A GTP-dependent Vertebrate-type Phosphoenolpyruvate Carboxykinase from Mycobacterium smegmatis

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This is the first report on a bacterial vertebrate-type GTP-dependent phosphoenolpyruvate carboxykinase (PCK). The pck gene of Mycobacterium smegmatis was cloned. The recombinant PCK was overexpressed in Escherichia coli in a soluble form and with high activity. The purified enzyme was found to be monomeric (72 kDa), thermophilic (optimum temperature, 70 °C), very stable upon storage at 4 °C, stimulated by thiol-containing reducing agents, and inhibited by oxalate and by α-ketoglutarate. The requirement for a divalent cation for activity was fulfilled best by Mn2+ and Co2+ and poorly by Mg2+. At 37 °C, the highest Vm value (32.5 units/mg) was recorded with Mn2+ and in the presence of 37 mM dithiothreitol (DTT). The presence of Mg2+ (2 mM) greatly lowered the apparent Km values for Mn2+ (by 144-fold in the presence of DTT and by 9.4-fold in the absence of DTT) and Co2+ (by 230-fold). In the absence of DTT but in the presence of Mg2+ (2 mM) as the co-divalent cation, Co2+ was 21-fold more efficient than Mn2+. For producing oxaloacetate, the enzyme utilized both GDP and IDP; ADP served very poorly. The apparent Km values for phosphoenolpyruvate, GDP, and bicarbonate were >100, 66, and 8300 μM, respectively, whereas those for GTP and oxaloacetate (for the phosphoenolpyruvate formation activity) were 13 and 12 μM, respectively. Thus, this enzyme preferred the gluconeogenesis/glycogen biosynthetic direction. This property fits the suggestion that in M. smegmatis, pyruvate carboxylase is not anaplerotic but rather gluconeogenic (Mukhopadhyay, B., and Purwantini, E. (2000) Biochim. Biophys. Acta 1475, 191–206). Both in primary structure and kinetic properties, the mycobacterial PCK was very similar to its vertebrate-liver counterparts and thus could serve as a model for these enzymes; examples for several immediate targets are presented.

Phosphoenolpyruvate carboxykinase (PCK) catalyzes the conversion of oxaloacetate (OAA) and phosphoenolpyruvate (PEP) (1).

$$\text{OAA} + \text{ATP or GTP} \leftrightarrow \text{PEP} + \text{CO}_2 + \text{ADP or GDP}$$

REACTION 1

Based on the nucleotide substrate specificity the PCKs have been traditionally classified into two groups (1): 1) GTP-dependent enzymes (GTP:PEP:oxaloacetate carboxylyase (trans-phosphorylating); EC 4.1.1.32) that use both GDP and IDP in the OAA synthesis reaction and use ADP very poorly with very high and nonphysiological Km values and 2) ATP-dependent enzymes (ATP:oxaloacetate carboxylyase (trans-phosphorylating); EC 4.1.1.49) that do not use GTP or ITP (or GDP and IDP). Barring four exceptions, all known bacterial PCKs are ATP-dependent (2–4). The enzyme from Ruminococcus flavefaciens is a GTP-dependent PCK (GTP-PCK), although unlike others in this class it cannot use IDP (3). GTP-PCK activities have been identified in the cell extracts of Treponema pallidum (2) and Arthrobacter globiformis (4). Chlorobium limicola possesses a putative GTP-PCK gene (5). The PCKs from yeast, trypanosomatid parasites, and plants are ATP-dependent, but the mammalian and many other eukaryotic enzymes are GTP-dependent (1). It has been suggested that this difference could form the basis for designing therapeutic drugs against parasitic nematodes (1, 6) and trypanosomatid parasites (7). In most cases, the PCK activity has been postulated to catalyze the first committed step in gluconeogenesis, the formation of PEP from OAA (8, 9). In adipose tissue, GTP-PCK primes glycogen synthesis, especially under fasting conditions, when pyruvate, lactate, and amino acids serve as the precursors for PEP (9); this tissue does not synthesize glucose. It is the gluconeogenesis role that makes GTP-PCK a potential target in the treatment for noninsulin-dependent diabetes mellitus (10). In parasites and in certain bacteria, GTP-PCK fulfills an anaplerotic role by carboxylating PEP to OAA (2, 3, 11, 12). For all of these reasons, this enzyme has been a focus of intense mechanistic studies since its discovery (1, 9). The ATP- and GTP-dependent PCKs show very little primary structure similarity to each other (1, 13). Recently, the crystal structure for the PCK from Escherichia coli, an ATP-dependent enzyme, has been solved (14). But until now the GTP-dependent enzymes have been refractory to such studies (13). Also, a bacterial model for a GTP-dependent PCK has not been presented.

