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# Structural and Functional Analysis of Pyruvate Kinase from *Corynebacterium glutamicum*

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**Pyruvate kinase activity is an important element in the flux control of the intermediate metabolism. The purified enzyme from *Corynebacterium glutamicum* demonstrated a marked sigmoidal dependence of the initial rate on the phosphoenolpyruvate concentration. In the presence of the negative allosteric effector ATP, the phosphoenolpyruvate concentration at the half-maximum rate ($S_{0.5}$) increased from 1.2 to 2.8 mM, and cooperation, as expressed by the Hill coefficient, increased from 2.0 to 3.2. AMP promoted opposite effects: the $S_{0.5}$ was decreased to 0.4 mM, and the enzyme exhibited almost no cooperation. The maximum reaction rate was 702 U/mg, which corresponded to an apparent $k_{cat}$ of 2,540 $s^{-1}$. The enzyme was not influenced by fructose-1,6-diphosphate and used $Mn^{2+}$ or $Co^{2+}$ as cations. Sequence determination of the *C. glutamicum pyk* gene revealed an open reading frame coding for a polypeptide of 475 amino acids. From this information and the molecular mass of the native protein, it follows that the pyruvate kinase is a tetramer of 236 kDa. Comparison of the deduced polypeptide sequence with the sequences of other bacterial pyruvate kinases showed 39 to 44% homology, with some regions being very strongly conserved.**

*Corynebacterium glutamicum* is widely used for the industrial production of the amino acids, lysine, and glutamate (26). Although progress has been made in the understanding of the molecular organization of genes involved in lysine biosynthesis, relatively little is known about the structure and function of enzymes involved in the intermediate metabolism of *C. glutamicum* (15). Flux analysis of glucose metabolism in *C. glutamicum* suggested that regulation of the phosphoenolpyruvate (PEP) branch point is the limiting factor in lysine production (26). At this branch point, PEP carboxylase and pyruvate kinase compete for the available PEP (15). Recently, it was shown that amplification or disruption of the PEP carboxylase gene had little influence on lysine production (13). However, disruption of the single copy of the *pyk* gene resulted in a dramatic decrease in lysine biosynthesis, with concomitant accumulation of dihydroxyacetone, glyceraldehyde, and acetate (12).

The reaction catalyzed by pyruvate kinase (EC 2.7.1.40) is essentially irreversible in vivo and appears to be a control point for the regulation of glycolytic flux (10). Several mechanisms exist to control pyruvate kinase activity. In mammals, isoenzymes with different kinetic properties are found in various tissues (14). *Escherichia coli* and *Salmonella typhimurium* possess two types of pyruvate kinase, one inducible and activated by fructose-1,6-diphosphate, and the other constitutive and activated by AMP (11, 24, 28, 29). However, in many single-celled eukaryotes and prokaryotes, only one type of allosteric pyruvate kinase is found (3, 6, 17). Almost all bacterial pyruvate kinases are activated by nucleoside monophosphates and a number of phosphorylated sugar-phosphates (21). In *C. glutamicum*, pyruvate kinase is also influenced positively by AMP and negatively by ATP (15, 20). We recently isolated the pyruvate kinase (*pyk*) gene by applying a combination of PCR, site-directed mutagenesis, and complementation (12). With the cloned *pyk* gene now available, the experiments reported here were designed to provide molecular data for the *pyk* gene and to characterize the properties of the purified enzyme in more detail.

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## MATERIALS AND METHODS

**Strains, plasmids, and media.** All bacterial strains and plasmids used are listed in Table 1. *E. coli* and *C. glutamicum* M2 were grown on complex medium supplemented with 20 g of glucose per liter as described previously (12, 13). When appropriate, kanamycin (50 μg/ml), chloramphenicol (12 μg/ml), or ampicillin (100 μg/ml) was added.

**DNA techniques.** DNA isolations, digestions, electrophoresis, and transformations were performed as described elsewhere (9, 22). DNA sequence analysis was performed by the dideoxy chain termination methods of Sanger et al. with the Sequenase kit from United States Biochemicals (Cleveland, Ohio) (23). Oligonucleotides used for sequencing were provided by Midland Certified (Dallas, Tex.). Sequence data were compiled and analyzed by use of the Genetics Computer Group program package. Multiple alignments were carried out by use of the algorithm of Lipman and Pearson (16).

