other mutants (15), to give the order *pfkA-glpK-metB* (Table 2). All the mutants, as isolated, were assayed for phosphofructokinase and found to have the typical low activities characteristic of *pfkA⁻ pfkB⁺* strains. They were also assayed after transduction (*metB⁺*) into a common recipient, RT142, with the same results (specific activities): *pfkA⁺*, 0.804; *pfkA4*, 0.045; *pfkA5*, 0.047; *pfkA7*, 0.038; *pfkA8*, 0.045; *pfkA9*, 0.025; *pfkA10*, 0.068; *pfkA11*, 0.040; *pfkA12*, 0.023; *pfkA13*, 0.042; *pfkA14*, 0.030; *pfkA21* (12), 0.034.

Thus, there are now 15 independent mutants of similar phenotype with lesions at *pfkA*, all with low phosphofructokinase activities.

Temperature-sensitive revertants at pfkA. Since none of the new mutants was temperature sensitive in growth, we looked for temperature-sensitive revertants. As in earlier experiments (16), most revertants selected at low temperature were also normal at high temperature. (We tested two to four independent revertants of strains carrying *pfkA1-11*, including both large and small colonies.) One strain (RT65, *pfkA9*) was an exception, yielding some temperature-sensitive revertants (strains RT116, RT117, and 126); the lesion giving rise to the temperature-sensitive phenotype lies at *pfkA* (Table 2). The enzyme in these strains was heat labile (Fig. 1). These results accord best with *pfkA* being a structural gene for the enzyme and a strain such as RT117 carrying two mutations in it (*pfkA9*, *23*). However, we must note that *pfkA9* turned out to be an unusual allele in several respects. It was the only mutation giving temperature-sensitive phosphofructokinase revertants. There was also indication of another mutation in this strain, linked by transduction with *pfkA9* but separable from it, which affected growth on certain rich media, but did not obviously affect phosphofructokinase. Furthermore, allele *pfkA9* was unique in being the only one that yielded partial phenotypic revertants by loss of phosphoglucose isomerase (*pgi*) (see below).

Nonsense mutations at pfkA. For further knowledge about the *pfkA* locus, as well as to search for its complete inactivation, many of the *pfkA* alleles were tested for being nonsense mutations. Five independent mannitol-positive revertants of strains carrying *pfkA1-9* were

2. *Transductional mapping of new phosphofructokinase mutations*[a]

Phage donor	Recipient strain	No. of Met⁺ transductants scored	Unselected markers			
			glpK pfkA⁺	*glpK⁺ pfkA⁺*	*glpK⁺ pfkA*	*glpK pfkA*[b]
RT65 (*pfkA9*)	161 (*glpK metB*)	358	185	75	95	3
RT26 (*pfkA11*)	161 (*glpK metB*)	126	64	19	42	1
K10-15-16 (*pfkA21*)	161 (*glpK metB*)	74	30	8	35	1
RT116, 117, 126[c]	161 (*glpK metB*)	209	118	44	46	1

[a] Transduction was to methionine independence using appropriately supplemented minimal plates with gluconate or glucuronate as carbon source. Purified transductants were scored for growth on glycerol (*glpK*) and on mannose or sorbitol (*pfkA*). These are typical results; most of the other new mutations have been similarly mapped.

[b] This is the quadruple cross-over class if the order is *metB glpK pfkA*.

[c] These strains are temperature-sensitive revertants of RT65 *pfkA9* (*pfkA22*, *pfkA23*, *pfkA24*, respectively), and the data are pooled for the three transductions. In this cross the recipient was an Mtl⁺ derivative of stain 161 and scoring of *pfk* was on mannitol at 42 C; growth was wild type at room temperature.

