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# Temperature-Sensitive Mutation Affecting Synthesis of Phosphoenolpyruvate Carboxykinase in *Escherichia coli*

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A mutation has been characterized in *Escherichia coli* which results in temperature-sensitive expression of phosphoenolpyruvate carboxykinase activity and antigen. The enzyme produced by the mutant strain at a permissive temperature or by cells treated with chloramphenicol at nonpermissive temperatures had normal activity and stability in extracts. Since phosphoenolpyruvate carboxykinase has a monomeric structure, the mutation probably affects the synthesis, rather than the structure or assembly, of the enzyme.

Phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.49) is subject to allosteric activation by calcium in *Escherichia coli* (4) and is regulated by catabolite repression (5, 7). Also, levels of this enzyme increase in cells during the stationary phase of growth on a variety of media in the presence or absence of exogenous cyclic AMP (1).

Stationary-phase induction has been observed also for the enzymes of glycogen biosynthesis (1), for proteolysis (11), and for a number of Krebs cycle enzymes (3). No central mechanism has been shown to coordinate these stationary-phase indictions at present. The *relA* gene product has been implicated in the stationary-phase induction of glycogen biosynthesis (1) and proteolysis (11), but the induction of proteolysis is independent of *relA* when the stationary phase is induced by starvation for a carbon source, phosphate, potassium, or energy (11). Stationary-phase induction of PEP carboxykinase is also independent of *relA* (5).

We had earlier (5) isolated *pck* mutants lacking activity of PEP carboxykinase from *pps* strains lacking activity of PEP synthetase. These double mutants (*pck pps*) were unable to utilize succinate and other four-carbon metabolites as carbon sources (5). Strains deficient in PEP carboxykinase alone (*pck*) grew well on all carbon sources, whereas those deficient in PEP synthetase alone (*pps*) were unable to utilize pyruvate and other three-carbon metabolites, but could grow on succinate and other carbon sources (5). Most of the *pck* strains picked up in our earlier study (5) were found to produce an enzymatically inactive antigen and were structural gene mutants. To investigate whether regulatory genes are also involved in the synthesis of PEP carboxykinase, we undertook isolation of mutants which have temperature-sensitive lesions in the synthesis of the enzyme.

A number of *pck pps* strains were plated on succinate minimal medium (5) at 30°C to select revertants. Succinate-positive, pyruvate-negative isolates (*pck*$^+$ *pps*) were purified and retested at 42 and 30°C on glucose, pyruvate, and succinate minimal plates. One of the strains tested, HG5 (*pck 3 pps*, derived by mutagenesis of DF1651 [5]), gave rise to a spontaneous revertant which was temperature sensitive for growth on succinate (Table 1). This revertant, HG40 (*pck-6 pps*), was selected for further study.

Levels of PEP carboxykinase and antigenically cross-reacting material (CRM) in strain HG40 are shown in Table 2. This strain had normal levels of enzyme activity and CRM at 30°C, but lower levels (less than 30%) at 42°C. The amount of CRM at 42°C was equivalent to the low level of enzyme activity, indicating that this strain did not contain inactive PEP carboxykinase molecules at 42°C.

When strain HG40 was incubated in succinate plates at 42°C, succinate-positive revertants which had regained normal activity of PEP carboxykinase (e.g., strain HG40-1, Table 1) were obtained at a frequency of about $10^{-8}$. Succinate-positive, pyruvate-positive revertants, which were probably *pck-6 pps*$^+$, were obtained at a slightly higher frequency. The results suggest that the temperature-sensitive *pck-6* is probably a point mutation at the same site as the original

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TABLE 1. *Growth of mutant strains on various carbon sources*

| Strain | Growth[a] | | | | | |
|---|---|---|---|---|---|---|
| | Glucose[b] | | Pyruvate | | Succinate[c] | |
| | 30° C | 42° C | 30° C | 42° C | 30° C | 42° C |
| DF1651 *pck*$^+$ *pps* | + | + | − | − | + | + |
| HG5 *pck-3 pps* | + | + | − | − | − | − |
| HG40 *pck-6 pps* | + | + | − | − | + | − |
| HG40-1 *pck*$^+$ *pps* (revertant of HG40) | + | + | − | − | + | + |
| HG40-2 *pck-6 pps*$^+$ (revertant of HG40) | + | + | + | + | + | + |

[a] Growth was scored as the presence or absence of visible colonies after 48 h when strains were streaked on minimal medium A (5) plates.