**Pyruvate kinase assay.** Assays for pyruvate kinase were performed in methylacrylate cuvettes (Fisher, Pittsburgh, Pa.) at room temperature (22 ± 2°C) with a Hewlett-Packard 8452A diode array spectrophotometer. Pyruvate kinase activity was monitored by the decrease in absorbance of NADH ($\varepsilon$ = 6.22 $mM^{-1} \cdot cm^{-1}$) at 340 nm, with lactate dehydrogenase as the coupling enzyme. The assay mixture contained (in 1 ml) 80 μmol of Tris-HCl (pH 7.7), 20 μmol of KCl, 10 μmol of $MnCl_2$, 2 μmol of ADP, 5 μmol of PEP, 0.4 μmol of NADH, 10 U of lactate dehydrogenase, and an appropriate amount of enzyme. Two micromoles of AMP was added per milliliter of assay mixture to achieve maximal activity. One unit of pyruvate kinase activity is defined as the amount of enzyme which converts 1 μmol of PEP per min.

**Analysis of kinetic properties.** The kinetic properties of

TABLE 1. Bacterial strains and plasmids used

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* DH5α | *lacZ*ΔM15 *hsdR recA* | Bethesda Research Laboratories |
| *C. glutamicum* E12 | Restriction-deficient derivative of strain ASO19 | 9 |
| *C. glutamicum* M2 | $Cm^r$ $Pyk^-$ derivative of strain E12 | 12 |
| Plasmids | | |
| pBluescript $SK^+$ | $Amp^r$ derivative of pUC19 | Stratagene |
| pMF1014α | $Km^r$ derivative of pSR1 | 1 |
| pMG123 | $Km^r$, 2.8-kb *Pst*I-*Bam*HI *pyk*-containing fragment in pMF1014α | 12 |
| pMG125 | pBluescript $SKII^+$ containing 1.0-kb *Eco*RI-*Sal*I *pyk* fragment | This work |
| pSL2 | pBluescript $SKII^+$ containing 1.3-kb *Pst*I-*Xho*I *pyk* fragment | This work |
| pSL3 | pBluescript $SKII^+$ containing 1.5-kb *Xho*I-*Bam*HI *pyk* fragment | This work |

pyruvate kinase were determined by varying the substrate, cofactor, and effector concentrations in the assay over at least a 100-fold range bracketing the $K_m$. The $K_m$, $K_i$, $V_{max}$, and Hill coefficients of the enzymes were estimated by using the nonlinear fitting program GRAFIT from Erithacus Software Ltd. (Staines, United Kingdom) with either the Michaelis-Menten ($V = V_{max} \times [S]/K_m + [S]$) or Hill ($V = V_{max} \times [S]^n/K_m + [S]^n$) equation, where $[S]$ is the substrate concentration and $n$ is the Hill coefficient.

**Protein purification.** Pyruvate kinase was purified from *C. glutamicum* M2 harboring plasmid pMG123, which contains the *pyk* gene (12). Expression of the pyruvate kinase in this strain is approximately 19-fold higher than in the wild type (12). Cells were grown overnight in complex medium with 20 g of glucose and 50 mg of kanamycin per liter, harvested at the late log phase by centrifugation (10 min at 10,000 × *g*), washed with buffer A (50 mM Tris-HCl [pH 7.7] containing 20 mM KCl, 10 mM $MnCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol), and stored as pellets at −20°C. Unless stated otherwise, all purification procedures were carried out at 4°C. Cell pellets (4 g) were resuspended in 20 ml of buffer A and disrupted by ultrasonication on ice at 40 W output power (XL2020 sonicator; Heat Systems, Farmingdale, N.Y.) 10 times for 30 s each with alternating cooling periods of 30 s. The cell debris was removed by centrifugation for 45 min at 47,000 × *g*. The supernatant was used as the crude extract and contained 6 to 10 mg of protein per ml, as determined by the method of Bradford (2).

A saturated $(NH_4)_2SO_4$ solution in 50 mM Tris-HCl (pH 7.7) was added to the crude extract until 45% saturation was obtained. The solution was centrifuged for 30 min at 47,000 × *g*, and the pellet was discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant until 75% saturation was reached. After being stirred on ice for 30 min, the solution was centrifuged for 20 min at 20,000 × *g*. The supernatant was discarded, and the pellet was dissolved in 20 ml of buffer A and dialyzed against 1 liter of this buffer containing 10 mM Tris-HCl (pH 7.7), 5 mM $MnCl_2$, and 0.5 mM dithiothreitol. The dialyzed protein solution was applied to a Q-Sepharose (Pharmacia, Piscataway, N.J.) column (2.5 by 20 cm), previously equilibrated with buffer A. The adsorbed proteins were eluted with a 480-ml linear gradient from 0.02 to 1 M KCl in buffer A. Fractions with pyruvate kinase activity, collected at 120 mM KCl, were pooled and concentrated in an Amicon ultrafiltration unit with an XM50 filter. The concentrated protein solution was mixed in a 1:1 ratio with glycerol and stored overnight at −20°C. This solution was thereafter diluted fourfold in buffer A and applied to a Cibacron blue F3GA column (Bio-Rad, Richmond, Calif.) composed of two Econo-cartridges, previously equilibrated with buffer A. The adsorbed proteins were eluted in a 100-ml linear gradient from 0.02 to 2 M KCl in buffer A. Fractions with pyruvate kinase activity, collected at 800 mM KCl, were pooled and concentrated in a Centricon YM-100 unit. The concentrated protein solution was diluted 10-fold with buffer A and applied to a mono-Q HR5/5 (Pharmacia) column previously equilibrated with buffer A. The adsorbed proteins were eluted in a 30-ml gradient from 0.02 to 1 M KCl. Pyruvate kinase was collected in a single fraction at 350 mM KCl, concentrated in a YM-100 unit, and used immediately or diluted 1:1 with glycerol and stored at −20°C until use. No significant loss of activity was observed after storage in 50% glycerol for 3 months. Storage in 10 or 25% glycerol resulted in 80 and 30% loss of activity, respectively, after 2 months.