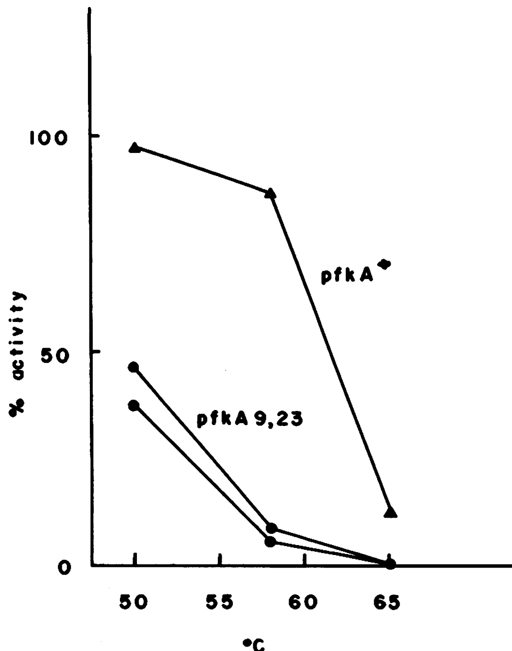

FIG. 1. *Heat inactivation. Extracts were prepared from cultures grown in broth containing fructose at 30 C, diluted into buffer A containing 14 mM mercaptoethanol at the temperature indicated, and left 2 min before chilling in ice and assaying. 100% means activity found in the same protocol without heating. The two strains used were RT142 (pfkA+) and RT235 (pfkA9, pfkA23) introduced by transduction into RT142 from RT117. The lower two curves are both for the mutant and differ in final protein concentration in the heating step (1.3 and 0.5 mg/ml); the concentration used for the pfkA+ strain was 0.7 mg/ml. Similar results were obtained with the original strains (K10 and RT117).*

tested for ability to yield plaques with a T4 amber mutant and none did. Alleles 10 and 11 had been selected in a sup^+ strain and could not be checked this way. Therefore, all the *pfkA* mutations (no. 1–14) were transduced into strain RT143 ($metB^-$, trp^-_{amber}, $\phi80^s$). One series (*pfkA* mutations no. 1–9, 12–14) was then tested for suppressibility by selection of revertants (two to six independent ones from each strain) on mannitol and then testing for simultaneous reversion to tryptophan independence (e.g., nonsense suppression of both the *pfkA* and *trp* loci). None of these revertants had lost the tryptophan requirement. We also tested the *pfkA* alleles 1 and 4–14 for suppressibility (growth on mannitol) by cross-streaking with $\phi80$*supF* and, as a control, $\phi80supF^+$. There was clear indication for suppression of the mannitol negativity with *pfkA11* and slight indication with *pfkA10*.

These two alleles were therefore retested, in the same *trp*-amber background, by phage P1 transduction to tryptophan independence, using a trp^+ *supF* donor, strain RT686. Since *trp* and *supF* are closely linked, the transductants might be trp^+ or *supF* or both. *supF* was screened according to suppression of a phage T4 nonsense mutation. All the transductants carrying *supF* (64 out of 70 for *pfkA10* and 60 out of 80 for *pfkA11*) showed restoration of growth on mannitol, and the others did not, which confirmed the two alleles as likely nonsense mutations.

The suppression of *pfkA10* by *supF* was rather weak, and this mutation has not been studied further. *pfkA11* has, however, been used in many experiments and for constructing various combinations with *pfkB* alleles. We will not describe all these combinations here, but will cite some general characteristics of strains with this mutation. Table 3 shows the phenotype of isogenic strains carrying $pfkA^+$, *pfkA11*, and *pfkA11 supF*. The strains carrying *pfkA11* (RT151 and -850) had a phenotype which did not differ markedly from any of the other *pfkA* mutants in this genetic background. The phenotype of the suppressed strain was not absolutely wild type again, but all the general growth characteristics were restored towards normal.

Phosphofructokinase in suppressed pfkA11. With a suppressible *pfkA* nonsense mutant, one might expect to find in the suppressed mutant a phosphofructokinase activity different from normal activity 1; this result would afford another demonstration that *pfkA* was the structural gene. Our initial search for phosphofructokinase activity in suppressed *pfkA11* failed to reveal it (Table 3); although RT849 contained *supF* and was suppressed for growth, it had the same enzyme activity as the isogenic $supF^+$ strain, RT850.