[b] Growth on glycerol minimal plates was similar to glucose for all strains.

[c] Growth on malate, fumarate, and acetate plates was similar to growth on succinate for all strains.

TABLE 2. *Levels of PEP carboxykinase and CRM in pck mutants*

| Strain | Relevant genotype | Temp (°C) | PEP carboxykinase (U/mg)[a] | CRM (U/mg)[b] |
|---|---|---|---|---|
| DF1651 | *pck*$^+$ *pps* | 37 | 33 | ND[d] |
| HG5 | *pck-3 pps* | 37 | 11 | 9.0 |
| HG40 | *pck-6 pps* | 30 | 31 | 29 |
| HG40 | *pck-6 pps* | 42 | 8.5 | 8.3 |

[a] Strains were grown to saturation in LB medium, extracts were prepared, and levels of PEP carboxykinase were assayed as described previously (4). Enzyme units are nanomoles of $^{14}CO_2$ exchanged per minute.

[b] Antigenically CRM was assayed as described previously (4) with antibodies raised against purified PEP carboxykinase. CRM units are equivalent to units of enzyme activity (enzyme-precipitating equivalents removed from antiserum per milligram of mutant protein added to the immunoassay).

[c] Similar values were obtained for DF1651 and other *pck*$^+$ strains grown at 30, 37, and 42°C.

[d] ND, Not determined.

*pck-3* mutation since *pck-3* (PCK$^-$) reverted to *pck-6* [PCK(Ts)], which in turn reverted to PCK$^+$; however, the possibility that *pck-3* and *pck-6* occurred at separate sites was not ruled out.

The *pck* mutation of strain HG5 (*pck-3*) is closely linked to the *pck* structural gene (5). Preliminary mapping of the temperature-sensitive revertant (*pck-6*) by F′ spot matings (5) showed that it was complemented by F′ KLF41, but not F′ KLF2, and therefore, was probably located in the same region of the *E. coli* chromosome as the *pck* structural gene (5).

Since HG40 and related strains were resistant to bacteriophage P1 (5), a lysogen was isolated by infection with P1 *CAM clr* 100 and selection for chloramphenicol resistance (5, 9). A lysate produced by inducing this lysogen at 42°C (9) was used to transduce strain HG49 (*asd pps*) to *asd*$^+$ since *asd* is linked to the *pck* structural gene (5). Of 621 *asd*$^+$ transductants, 589 were temperature sensitive for growth on all media and chloramphenicol resistant, suggesting that they were lysogens for P1 *CAM clr*100. Of the 32 nonlysogens, 10 had the PCK(Ts) phenotype of strain HG40, and 22 were PCK$^+$. This cross showed that *pck-6* was linked to the *asd* locus, a linkage previously demonstrated for the *pck* structural gene and the *pck-3* mutation (5). It is therefore likely that the *pck-6* mutation occurred, at, or very near, the site of the *pck-3* mutation since no PCK$^-$ transductants were obtained from the cross.

To investigate whether strain HG40 produced a thermolabile enzyme, which would explain its phenotype, the stability of PEP carboxykinase was measured at 42°C in extracts of mutant and wild-type (*pck*$^+$) cells grown at 30°C (Fig. 1A). Also, the stability of PEP carboxykinase was measured in cells of these strains grown to the late log phase at 30°C and shifted to 42°C in the presence of 200 μg of chloramphenicol per ml (Fig. 1B). It was ascertained that at the concentration used chloramphenicol abolished incorporation of $^3$H-labeled Casamino Acids into trichloroacetic acid-precipitable material (data not shown). The enzyme synthesized by strain HG40 was found to have a stability identical to that of wild-type PEP carboxykinase in extracts and in chloramphenicol-treated cells at 42°C. Also, the extracts of HG40 and DF1651 had identical profiles of PEP carboxykinase activity when assayed at different temperatures from 30 to 47°C. In addition, the enzymes from these strains had very similar $K_m$ values for the substrates PEP, ADP, and bicarbonate (data not shown).