Certain mycobacteria are major human and animal pathogens. They are also ubiquitous in nature, where they play a role in the conversion of oxaloacetate (OAA) and phosphoenolpyruvate (PEP) (1).

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‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF332191.

§ The abbreviations used are: PCK, phosphoenolpyruvate carboxykinase; PEP, phosphoenolpyruvate; α-KG, α-ketoglutarate; MDH, malate dehydrogenase; PYC, pyruvate carboxylase; MsmPCK, PCK from M. smegmatis; rMsmPCK-His10, recombinant and His10-tagged PCK expressed in E. coli; rMsmPCK, recombinant PCK with its His10 tag removed; GTP-PCK, GTP-dependent PCK; ATP-PCK, ATP-dependent PCK; PCK-C, cytosolic PCK; PCK-M, mitochondrial PCK; DTT, dithiothreitol; HPLC, high performance liquid chromatography; OAA, oxaloacetate; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid.

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Vertebrate-type PEP Carboxykinase of M. smegmatis

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Media, and Culture Conditions—** *Mycobacterium smegmatis* mc^155 (22) was cultivated as described previously (19). *E. coli* DH5α (supE44 histR17 recA1 endA1 gyrA96 thi-1 relA1) (23) was used as a general purpose cloning host, and *E. coli* C41(DE3) (24) was the overexpression host. These strains were grown in Luria-Bertani (LB) medium. The *E. coli* transformants were selected on plates or grown in liquid medium containing 100 μg of ampicillin per ml, except for the cultures raised for overexpression of PCK, where a level of 400 μg/ml was used.

**DNA Techniques**—Generally, all DNA manipulations were performed according to standard methods (25). Chromosomal DNA from *M. smegmatis* mc^155 was isolated as described (26). Each polymerase chain reaction was carried out by using the PfuTurbo® DNA polymerase from Stratagene (La Jolla, CA), and a typical reaction mixture (100 μl in volume) had the following composition: reaction buffer (Stratagene), 0.189 μg of *M. smegmatis* chromosomal DNA, 10 pmol of each of the primers (10 μM), 2.5 mM MgSO_4_ (used instead of 2 mM provided by the buffer). The amplification conditions were as follows: initial melting at 95 °C for 5 min; 35 cycles comprising denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 7 min; and a final finishing extension at 72 °C for 7 min.

**Cloning and Sequencing of the pck Gene from M. smegmatis and the Construction of an Overexpression Plasmid**—The *pck* coding region from *M. smegmatis* chromosomal DNA was amplified by use of the primers 5’-TGACTCAGACAGGTCCCGCTGCTTGAATTGGACCGC-C3’ (MsmPCKF1) and 5’-CTAACTTACGGCTCTTTGGGCGT-GAATCATC-3’ (MsmPCKR2) and was cloned into the EcoRI site of the vector Bluescript II SK⁺ (Stratagene), generating the plasmid pBE129. The sequence for each strand of this clone was determined. The sequences of two more such clones, generated from the two independent amplification reactions, were also determined, and they were found to be the same as that for the first.