However, the activities in the unsuppressed strain were rather high, a characteristic of all *pfkA* mutants in this particular genetic background (strain X7187) and one which might obscure recognition of the suppressed product. To test whether the residual activity depended on *pfkB*, we introduced $pfkB^-$ into some of the above strains, giving strains RT853 (*pfkA11* $pfkB^-$ $supF^+$) and RT851 (*pfkA11* $pfkB^-$ *supF*). The former strain had the same phenotype as other $pfkA^-$ $pfkB^-$ strains, whereas the latter strain grew on carbon sources such as mannose (i.e., was suppressed). Nonetheless, the specific activities were 0.003 and 0.002, respectively, values typical of $pfkA^-$ $pfkB^-$ strains. Thus, even in the $pfkB^-$ background there was no suppressed activity evident.

TABLE 3. *pfkA-amber mutant*[a]

Strain	Genotype	Colony sizes (mm)						Phosphofructokinase activity
		Glucose	Mannose	Mannitol	Fructose	Glycerol	Galactose	
RT143	*pfkA*$^+$	1.8	1.8	1.6	1.8	2.0	1.8	0.204
RT151	*pfkA11*	1.0	1.1	0.3	1.7	2.1	2.3	0.059
RT850	*pfkA11 sup*$^+$	0.7	0.9	0.5	1.7	1.7	1.4	0.043
RT849	*pfkA11 supF*	1.6	1.5	0.9	1.9	2.2	2.0	0.042

[a] Minimal plates contained the indicated carbon sources (0.4%) and for RT143 and 151 were supplemented with methionine and tryptophan. Incubation was at 37 C for 48 h. For enzyme assay the strains were grown in broth supplemented with fructose.

One possible reason for not finding the suppressed product was that it might differ enough kinetically from activity 1 to only be revealed under unusual assay conditions. Our normal assay for phosphofructokinase uses 1 mM ATP and 1 mM fructose 6-phosphate, both saturating concentrations for activity 1. Accordingly, we varied the assay and indeed found with extracts of strains RT849 and RT851 (both *pfkA11 supF*) that increase of fructose 6-phosphate concentration to 6 mM revealed high phosphofructokinase activities. Unexpectedly, however, the new activity was also present in *pfkA11 supF*$^+$ strains. We discuss this "high K_m activity" in the following section and note here only that we have not yet found an activity dependent on *supF* in the *pfkA11* strains.

The high K_m activity. In this section we show that the high K_m phosphofructokinase in strains carrying *pfkA11* is related to activity 1. It was found only in strains carrying *pfkA11* (e.g., RT26, RT851, RT853). It was not found in strains 113 (*pfkA*$^+$, the parental strain of RT26), K10 (*pfkA*$^+$), DF1651 (*pfkA*$^+$), AM1 (*pfkA1*), DF86 (*pfkA2*), RT65 (*pfkA9*), or RT862 (*pfkA10*, the other nonsense mutant). The amount of activity, which we call activity 1*, was approximately 0.2 U/mg in crude extracts, a value similar to the specific activity of activity 1 in wild-type strains.

We will not present here a detailed kinetic comparison of activities 1* and 1. (Activity 1 has been studied in detail [3-5].) Using partially purified fractions from strain RT853 (*pfkA11 sup*$^+$) the special kinetic characteristics of activity 1* may be summarized as follows. The apparent affinity for fructose 6-phosphate was less, by a factor of about 10, for activity 1* than for activity 1; this is a difference opposite to that of activity 2 (Fig. 2). The curve is highly cooperative, like activity 1. (Because of lags during the assay these values are not accurate enough to determine Hill coefficients.) In several other kinetic properties activity 1* resembled activity 1. Thus, both depended on the NH_4^+ ion. Both were markedly activated, at nonsaturating fructose 6-phosphate concentrations, by ADP, with maximal activation at 0.5 mM ADP. (Indeed, our original finding of activity 1* using 6 mM fructose 6-phosphate likely depended on ADP formation in the assay; with an ATP-generating system the affinity was even less [Fig. 2]. Our present assay for activity 1* is to compare rates in the usual mixture containing 1 mM fructose 6-phosphate and that assay supplemented with 0.5 mM ADP.) Activity 1*, again like activity 1, was inhibited by phosphoenolpyruvate. Its affinity for ATP was similar to that of activity 1 and not cooperative.