Thus, it appears that the *pck-6* mutation causes temperature-sensitive synthesis or assembly of PEP carboxykinase. Since the enzyme has a monomeric structure (4, 8), it is unlikely that *pck-6* is an assembly mutation. It is possible that the mutation causes temperature-sensitive folding of the PEP carboxykinase monomer, so that monomers synthesized and folded at 30°C would be stable at 42°C; however, we know of no precedent for such a mutation. Temperature-sensitive assembly mutations have been isolated in bacteriophage P22 (10), but it is not clear whether folding of subunits or assembly of subunits into multimeric structures is involved.

Strain DF1651 had normal stationary-phase induction of PEP carboxykinase synthesis at 30 and at 42°C, whereas mutant strain HG40 had

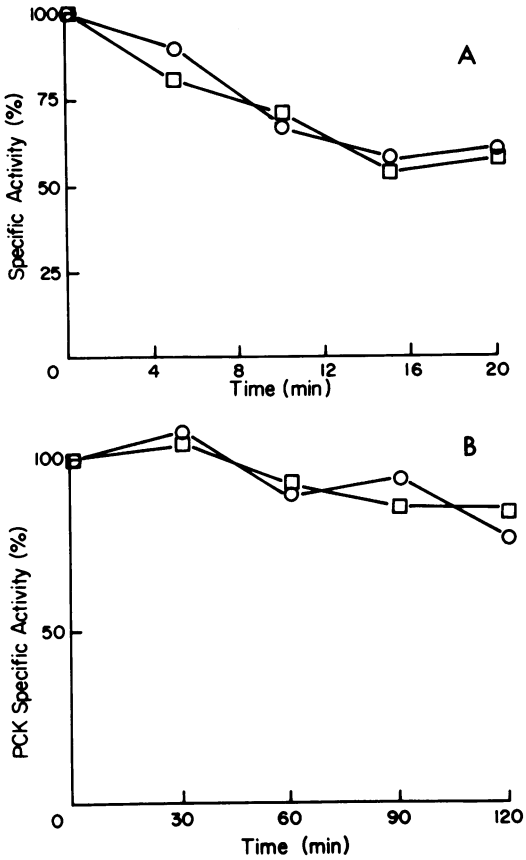


FIG. 1. *Stability of PEP carboxykinase in extracts and cells of strain HG40 (pck-6). (A) Extracts of DF1651 (pck⁺)* (□) *and HG40 (pck-6)* (○) *were incubated at 42°C. At various times 500-μl samples were removed, centrifuged at 8,000 × g for 10 min, and assayed for PEP carboxykinase activity. (B) Cells of DF1651 (pck⁺)* (□) *and HG40 (pck-6)* (○) *were grown to the mid-log phase (100 Klett units with a Klett-Summerson colorimeter and no. 66 filter) at 30°C and shifted to 42°C in the presence of 200 μg of chloramphenicol per ml. At various times, 5-ml samples were removed and assayed for PEP carboxykinase activity. Subsamples of each culture were labeled with $^3H$-Casamino Acids in the presence and absence of chloramphenicol to determine that protein synthesis (incorporation of $^3H$ into cold trichloroacetic acid-precipitable material) was inhibited by the chloramphenicol (data not shown).*