**Analytical procedures.** The purity of the enzyme after various chromatographic steps was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Miniprotean II system with Ready-gels of 4 to 15% polyacrylamide (Bio-Rad). The prestained standards (Bio-Rad) were phosphorylase (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), and lysozyme (18.5 kDa). Gels were stained with Coomassie blue R250. Native enzyme molecular mass was determined on a Superdex-200 column (Pharmacia) equilibrated with buffer A containing 150 mM KCl, with thyroglobulin (669 kDa), ferritin (445 kDa), catalase (232 kDa), and aldolase (158 kDa) as standards.

**Amino acid sequence determinations.** The amino acid sequences of the N terminus of pyruvate kinase and of internal peptide fragments were determined on an Applied Biosystems pulse liquid sequencer (model 477A/120A). To generate internal fragments, the enzyme (1 mg/ml in 10 mM $KH_2PO_4$, pH 7.0) was digested with trypsin. The resultant peptides were separated on a $C_{18}$ reverse-phase column (Vydac, Hesperia, Calif.), and some of the well-separated peaks were collected for the sequence determinations.

**Nucleotide sequence accession number.** The sequence data reported in this article will appear in the GenBank nucleotide sequence data library under accession number L27126.

## RESULTS

**Enzyme purification.** The purification of pyruvate kinase from *C. glutamicum* M2 harboring the *pyk* gene on multicopy plasmid pMG123 was carried out at 4°C and generally took 2 days. In four steps, a 50-fold-purified enzyme was obtained, with 27% recovery (Table 2). Affinity chromatography on Cibacron blue proved to be very effective for removing contaminating polypeptides.

**Characteristics of the purified enzyme.** The molecular mass of the native pyruvate kinase was estimated by gel filtration on

TABLE 2. Purification of pyruvate kinase from *C. glutamicum* M2 containing the *pyk* gene on multicopy plasmid pMG123

| Step | Protein (mg) | Sp act (μmol/min/mg) | U | % Recovery | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract | 188 | 13.2 | 2,475 | 100 | 1 |
| 45–75% $(NH_4)_2SO_4$ | 138 | 18.7 | 2,571 | 105 | 1.4 |
| Q-Sepharose | 20 | 85.2 | 1,704 | 69 | 6.5 |
| Cibacron blue | 5 | 278 | 1,390 | 56 | 21 |
| Mono-Q | 1 | 658 | 658 | 27 | 50 |

Superdex-200 and appeared to be 236 ± 12 kDa (data not shown). SDS-PAGE of the purified enzyme revealed one subunit with a relative molecular mass of 58 ± 4 kDa, which suggests an $\alpha_4$ subunit structure (Fig. 1). This size and subunit structure are similar to those reported for the enzyme from *E. coli*, *Bacillus stearothermophilus*, and *Lactococcus lactis* (17, 21, 29).

**Substrate specificity.** In addition to the ADP ($K_m$ = 0.07 mM)-dependent conversion of PEP, the purified enzyme also used GDP ($K_m$ = 0.25 mM) or IDP ($K_m$ = 0.12 mM) as nucleotides. The enzyme did not use $Mg^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Ca^{2+}$, or $Fe^{2+}$ as cations but showed an absolute dependence on either $Mn^{2+}$ ($K_m$ = 0.5 mM) or $Co^{2+}$ ($K_m$ = 1.8 mM). Ammonium or $K^+$ ions were not required for activity, nor did they inhibit the activity. The enzyme was slightly inhibited by $PO_4^{3-}$ ($K_i$ = 12 mM). Activity in Tris-HCl, triethanolamine-HCl, or Tricine-KOH buffer was comparable, with an optimum between pH 7.6 and 7.9.