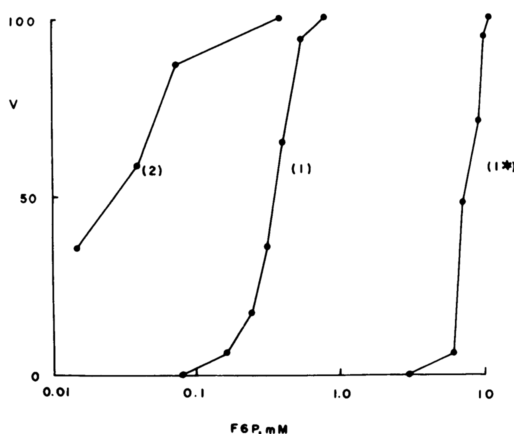

FIG. 2. *Kinetics of activity 1*. The enzyme was purified approximately 10-fold (pH step) from strain RT853 (pfkA11 pfkB⁻). The assay was described earlier and included creatine-P and creatin phosphokinase to keep ADP concentration low (9). The values are given as percentage of maximum activity. For comparison, data for activities 1 and 2 are replotted from the earlier paper (9).*

We purified activity 1* using a procedure only slightly modified from that for activity 1 (see Materials and Methods). Thus, their physical characteristics were similar, including affinity

for the dye Cibacronblau F3GA. But activity 1* was much more labile than activity 1, particularly with respect to dilution. Our purest preparation lost all its activity within 10 days at 4 C; activity 1 was stable for months.

Activity 1 is known to be an oligomer of four approximately 36,000-dalton subunits (3). In Fig. 3, gels 1 and 2 are pure preparations of activities 1 and 1*. Sodium dodecyl sulfate electrophoresis of the same preparation is shown also (gels 3 and 4), and both together are shown (gel 5). Gel 6 shows two standards (36,000 and 40,000 daltons), and gel 7 shows that activity 1 migrated with the smaller standard. Thus, the subunit molecular weight of activity 1* is not much smaller, if at all, than that of activity 1.

Antiserum to activity 1. Rabbit antiserum to activity 1 was prepared. Experiments using constant enzyme versus increasing antiserum (see Materials and Methods) showed similar amounts of activity precipitated from pure preparations of activity 1 and activity 1* (ca. 30 and 40 U, respectively, per ml of antiserum). With mixtures of activity 1 and 1*, inhibited units were additive, and if only activity 1 was measured in such a mixture then activity 1* appeared to inhibit its precipitation by competition (see below). All these results suggested that the two activities were antigenically similar, and, indeed, Fig. 4 shows that in a double diffusion plate they formed a reaction of identity.

We did some experiments on the antigenicity of crude extracts. With titrations using a constant amount of crude extract from wild-type strains (we tried both K10 [*pfkA*$^+$] and DF1651 [*pfkA*$^+$]), the ratio of activity precipitated per antiserum was the same as with the pure enzymes. We surveyed some other *pfkA* mutants for cross-reacting material (CRM) by titration of the inhibition of precipitation of pure activity 1 by crude extracts of the mutants (see Materials and Methods). Expressed as enzyme equivalent units per milligram of protein in the crude extract, all the strains surveyed contained CRM: DF85 (*pfkA1*), 0.075; DF86 (*pfkA2*), 0.214; DF87 (*pfkA3*), 0.228; RT65 (*pfkA9*), 0.165; RT116 (*pfkA9,22*), 0.256;