For obtaining an overexpression construct, the *pck* coding sequence was amplified from pBE129 by use of 5’-GGGCTGCAGGAATTCATAT-GACCTCAGACAGGTCCCGCTGCTTGAATTGGACCGC-C3’ (MsmPCKF1) oligonucleotide as the 5’-primer (the engineered *NdeI* restriction site is underlined) and 5’-GATCTAACCTTACGGCTCTTTGGGCGTGAAATCATC-3’ (MsmPCKR4) as the 3’-primer (the BamHI site is underlined). After digestion with *NdeI* and BamHI, the polymerase chain reaction product was cloned into similarly restricted pET19b (Novagen, Inc., Madison, WI). In the resultant plasmid, pBE129–19b, the *pck* coding sequence was placed under the control of the T7 promoter, and the corresponding open reading frame was fused to an NH₂-terminal His tag sequence via an enterokinase cleavage site. *E. coli* DH5α (supE44 histR17 recA1 endA1 gyrA96 thi-1 relA1) (24) was transformed with pBE129–19b to obtain the strain *E. coli* BE129–19b. The *pck* coding sequence in pBE129–19b was found to be identical to that in pBE129. This sequence has been submitted to GenBank™ with the accession number AF332191.

**Overexpression in *E. coli* and Purification of the Recombinant Phosphoenolpyruvate Carboxykinase** (rMsmPCK-His tag and rMsmPCK)—*E.

coli BE129–19b was grown under vigorous agitation in LB supplemented with ampicillin (400 μg/ml). When the *A₂₆₀* of the culture reached a value of ~0.6 (as measured by using a model DU640 UV-visible spectrophotometer; Beckman Coulter, Inc., Fullerton, CA), the PCK expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. After lysis of the cell wall at 37 °C, the cell slurry was allowed to proceed for an additional 3 h. Then the cells were harvested by centrifugation at 4,000 × g at 4 °C and stored at −20 °C. Each enzyme purification step was performed at 4 °C. Typically, 6.5 g of *E. coli* BE129–19b cell paste was thawed in 6.5 ml of 100 mM potassium phosphate buffer, pH 7. The cells were disrupted by three passages through a prechilled (−18 °C) French press (Hydranautics) at 1.28 × 10⁸ pascals. The broken cell slurry was centrifuged at 18,000 × g for 30 min at 4 °C, and 9 ml of supernatant was recovered. (For preliminary trials, as indicated under “Results,” this supernatant was further clarified via centrifugation at 100,000 × g). The supernatant was mixed with 0.74 ml of 4 mM NaCl and 0.1 ml of 1 mM imidazole-HEC buffer, pH 7 (final concentrations: 300 mM NaCl and 10 mM imidazole). This solution was loaded onto a 1-ml column bed (13 mm) of Superflow Ni^2+^-nitrilotriacetic acid-agarose (Qiagen, Inc.) under a gravity flow; before use, the column bed was equilibrated with 10 ml of a solution containing 50 mM potassium phosphate buffer, pH 7, 300 mM NaCl, and 10 mM imidazole. To remove unbound and weakly bound proteins, the matrix was washed with 10 ml of equilibration solution and then with four 1-ml washes containing 50 mM potassium phosphate buffer, pH 7, and 10 mM imidazole; 2 ml of 0.5 M NaCl; 2 ml of 1 x NaCl; 3 ml of 2 x NaCl; and 2 ml of 0.3 x NaCl (to lower the NaCl concentration). The elution was carried out in steps with the following solutions (each containing 50 mM potassium phosphate buffer, pH 7, and 0.3 M NaCl): 5 ml of 50 mM imidazole; 2 ml of 100 mM imidazole; 2 ml of 150 mM imidazole; 2 ml of 200 mM imidazole; and 5 ml of 250 mM imidazole. The fractions collected from these applications were assayed for PCK activity, and the active fractions (100, 150, and 200 mM imidazole washes) were pooled. The proteins in this pool were fractionated by ammonium sulfate precipitation. The precipitate obtained at each step (corresponding to ammonium sulfate concentrations of 40, 50, 60, and 70% of saturation at 0 °C) was recovered by centrifugation at 14,000 × g and was dissolved in 50 mM Tris-HEC, pH 7. The solution corresponded to the pellet obtained between 50 and 60% ammonium sulfate saturation contained about 81% of the total activity. It was diluted with an equal volume of a solution containing 20 mM potassium phosphate buffer, pH 7, and 2 mM ammonium sulfate. The diluted solution was centrifuged at 14,000 × g to remove particulate matter. The enzyme in this supernatant was concentrated in a Microcon 10 concentrator (Amicon, Beverly, MA). To obtain the enzyme with an intact N-terminal amino acid sequence, the ammonium sulfate precipitation was carried out in a Microcon 10 concentrator (Amicon, Beverly, MA) at 4 °C. To capture the uncleaved or His-tagged protein from the enzyme solution, it was passed through a 1-ml Superflow Ni^2+^-nitrilotriacetic acid-agarose column bed that was precolumn with the rEK cleavage/capture buffer. Then the column bed was washed with 3 ml of the same buffer. The effluents from the loading and wash steps, containing the recombinant enzyme without the His tag (rMsmPCK), were combined. The pooled protein was concentrated, washed with the gel filtration buffer (see above), and stored in the same buffer at 4 °C.