**Kinetic properties.** The kinetic properties of the purified pyruvate kinase of *C. glutamicum* were determined by creating substrate saturation curves. Figure 2 shows that the activity of the enzyme was sigmoidally dependent on the PEP concentration. By nonlinear regression analysis, a substrate concentration at the half-maximum rate ($S_{0.5}$) of 1.2 mM, a $V_{max}$ of 598 U/mg, and a Hill coefficient of 2.0 were obtained. The enzyme was not activated by fructose-1,6-diphosphate. However, in the presence of AMP, the dependence of the initial velocity on the PEP concentration was hyperbolic (Fig. 2), as has been described for pyruvate kinases from other sources (7, 24, 25, 29). Accordingly, the Hill coefficient was 1.1, indicating no cooperativity. The $S_{0.5}$ was reduced to 0.4 mM, and the $V_{max}$ was 702 U/mg. Assuming a molecular mass of 236 kDa, this would result in an apparent $k_{cat}$ of 2,540 $s^{-1}$. The effect of ATP on the initial velocity of the enzyme is shown in Fig. 2. At low PEP concentrations, the enzyme had only weak activity, and high PEP concentrations were required to obtain maximum velocity. Analysis of these data yielded an $S_{0.5}$ of 2.8 mM, a $V_{max}$ of 487 U/mg, and a Hill coefficient of 3.2, indicating increased cooperativity.

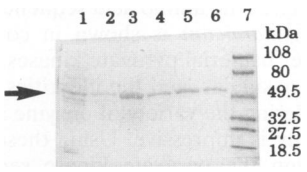



FIG. 1. SDS-PAGE of samples from different steps in the procedure to purify pyruvate kinase from *C. glutamicum*. Lane 1, crude extract of strain M2 harboring pMG123 (8 μg); lane 2, $(NH_4)_2SO_4$ concentrate (2 μg); lane 3, pooled fractions of Q-Sepharose (12 μg); lane 3, pooled fractions of Cibacron blue (4 μg); lane 4, fraction 27 of Mono-Q (6 μg); lane 6, fraction 28 of Mono-Q (4 μg); lane 7, prestained molecular mass standards. The arrow indicates pyruvate kinase.

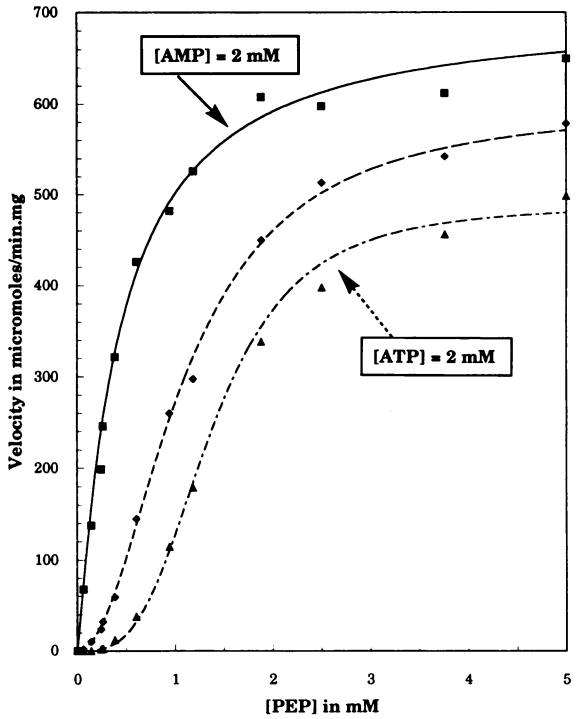



FIG. 2. Steady-state kinetics of pyruvate kinase. Assay mixes contained 2 mM ADP, 10 mM $MnCl_2$, and 2 μg of purified enzyme. ■, kinetics in the presence of 2 mM AMP; ◆, kinetics in the absence of any activator or inhibitor; ▲, kinetics in the presence of 2 mM ATP. Symbols show actual determinations; curves were calculated by nonlinear regression.