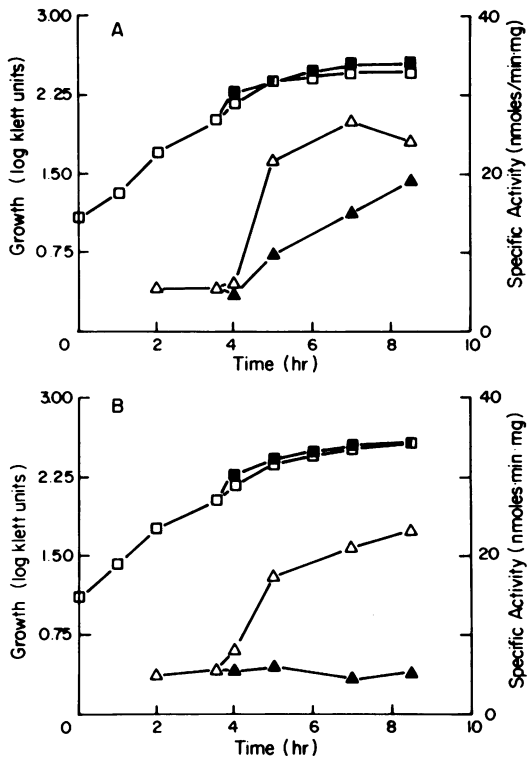


FIG. 2. *Stationary-phase induction of PEP carboxykinase in strains DF1651 and HG40. Cells were grown at 30°C on LB medium (without glucose). After 3.5 h, half of the culture was shifted to 42°C. (A) DF1651 (pck⁺). Growth at 30°C* (□) *and 42°C* (■). *PEP carboxykinase levels at 30°C* (△) *and 42°C* (▲). *(B) HG40 (pck-6). Growth at 30°C* (□) *and 42°C* (■). *PEP carboxykinase levels at 30°C* (△) *and 42°C* (▲).

normal induction at 30°C, but no induction at 42°C (Fig. 2). Although most temperature-sensitive mutations alter the stability of a protein (2, 6), the enzyme levels in both strains were very similar before induction at 42°C, which is consistent with the hypothesis that the *pck-6* mutation affects induction, rather than the stability, of the enzyme. Further, complementation by an F′ factor implies that the *pck-6* mutation is recessive and represents a loss of function. It is possible, therefore, that the *pck-6* mutation causes an alteration of an activator of transcription. This mutation, however, does not involve the *cya* or *crp* protein since strain HG40 fermented arabinose and maltose at 42°C on MacConkey plates (5).

Further proof for the hypothesis that *pck-6* affects induction, rather than the stability or activity, of the enzyme is provided in Table 3. The strain containing a mutation in the structural gene for PEP carboxykinase in the chromosome (5) and the wild-type gene on the F′ factor (strain KLF41/HG89, Table 3) had lower levels of enzyme activity than a *pck⁺/pck⁺* partial diploid strain (KLF41/HG87). However, the *pck⁺/pck-6* partial diploid strain (KLF41/HG88) had levels of enzyme similar to those of the *pck⁺/pck⁺* strain. This suggests that the *pck⁺/pck-6* strain has active copies of the struc-

TABLE 3. *Levels of PEP carboxykinase activity in partial diploid strains*

| Strain[a] | Relevant genotype | PEP carboxykinase (U/mg)[b] |
|---|---|---|
| HG87 | *pck*$^+$ *recA56* | 31 |
| KLF41/HG87 | F′ *pck*$^+$/*pck*$^+$ *recA56* | 69 |
| HG88 | *pck-6 recA56* | 7.5 |
| KLF41/HG88 | F′ *pck*$^+$/*pck-6 recA56* | 66 |
| HG89 | *pck-2 recA56* | 0.2 |
| KLF41/HG89 | F′ *pck*$^+$/*pck-2 recA56* | 42 |

[a] Strains HG87, HG88, and HG89 were constructed from strains DF1651 (*pck*$^+$), HG40 (*pck-6*), and HG4 (*pck-2*), respectively. The *pck-2* mutation affects the structural gene for PEP carboxykinase (5). These strains were made *argG*$^-$ by P1 transduction of an *argG*::Tn*5* mutation from strain DB6924 (David Botstein, unpublished data) and were then made *recA*$^-$ by cotransduction of *srl*::Tn*10* and *recA56* from strain DR7002 (David Rothstein, unpublished data). The F′ factor, KLF41, was introduced by spot matings on media lacking arginine (5). Genotypes of the F′ strains were confirmed by curing with sodium dodecyl sulfate (9) and by spot matings with appropriate *pck* strains (5).

[b] Strains were grown to saturation in LB medium at 42°C, and extracts were prepared and assayed as described previously (5). Enzyme units are given as nanomoles of $^{14}CO_2$ exchanged per minute.

tural gene on both the chromosome and the F′ factor and that the mutation in *pck-6* is not in the structural gene.

If the *pck-6* mutation affects the regulation of PEP carboxykinase synthesis, it may be possible to isolate mutants which overproduce enzyme from the revertants of HG40. It is anticipated that further examination of this strain will provide insight into the mechanisms regulating the synthesis of PEP carboxykinase and the role that this enzyme plays in gluconeogenesis.

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