The His tag in the rMsmPCK-His tag was removed by the action of recombinant enterokinase (Novagen, Inc.) in the rEK cleavage/capture buffer (20 mM Tris-HEC, pH 7.4, 50 mM NaCl, 2 mM CaCl₂), and the enzyme in the digest was purified by use of the rEK cleavage/capture buffer. Then the enzyme was purified by Superflow Nitrilotriacetic Acid-Agarose (Novagen, Inc.) at 4 °C. To capture the uncleaved or His-tagged protein from the enzyme solution, it was passed through a 1-ml Superflow Ni^2+^-nitrilotriacetic acid-agarose column bed that was precolumn with the rEK cleavage/capture buffer. Then the column bed was washed with 3 ml of the same buffer. The effluents from the loading and wash steps, containing the recombinant enzyme without the His tag (rMsmPCK), were combined. The pooled protein was concentrated, washed with the gel filtration buffer (see above), and stored in the same buffer at 4 °C.

**Determination of the Molecular Characteristics—** SDS-PAGE was performed with a slab gel according to Laemmli (27). The Tris-HEC Ready Gels from Bio-Rad were used for nondenaturing gel electrophoresis. The methods for size exclusion chromatography, matrix-assisted laser desorption/ionization time-of-flight mass spectrometric measurements, and the determination of the N-terminal amino acid sequence have been described previously (19).

**Assays and Data Analysis—** Protein was assayed according to Bradford (28) by using the dye reagent from Bio-Rad. Two methods were used for assaying the phosphoenolpyruvate carboxykinase activity, and the methods for size exclusion chromatography, matrix-assisted laser desorption/ionization time-of-flight mass spectrometric measurements, and the determination of the N-terminal amino acid sequence have been described previously (19).

**Vertebrate-type PEP Carboxykinase of M. smegmatis**
mm HEPES-NaOH buffer, pH 7.2, 100 mM KHCO₃, 2 mM PEP, 2 mM IDP, 2 mM MgCl₂, 0.1 mM MnCl₂, 37 mM dithiotreitol (DTT), 2 units/ml MDH, and 0.25 mM NAD⁺ + H⁺. Each assay was initiated with enzyme addition and conducted at 37 °C. The other assay was performed in the direction of PEP formation (30, 31). The standard assay mixture contained 100 mM HEPES-NaOH buffer, pH 7.2, 3 mM malate, 0.2 mM GTP, 1 mM NAD⁺, 2 mM MgCl₂, 0.1 mM MnCl₂, 37 mM DTT, and 6 units/ml MDH. To a temperature-equilibrated reaction mixture MDH was added, and the absorbance at 340 nm was allowed to increase and stabilize. At this stage, PCK was added and progress of the reaction was followed by a further increase in the absorbance at 340 nm. Here, the PCK reaction tends to disturb the established equilibrium of the MDH reaction by consuming OAA, and to counter this effect MDH makes more OAA from malate by reducing NADH, and to counter this effect MDH makes more OAA from malate by reducing NAD⁺, and to counter this effect MDH makes more OAA from malate by reducing NAD⁺. This assay was also conducted at 37 °C. For pH studies, the HEPES-NaOH buffer was replaced with a buffer that was obtained by adjusting a solution of 50 mM MES, 100 mM Tris, and 50 mM glacial acetic acid to the desired pH value with NaOH; over the pH range used (pH 6–9), the ionic strength of this buffer remained essentially constant (32). Unless mentioned otherwise, values of the PCK activity are for the OAA formation reaction. All initial rate data were analyzed by using the KinetAsyst program version 1.01 (IntelliKinetics, State College, PA). A multiple alignment was generated by use of the ClustalW program (33) at the PBIL (Pôle Bio-Informatique Lyonnais) site on the World Wide Web. The same program was used for comparing a pair of primary structures to each other.