**Sequencing and nucleotide sequence analysis.** The pyruvate kinase gene *pyk* was cloned from *C. glutamicum* previously by a combination of PCR, site-specific mutagenesis, and complementation (12). By this procedure, a 2.8-kb *Pst*I-*Bam*HI genomic DNA fragment harboring the *pyk* gene was isolated. For sequencing of the *pyk* gene, a 1.3-kb *Pst*I-*Xho*I, a 1.5-kb *Xho*I-*Bam*HI, and a 1.0-kb internal *Eco*RI-*Sal*I fragment were recloned into pBluescript SKII$^+$ to yield pSL2, pSL3, and pMG125, respectively. A detailed restriction map was made (Fig. 3), and after preliminary analysis, a contiguous sequence of 2,795 nucleotides (nt) for both strands was determined with overlapping sequences. The resulting nucleotide sequence is shown in Fig. 4. Coding region analysis revealed an open reading frame with possible translation start sites at nt 740 (ATG) and nt 746 (GTG). Sequence analysis of the N-terminal amino acids of the purified protein showed MDRRTKIVCTL GPAVASADGIL as the N-terminal peptide sequence, which indicated that the GTG start codon at nt 746 is most likely used as the initiation site. A potential ribosome-binding site, AG GCT, was located 7 bp upstream of the GTG translation initiation codon. As for other known *Corynebacterium* genes, TAA was identified as the stop codon. The codon preference and the G+C content (55.6%) of *pyk* were very similar to those for previously reported *C. glutamicum* genes (8). The amino acid sequence of several peptide fragments obtained by trypsin treatment of the purified enzyme showed 100% homology with the deduced amino acid sequences of the open reading frame starting at nt 746. By using this initiation site and the stop

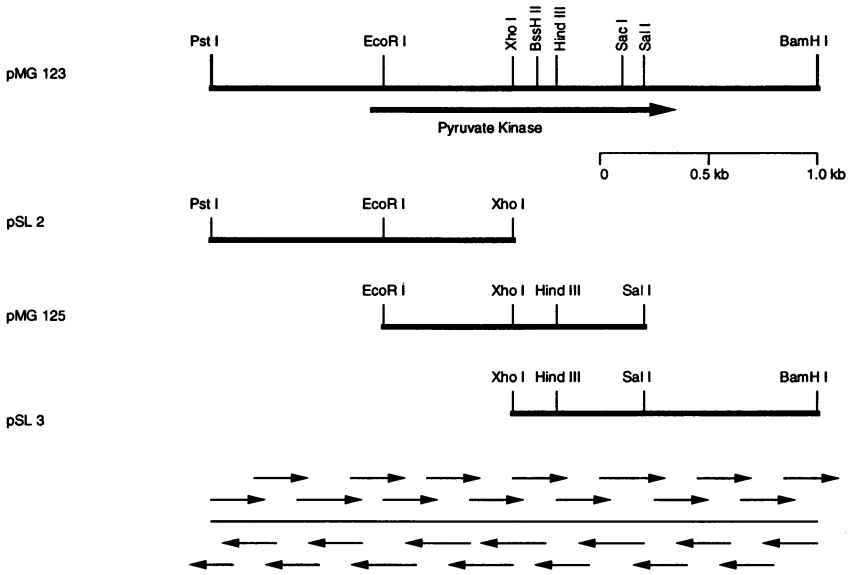



FIG. 3. Restriction map and sequencing strategy for the chromosomal *Pst*I-*Bam*HI fragment of pMG123. The location and direction of transcription of *pyk* are also shown. The arrows indicate the extents and directions of nucleotide sequence determinations.

codon at nt 2171, the nucleotide sequence predicted a chain length of 475 amino acids and a subunit molecular mass of 59,912 Da for the *C. glutamicum* pyruvate kinase. This value coincided very well with the molecular mass of 58 kDa determined for the purified enzyme by SDS-PAGE. Downstream of the *pyk* gene, centered around nt 2208, a region of dyad symmetry was identified with a structure similar to that of rho-independent transcription terminators, a result indicative of the transcription termination of the *pyk* mRNA at this site.