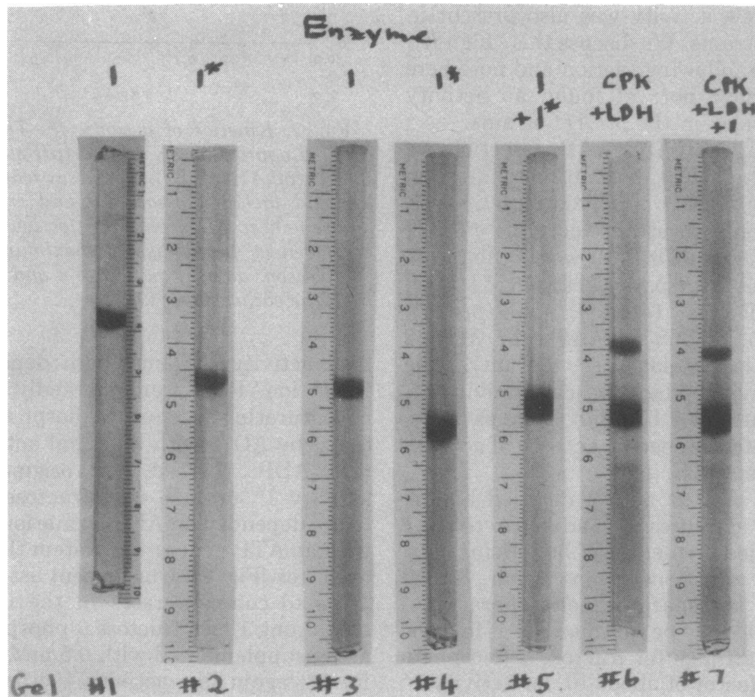

FIG. 3. Gels of activities 1 and 1. Preparation of the enzyme is described in Materials and Methods. Gels 1 and 2 are 7.5% polyacrylamide gels with native enzyme, approximately 20 and 10 μg, respectively, of activities 1 and 1*. Gels 3–7 are SDS gels and contained: gel 3, 10 μg of activity 1; gel 4, 10 μg of activity 1*; gel 5, 10 μg each of activities 1 and 1*; gel 6, 10 μg of dog muscle lactic dehydrogenase (LDH; Boehringer, 36,000-dalton subunits) and 5 μg of rabbit muscle creatine phosphokinase (CPK; Sigma; 40,000-dalton subunit; gel 7, activity 1, LDH, and CPK. To emphasize any separation, sodium dodecyl sulfate gel electrophoresis was for 19 h.*