RESULTS
Cloning, Expression, Purification, and Storage Stability of Recombinant Phosphoenolpyruvate Carboxykinase—The phosphoenolpyruvate carboxykinase gene (pck) from M. smegmatis was cloned by polymerase chain reaction by use of the primers that were designed based on the putative pck gene of M. tuberculosis (20). The recombinant enzyme was overproduced in E. coli under the control of the T7 promoter. It was synthesized with activity in an NH₂-terminal His₁₀-tagged form (rMsmPCK-His₁₀), Fig. 1A shows SDS-PAGE patterns for the whole cell lysate, low spin cell extract (18,000 × g supernatant) and high spin extract (150,000 × g supernatant) of E. coli (pBE129–19b) cells that had been induced with isopropyl-1-thio-β-galactopyranoside. These centrifugation steps were sequential (the low spin step followed by the high spin), and no concentration or volume reduction step was involved. Through these steps the protein concentration in the extract dropped 2-fold, but, as seen in Fig. 1A (analyzes with 5 µl of extract from each step), the level for the soluble fraction of heterologously expressed enzyme apparently did not decrease to the same extent. Thus, most of the synthesized rMsmPCK-His₁₀ was present in the cell in a soluble form.

Starting from either low-spin (18,000 × g) or high spin (100,000 × g) cell extracts, the recombinant enzyme was purified by use of a Ni²⁺-affinity-based chromatographic step and ammonium sulfate precipitation. The use of a high spin extract prevented clogging of the column and provided a higher flow rate during chromatography. Nevertheless, both types of extracts provided final products of the same quality. Typically, from 10 g of cell paste, 33 mg of His-tagged enzyme with specific activities of 18–22 OAA-forming units/mg of protein was obtained. Such a preparation exhibited a single band in a native gel (Fig. 1B) and thus was considered homogeneous. The corresponding SDS-PAGE pattern (Fig. 1C) showed that the enzyme was composed of one or more subunits of an apparent mass of 74 kDa.

The His tag was removed from homogeneous rMsmPCK-His₁₀ by the action of enterokinase. Fig. 1B shows a native-PAGE pattern for the non-His-tagged recombinant protein (rMsmPCK), and Fig. 1D shows the corresponding SDS-PAGE pattern. The specific activities (OAA forming) of such rMsmPCK preparations were in the 17–20 units/mg range.

Upon storage for 2 months at 4 °C in 100 mM sodium phosphate buffer, pH 7, containing 100 mM NaCl, the purified rMsmPCK-His₁₀ retained about 75% of the original activity, and the corresponding value for rMsmPCK after a 1-month storage was 76%.

Very recently, we obtained a rMsmPCK-His₁₀ preparation with an initial specific activity of 41 units/mg, and it retained 85% of its activity after 5 months of storage at 4 °C. The data in Fig. 5B were obtained with this enzyme.

Molecular Properties of the Recombinant Enzyme—The NH₂-terminal sequence for the rMsmPCK was determined to be HMTSATIPGLDTAP. Of this sequence, the first His residue originated from the vector sequence, and the MTSAT sequence
that followed was from the polymerase chain reaction primer (designed based on the NH₂ terminus of putative *M. tuberculosis* PCK; accession number, P96393; Ref. 20); the MTSAT element is also present at the equivalent position in the putative *Mycobacterium leprae* PCK (accession number, CAB08805).

The matrix-assisted laser desorption/ionization time-of-flight mass spectrum of the purified rMsmPCK showed three major peaks at the m/z values of 71,209 (M + H⁺), 35,711 (M + 2H⁺), and 23,917 (M + 3H⁺). Hence, the subunit molecular mass for the rMsmPCK was 71.2 kDa. From the gel filtration chromatography data, the Stokes radii of the native forms of rMsmPCK and rMsmPCK-His₁₀ were determined to be 38.7 and 39.2 Å, respectively. From the same set of data, the apparent native molecular masses for the non-His-tagged and His-tagged forms of the recombinant enzyme were estimated to be 83.2 and 85.1 kDa, respectively. Thus, both these forms appeared to be monomers. However, a final conclusion in this line must await an accurate determination of the native molecular mass for these proteins by use of a more appropriate method; the molecular mass data derived solely from a gel filtration data set are unreliable (34).