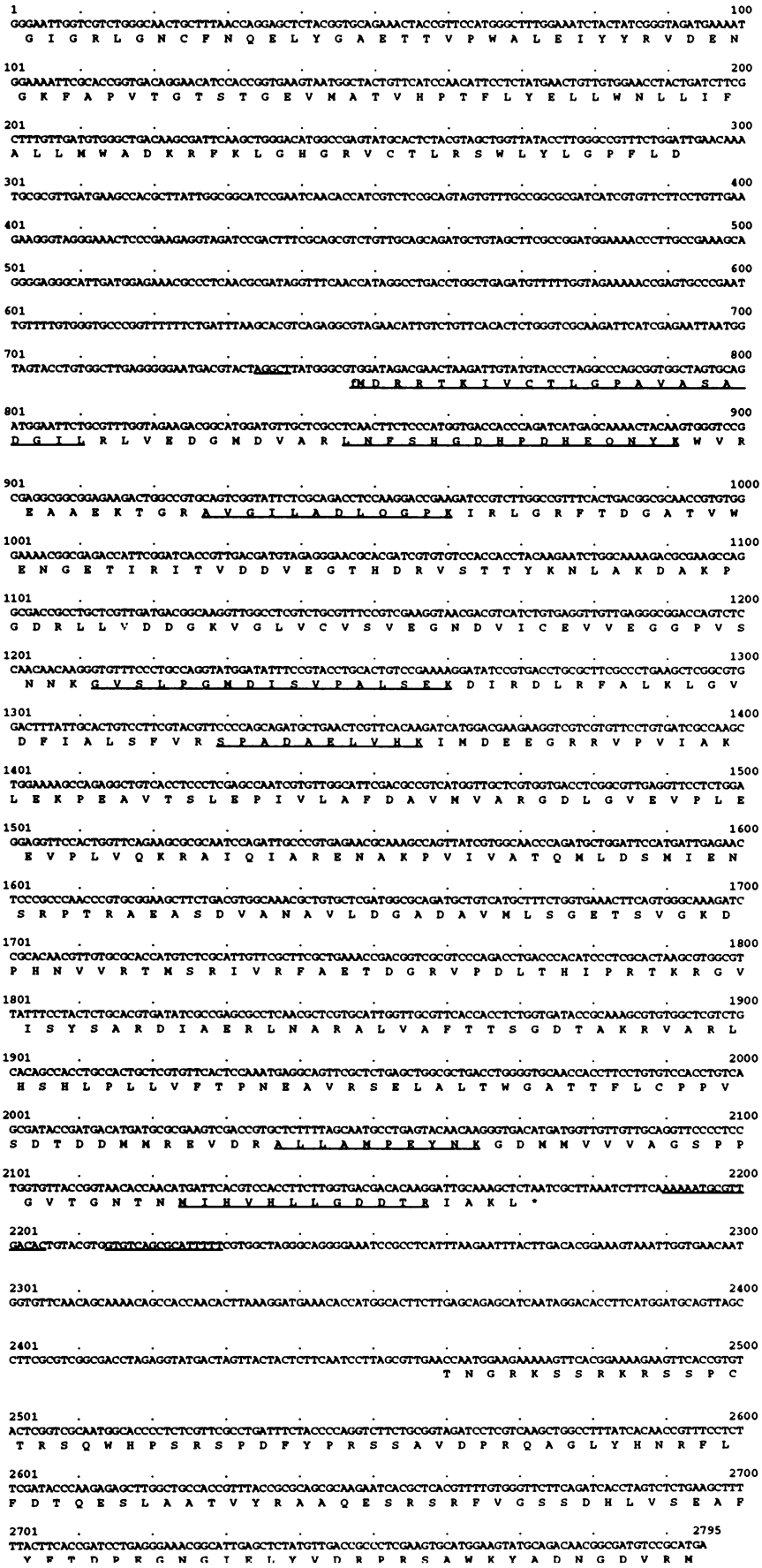
The homologies of the primary amino acid sequence over the total length of the *C. glutamicum pyk* gene to the corresponding sequences of the *B. stearothermophilus*, *E. coli pyk* I, *E. coli pyk* II, and *L. lactis* genes were 44, 42, 40, and 39%, respectively, with some very conserved regions (Fig. 5).

## DISCUSSION

In *C. glutamicum*, pyruvate kinase is a key enzyme in the central pathway of energy production (15). The enzyme is a target for regulation by metabolites and plays a major role in the rate of energy synthesis, growth, and lysine production (12, 15, 20). The pyruvate kinase was isolated and purified to homogeneity. The activity of the purified enzyme was dependent on several factors in vitro. The pyruvate kinase was activated by the substrate PEP and by AMP, whereas ATP and $P_i$ were inhibitory, as reported for several other pyruvate kinases (7, 25, 28). No evidence was obtained for the activation of the *C. glutamicum* pyruvate kinase by fructose-1,6-diphosphate, although such action has been reported for the enzymes of *E. coli pyk* I, *L. lactis*, and *Bacillus licheniformis* (4, 5, 25, 29). Similar to the *E. coli* fructose-1,6-diphosphate-activated pyruvate kinase, the enzyme from *C. glutamicum* uses both ADP and GDP as a phosphate acceptor (29). However, the *C. glutamicum* enzyme showed a higher affinity for ADP than for GDP, whereas the *E. coli* enzyme had a preference for GDP. In contrast to most other pyruvate kinases, the enzyme from *C. glutamicum* did not use $Mg^{2+}$ as a cation but depended completely on the presence of either $Mn^{2+}$ or $Co^{2+}$. The specific activity (702 U/mg) of the *C. glutamicum* enzyme was very high compared with the activity of other bacterial enzymes (130 to 240 U/mg) but is in the same range as mammalian enzyme preparations (400 to 800 U/mg) (4, 5, 7, 14, 29). The pyruvate kinase of *C. glutamicum* showed complex steady-state kinetics. At a saturating PEP concentration, normal Michaelis-Menten kinetics were observed with respect to ADP. With various lower PEP concentrations, especially in the presence of ATP, the enzyme showed sigmoidal kinetics. The Hill plot gave a slope of 3.2, which, together with the molecular mass determinations, is a good indication of the $\alpha_4$ subunit structure.