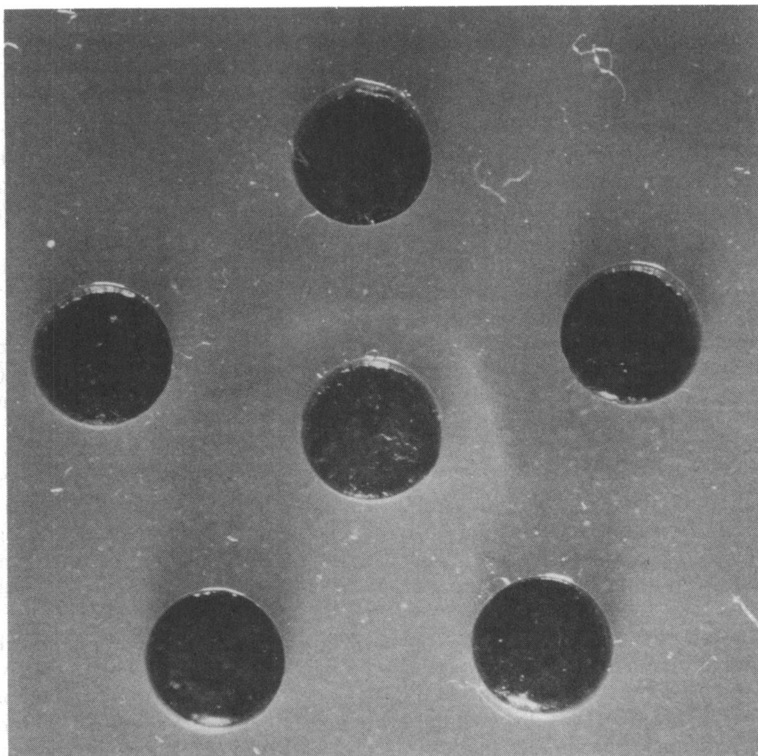

FIG. 4. *Double diffusion in agar. The center well contained 5 μl of antiserum, the top well contained approximately 5 μg of activity 1, and the upper right well contained approximately 5 μg of activity 1* (same preparations shown in Fig. 3). Control antiserum from the same rabbit before immunization gave no bands (not shown).*

and RT229 (*pfkA11*), 0.118 (this last value is activity 1*, here measured as blocking power). Of course, since even the nonsense mutation *pfkA11* contained CRM, it was not surprising that the other *pfkA* mutants did too.

Does pfkB1 suppress all pfkA mutants? One reason for selecting many *pfkA* mutants was to assess whether they were all suppressed by *pfkB1*. Double mutants were prepared by two transductions, starting with strain RT142, first putting in the various *pfkA* mutations by co-transduction with *metB* and then adding *pfkB1* by co-transduction with *pps*. Suppression by *pfkB1* was scored on glucose and mannose and was found with all *pfkA* alleles tested, no. 4–14. (It was previously shown for A1–3 [16].) We have not yet assayed phosphofructokinase activity in all these strains, but those assayed to date have shown it (i.e., a substantial increase over the activity in the $pfkA^-$ $pfkB^+$ parent). In particular we note that the amber allele, *pfkA11*, was phenotypically suppressible by *pfkB1*, just as all the others, including *pfkA12*,-*13*, and -*14*, alleles selected in a $pfkB^-$ background.

Might a conventional pfkA nonsense mutant not have the negative phenotype? Perhaps the simplest explanation for our failure to easily find conventional *pfkA* nonsense mutants (termination anywhere in the gene) would be that complete gene inactivation is lethal. However, for reasons having to do with the characteristics of enzyme in *pfkB1* suppressed strains (to be reported separately), we have also considered a different possibility: that complete loss of the *pfkA* gene product might not result in phosphofructokinase negativity. One such model (developed through discussion with Richard E. Wolf, Jr.), involving inactive *pfkA* product as an inactivator (e.g., a dominant negative subunit) of a second activity, would predict that reversion of *pfkA* mutants could occur by nonsense mutation at *pfkA*. Such revertants could be sensitive to a nonsense suppressor such as *supF*, which might then return their phenotype to phosphofructokinase negativity. We screened for this situation using strains carrying *pfkA* alleles (1 and 4–9), testing 12 independent

mannitol-positive revertants of each strain for sensitivity to ϕ80*supF* (decreased growth on mannitol). One such revertant was found with strain RT534 (*pfkA9*). This revertant (strain RT559) did not have more phosphofructokinase activity than RT534, and transduction showed *pfkA9* still present in normal linkage. Thus, the phenotypic reversion was by nonsense mutation in an unlinked gene. Considering mechanisms for such phenotypic reversion, one possibility was that if a phosphofructokinase mutant were relatively "leaky" it might be that closing off another pathway of hexose phosphate metabolism, such as the hexose-monophosphate shunt, could restore growth, perhaps by allowing higher fructose 6-phosphate levels and consequent use of the altered enzyme. We therefore assayed phosphoglucose isomerase and glucose 6-phosphate dehydrogenase and found strain RT599 to carry a *supF*-suppressible nonsense mutation in phosphoglucose isomerase. This evidence is reported in an accompanying paper (23).