**Effect of Temperature, pH, Reducing Agents, and Salts on the Activity of rMsmPCK**—The recombinant mycobacterial PCK was found to be thermostable. When tested at a reaction pH of 7.2, the optimum temperature for rMsmPCK was found to be 70 °C (Fig. 2). At the optimum temperature, a very high activity value (100 units/mg) was recorded. From the linear segment (between 28 and 60 °C) of the corresponding Arrhenius plots, the value of the activation energy (for OAA formation) for the enzyme was calculated to be 54 kJ/mol (Fig. 2). In the same range, the Q₁₀ value was 1.9. At 37 °C, the optimum pH for this enzyme was 7.0–7.4. The behavior of rMsmPCK-His₁₀ with respect to reaction pH and temperature was identical to that of rMsmPCK.

The enzyme was stimulated by reducing agents. With DTT, the stimulation was maximum in the range of 10–40 mM and amounted to about 30% for both rMsmPCK and rMsmPCK-His₁₀. Reduced glutathione and 2-mercaptoethanol provided maximum stimulation at concentrations of 20 and 75 mM, respectively, and in each case the peak value was about 95% of that obtained with DTT. The activity was inhibited by NaCl and KCl; at a concentration of 0.5 M, about 50% activity was lost. A similar observation was made with Na₂SO₄.

**Divalent Cation Requirements for the Activity of rMsmPCK**—Preliminary screening showed that the addition of Co²⁺ or Mn²⁺, but not Ca²⁺, Zn²⁺, Cu²⁺, or Ni²⁺, to an assay mixture containing 1 mM Mg²⁺ enhanced PCK activity; each of these additional divalent ions was tested up to the 100 μM level. Fig. 3 shows the data from a more detailed study with Mg²⁺, Co²⁺, and Mn²⁺. Each of these data sets fits the standard Henri-Michaelis-Menten kinetics. The values for the kinetic constants derived from these fits are presented in Table I. When tested singly and in the absence of DTT, Mg²⁺ gave a very low activity (Table I), whereas, under the same conditions, Co²⁺ and Mn²⁺ activated the system well, and the apparent K₉₅, V₉₅, and V₉₅/K₉₅ values for Mn²⁺ were comparable with those for Co²⁺ (Fig. 3A, Table I). In an assay with DTT and only one divalent cation, Mn²⁺ and Mg²⁺ showed similar apparent K₉₅ values, but in terms of activity and catalytic efficiency Mn²⁺ was about 6-fold superior to Mg²⁺. Observation with Co²⁺ in the presence of DTT could not be made because of intense color development under these conditions. The presence of Mg²⁺ (2 mM) in the assay greatly lowered the apparent K₉₅ values for Mn²⁺ (by 144-fold in the presence of DTT and by 9.4-fold in the absence of DTT) and Co²⁺ (by 230-fold) (Fig. 3B and Table I). When such an assay was conducted without DTT, Co²⁺ exhibited about 16-fold lower apparent K₉₅ value and 21-fold higher apparent catalytic efficiency or V₉₅/K₉₅ than Mn²⁺, although the corresponding apparent V₉₅ values offered by these cations were comparable. However, in the presence of both DTT and Mg²⁺, Mn²⁺ provided a high value for the apparent V₉₅ (Table I); the corresponding apparent K₉₅ and V₉₅/K₉₅ values were comparable with that exhibited by Co²⁺ in the absence of DTT (Table I). In the presence of 2 mM Mg²⁺ in the assay, Co²⁺ was inhibitory above a level of 10 μM (data not shown). When all of these data were considered together, it was seen that the apparent V₉₅ value was maximum when Mn²⁺ was the sole divalent cation and DTT was present in the assay. But with Mn²⁺, high values for both the V₉₅ and V₉₅/K₉₅ were obtained only if the assay contained 2 mM Mg²⁺ and DTT (Fig. 3B, Table I).