Given the functional identities of the various pyruvate kinases as well as similar regulatory characteristics, it is not surprising that their globular structures also appear to be similar. The *C. glutamicum* enzyme consists of four subunits with a total mass of 236 kDa. Similar quaternary structures have been observed for the *B. stearothermophilus*, *L. lactis*, and *E. coli pyk* I enzymes (17, 20, 28). These biochemical criteria suggest that the primary structures of the proteins are also comparable. In Fig. 5, the amino acid sequence of the pyruvate kinase from *C. glutamicum* is shown in comparison to the sequences of other bacterial pyruvate kinases (17, 19, 20). The sequences are very similar, and the identities vary between 39 and 44%. Considering the variety of enzyme sources, the high sequence similarity is impressive. Using these similarities, we were able to align the pyruvate kinase sequences. Studies involving chemical modifications and elucidation of the three-

FIG. 4. Nucleotide sequence of *pyk* and adjacent DNA and deduced amino acid sequence. The nucleotide sequence is shown from the *Pst*I site (position 1) to the *Bam*HI site (position 2795). Numbers indicate the number of nucleotides from the *Pst*I site. The putative ribosome-binding site and potential transcription terminator are underlined. Underlined amino acid sequences indicate sequences determined by peptide sequence analysis.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101 200
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G K F A P V T G T S T G E V M A T V H P T F L Y E L L W N L L I F
201 300
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G V T G N T N M I H V H L L G D D T R I A K L *
2201 2300
GACACTGTACGTGGTGTCAGCGCATTTTTCGTGGCTAGGGCAGGGGAAATCCGCCTCATTTAAGAATTTACTTGACACGGAAAGTAAATTGGTGAACAAT
2301 2400
GGTGTTCAACAGCAAAACAGCCACCAACACTTAAAGGATGAAACACCATGGCACTTCTTGAGCAGAGCATCAATAGGACACCTTCATGGATGCAGTTAGC
2401 2500
CTTCGCGTCGGCGACCTAGAGGTATGACTAGTTACTACTCTTCAATCCTTAGCGTTGAACCAATGGAAGAAAAAGTTCACGGAAAAGAAGTTCACCGTGT
T N G R K S S R K R S S P C
2501 2600
ACTCGGTCGCAATGGCACCCCTCTCGTTCGCCTGATTTCTACCCCAGGTCTTCTGCGGTAGATCCTCGTCAAGCTGGCCTTTATCACAACCGTTTCCTCT
T R S Q W H P S R S P D F Y P R S S A V D P R Q A G L Y H N R F L
2601 2700
TCGATACCCAAGAGAGCTTGGCTGCCACCGTTTACCGCGCAGCGCAAGAATCACGCTCACGTTTTGTGGGTTCTTCAGATCACCTAGTCTCTGAAGCTTT
F D T Q E S L A A T V Y R A A Q E S R S R F V G S S D H L V S E A F
2701 2795
TTACTTCACCGATCCTGAGGGAAACGGCATTGAGCTCTATGTTGACCGCCCTCGAAGTGCATGGAAGTATGCAGACAACGGCGATGTCCGCATGA
Y F T D P R G N G I E L Y V D R P R S A W K Y A D N G D V R M