It should be noted that we have not proven that loss of phosphoglucose isomerase in a strain carrying *pfkA9* restored growth on mannitol and mannose by increasing fructose 6-phosphate levels, but it does seem a reasonable model in view of the complexities of sugar metabolism in a strain forced to use the hexose-monophosphate shunt (see ref. 21). This is not a general phenomenon for suppression of the phosphofructokinase phenotype, however. *pfkA1* was not suppressed by *pgi-2*. Likewise, it has been reported that loss of glucose 6-phosphate dehydrogenase weakens growth on glucose of a *pfkA* mutant (12), and we confirmed this for *pfkA1* (21). The situation with RT559 is an interesting case of unlinked phenotypic suppression of phosphofructokinase mutation, which serves also to illustrate the unusual nature of the *pfkA9* mutation.

DISCUSSION

The original questions were: is *pfkA* the structural gene for phosphofructokinase, and can this gene be completely inactivated?

For the first question, the answer is almost certainly yes, by several criteria. (i) The enzyme is known to be a tetramer of one size subunit (3), and kinetic analysis and binding studies (4, 5) suggest the subunits are equivalent. All 15 of the independent phosphofructokinase mutants isolated to date lie in *pfkA*. (ii) There is alteration at this locus (e.g., *pfkA9 pfkA22*) which confers on the cell a temperature-sensitive phosphofructokinase mutant growth phenotype and temperature-labile phosphofructokinase activity in vitro. (iii) There is an amber mutation at the locus, *pfkA11*, resulting in a product clearly related to the normal enzyme physically, kinetically, and antigenically. (iv) The few mutants tested for material cross-reacting with antiserum prepared against the normal enzyme contained it. Although this test was a relatively crude one, inhibition of precipitation of normal enzyme, the amounts of CRM were in the range of the amounts of normal enzyme; thus, the mutants, which were usually approximately 90% deficient in enzyme activity, were not 90% deficient in CRM. (v) One more experimental result bearing on the question of whether *pfkA* is the structural gene has been documented elsewhere (Morrissey, Ph.D. thesis, Harvard University, 1971); a specialized transducing phage carrying the locus (ϕ80*dpfkA*) gave, upon induction of the lysogen, approximately a fivefold increase in enzyme activity.

All these results strongly suggest that *pfkA* is the structural gene for the enzyme. However, for several reasons the matter cannot be considered proven. The temperature-sensitive phenotype was very rare and only obtained as reversion of one particular allele, *pfkA9*, which showed several peculiarities. Likewise, the amber mutation had unusual characteristics, evidently producing a fragment of near normal size, and no in vitro activity could be found ascribable to suppression of this mutation. There is no doubt but that *pfkA* affects the structure of the enzyme; whether it actually codes for the primary sequence of the polypeptide, however, awaits demonstration that a missense mutation causes an amino acid substitution. Other possibilities are that *pfkA* specifies one of two dissimilar subunits of the enzyme or is involved in covalent modification of the primary subunit. It should be emphasized that in this paper we have referred to *pfkA* as a single locus to distinguish between it and *pfkB* and *pfkC* (22) as the possible structural genes. In fact, there is no direct evidence that all the *pfkA* mutations are in a single cistron, and complementation and fine structure analysis are logical next steps in our work.

As to the second question, can *pfkA* be completely inactivated (e.g., by deletion or early amber mutation), the answer still is uncertain. We have not yet succeeded in finding such mutations, among relatively few mutants, so we have naturally been led to speculate that such inactivation either is lethal or might not give the phosphofructokinase mutant phenotype. Models could be made for either suggestion. For the first, one might imagine that the

pfkA gene product has another function, perhaps enzymatic, or that total loss of phosphofructokinase activity is not compatible with growth. Certain results even restrict these possibilities. For example, $pfkA^-$ $pfkB^-$ strains are particularly "negative" phosphofructokinase mutants with respect to growth and assay, but they nonetheless grew on the usual permissive carbon sources such as glycerol. The temperature-sensitive mutant (e.g., *pfkA9 pfkA22*) likewise grew on permissive media at 42 C; it was not a temperature lethal, and we have not found strains with the usual mutant phenotype at 30 C but unable to grow on any medium at 42 C. Finally, we note that whatever the restriction might be on *pfkA* mutations it is clear that the ones we have found are not all the same.

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