**The Michaelis Constants for the Substrates and the Effects of Inhibitors**—Fig. 4, A and B, shows the initial velocity data over a range of PEP concentration (0.025–2 mM) with either IDP or GDP and in the presence of various combinations of divalent cations (with or without DTT) for rMsmPCK. These data fit the Henri-Michaelis-Menten relationship well. The values for the kinetic constants derived from these fits (Table I) helped to draw the following conclusions. 1) When GDP served as the
nucleotide substrate and the assay mixture contained 2 mM MgCl₂ but lacked DTT, Co²⁺ (5 μM) provided an apparent Kₘ value for PEP that was 2-fold lower than that obtained with Mn²⁺ (100 μM). However, the apparent Vₘ values under these two conditions were comparable (Fig. 4A, Table I). A similar effect was also seen when IDP was used in place of GDP (Fig. 4B, Table I). 2) In an assay with 2 mM MgCl₂ and 0.1 mM Mn²⁺, the presence of DTT improved the Vₘ values substantially (2-fold with GDP and 2.6-fold with IDP), although the Kₘ for PEP increased (Fig. 4, A and B, Table I).

Fig. 4C presents the initial velocity data for IDP and GDP. The apparent Kₘ value for GDP was found to be lower than that for IDP (Table I). Similarly, GDP provided more activity and catalytic efficiency than IDP (Table I). At an ADP concentration of 25 or 50 mM, the specific activity value was only about 5% of that obtained with 2 mM IDP. The apparent Kₘ value for bicarbonate was fairly high (8.3 mM; Fig. 4D and Table I). Fig. 4, E and F, shows the initial velocity data for the PEP formation activity of rMsmPCK. For both substrates, GTP and OAA, the apparent Kₘ values were in the low micromolar range.

A comparison between MsmPCK and E. coli ATP-PCK (43) yielded values of 14 and 18% for the identity and strong similarity.

A primary structural alignment of MsmPCK with several GTP-PCKs (Fig. 6) revealed the following features. 1) For the vertebrate enzymes, the previously proposed PEP-binding site PX₃-A, with the aid of the crystal structure of rMsmPCK, we were able to show that this site was the amino acid 13-Ala²⁷-Glu⁴⁴, and it lacked the Cys residue (Fig. 6). 2) The PCK-specific domain (1) and the overlapping GTP phosphorylation site (38) were found conserved in the mycobacterial enzymes (the stretches 217–229 and 222–228, respectively, in MsmPCK). 3) The kinase 1a site of ATP-PCKs (1) was found to be fully conserved in the mycobacterial PCKs (residues 269–276 in MsmPCK). 4) The chicken liver GTP-PCK-M possesses two Co²⁺ or Mn²⁺ binding sites (13, 44, 45), and one of these is
equivalent to the kinase 2 site of the E. coli ATP-PCK (1). The metal binding residues in both of these sites were conserved in the mycobacterial PCKs (Asp75, Asp78, and Glu83 at site 1 and Asp295 and Asp296 at site 2). However, we found that in the putative bacterial GTP-PCKs from C. pneumoniae and T. pallidum, the first Asp residue of site 1 has been replaced with a Ser and a Gln, respectively (40, 41).

5) The proposed GTP phosphorylation site 2 (the 318DELG321 and 318DAQG321 sequences in the chicken and rat liver GTP-PCK-Cs, respectively (38)) corresponded to the 303GKDG306 in MsmPCK.

6) The putative guanine binding sequence (388NKDW391 and 388NKEW391 elements in the chicken and rat liver GTP-PCK-Cs, respectively (38)) corresponded to the 371GNDW374 sequence of the mycobacterial enzymes.

7) The His249 and Arg388 of MsmPCK belonged to two highly conserved elements and corresponded to, respectively, the catalytically active His232 and Arg233 of E. coli ATP-PCK (1).

8) The Cys-Lys pairs of chicken liver PCK-M (Cys64/Lys72 and Cys93/Lys89 for the unprocessed protein (46)) corresponded to the Ala38/Glu46 and Ser69/Lys65 of MsmPCK (Fig. 6).

9) The hyperreactive Cys288 of rat GTP-PCK-C (47, 48) was seen conserved in each bacterial GTP-PCK and corresponded to Cys273 in MsmPCK (Fig. 6).