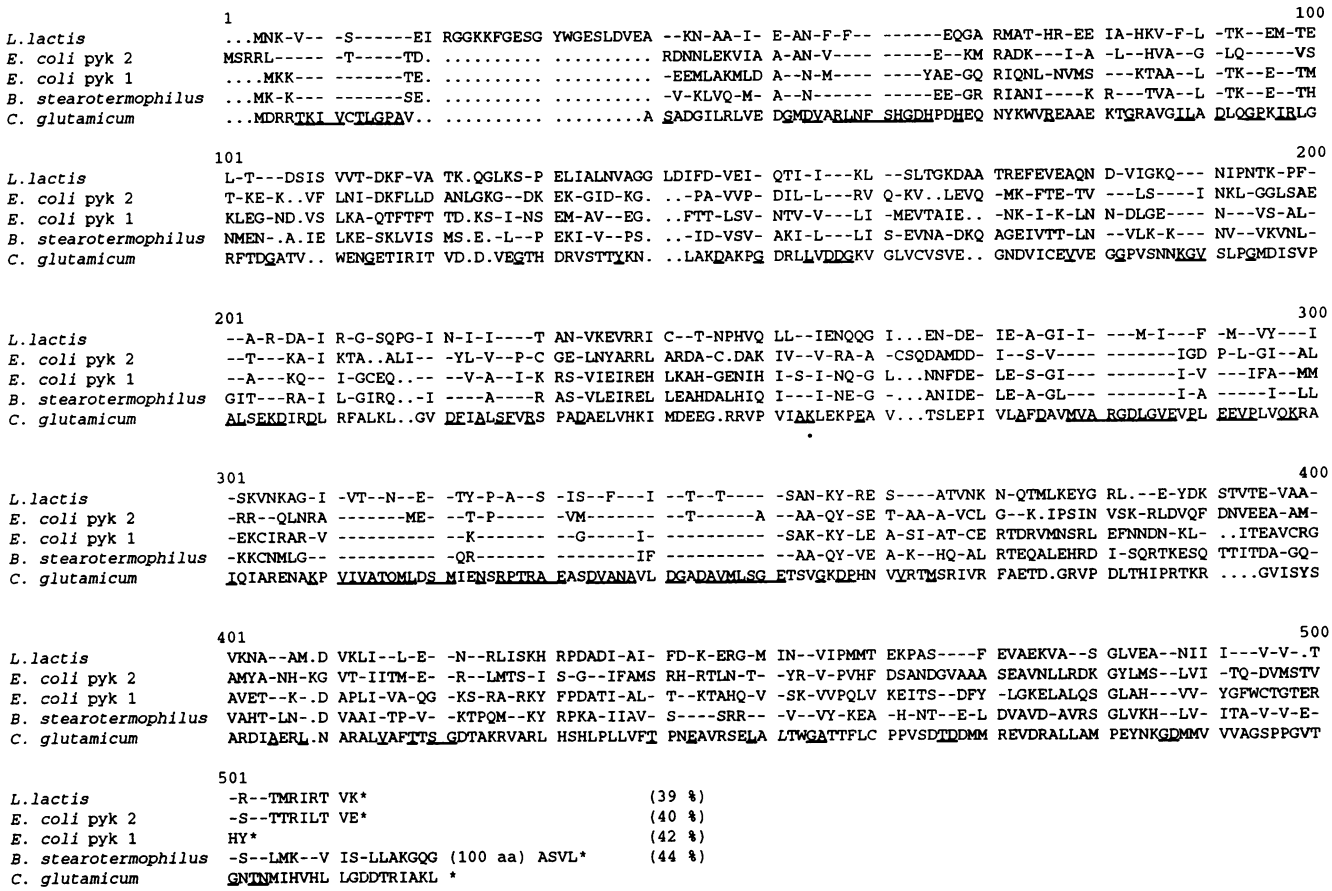


FIG. 5. Comparison of the amino acid sequence of *C. glutamicum* pyruvate kinase with the sequences of other bacterial pyruvate kinases. The sequences were aligned mainly by the algorithm of Lipman and Pearson. The first amino acid for each pyruvate kinase is the N-terminal amino acid. Dashes indicate amino acid residues which are identical to that of the *C. glutamicum* pyruvate kinase. Residues identical in four of the five sequences presented are underlined. The C-terminal end of each sequence (*) and the percent identity with the *C. glutamicum* sequence are indicated. The conserved lysine residue is marked (·). The pyruvate kinases examined were from *B. stearothermophilus* (GenBank accession number D13095), *E. coli pyk* II (M63703), *E. coli pyk* I (M24636), and *L. lactis* (L07920).

dimensional structure of cat muscle M1 and *B. stearothermophilus* pyruvate kinases have identified amino acids close to the active site and revealed an essential lysine residue in a very conserved sequence (17, 18, 21, 24, 27). Many of these amino acid residues are also conserved in the *C. glutamicum* pyruvate kinase (Fig. 5), including the lysine residue suggested to act as an acid-base catalyst in the active site (Lys-208). Furthermore, a large number of conserved glutamate and aspartate residues, which are thought to be involved in ADP and ATP binding, were found throughout the sequence (20).

Unlike the gene in *B. stearothermophilus* and *L. lactis*, the *pyk* gene in *C. glutamicum* does not seem to be part of an operon together with the genes for phosphofructokinase (*pfk*) or lactate dehydrogenase (*ldh*) (17, 20). Sequence analysis of the up- and downstream regions of the *pyk* gene did not reveal any homology with either *pfk* or *ldh* when analyzed with the *C. glutamicum* codon preference but revealed some homology to the RNA-directed RNA polymerase (<24%) of influenza virus (upstream region) and some homology to a sterol desaturase (<28%) from *Saccharomyces cerevisiae* and the *malD* (<27%) gene of *Streptococcus pneumoniae* (downstream region).

Previous reports (12, 13, 15) show that intermediate metabolism in *C. glutamicum* is not well understood. Molecular cloning and DNA sequence analysis of the genes involved in carbon metabolism, including the *pyk* gene, together with mutant studies and in vivo flux analyses are necessary to elucidate the regulatory network and gene structure in this biotechnologically important organism.

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