10) In rat PCK-C, Cys399 and Cys407 (or Cys413) are important for activity (48), and these correspond to the Cys417 and Cys425 (or Cys431) of chicken PCK-M (Fig. 6). The Cys431 of PCK-M was conserved in the bacterial GTP-PCKs, whereas Cys417 was replaced by an Ala or Ile residue (Fig. 6). The Cys425 of chicken PCK-M was conserved in the mycobacterial PCKs but was replaced with a Thr residue in C. pneumoniae and T. pallidum enzymes (40, 41).

DISCUSSION

This is the first report on a vertebrate-type GTP-dependent phosphoenolpyruvate carboxykinase (GTP-PCK) from a bacterium and on the synthesis of PEP, the first committed step in the gluconeogenesis and glycogenesis in a Mycobacterium. It sets the stage for mutational analyses of the structure-function aspects of this very important enzyme in an amenable host, E. coli.
The recombinant mycobacterial PCK was found to be GTP-dependent. It also used IDP but performed very poorly with ADP. Both Mn$^{2+}$ and Co$^{2+}$ acted as PEP activators for this enzyme (see below). A comparison of the apparent $K_m$ values for PEP, GDP, and bicarbonate (>100, 66, and 8300 µM, respectively) suggested that in vivo this enzyme could operate in the PEP formation direction. In contrast, the GTP-PCK from the bacterium *R. flavefaciens* is anaplerotic and does not use either Co$^{2+}$ or IDP (3). Similarly, the uncharacterized enzyme from *T. pallidum* (2) and the IDP- and Co$^{2+}$-utilizing GTP-PCK from the nematode *A. suum* (11) are anaplerotic. Thus, the characteristics of the mycobacterial enzyme paralleled that of the mammalian and avian GTP-PCKs (1, 9, 29, 49–53). This vertebrate-type nature of MsmPCK fits well with the suggestion that in *M. smegmatis* PYC does not fulfill a straightfor-

![Fig. 6. Primary structure alignment for several GTP-dependent phosphoenolpyruvate carboxykinases.](http://www.jbc.org/)

The sources for the enzymes compared are as follows (accession number; reference): MYCame, *M. smegmatis* (this work); MYCtube, *M. tuberculosis* (P96383; Ref. 20); TREPap, *T. pallidum* (O83159; Ref. 41); CHLpne, *C. pneumoniae* (BA98059; Ref. 40); HOMmap, *H. sapiens* or human liver mitochondria (Q16832; Ref. 36); HOMmap, *C. H. sapiens* or human liver cytosol (A55466; Ref. 37); Galgal, *G. gallus* or chicken liver mitochondria (QYCHGM; Ref. 39). The white letters on a black background indicate the identities or the residues that are fully conserved. The positions showing strong conservations are shown as white letters on a shaded background. The boldface letters on a shaded background show the residues that are weakly conserved. See “Results” and “Discussion” for the details of the marked roles and the sources for these pieces of information.
ward anaplerotic function (19); the PKC and PYC activities, in combination, could constitute the priming steps for the gluconeogenesis or glycerogenesis. Recently, it has been shown that in *M. tuberculosis* the protein level for the putative PKC is elevated if the cells are grown under low iron concentration conditions (54). The reason for this effect is unknown. The availability of enzyme property and nucleotide sequence data (Ref. 19 and this work) would now allow a genetic analysis of the physiological roles of PKC and PKC in *M. smegmatis*. We are currently pursuing this topic.

The requirement for a divalent cation for the rMsmPKC activity was met poorly by Mg$^{2+}$ (Table I). But in the presence of 2 mM Mg$^{2+}$, the $K_m$ values for Co$^{2+}$ and Mn$^{2+}$ dropped markedly. These observations suggested that there were two roles for a divalent cation. Following previously established mechanisms (52, 55, 56), it would be reasonable to assume that for activating PEP, rMsmPKC preferred to use Co$^{2+}$ and Mn$^{2+}$ over Mg$^{2+}$, and Mg$^{2+}$ was superior in complexing the nucleotide substrate (Fig. 3, A and B). The reported $K_m$ values for Mn$^{2+}$ and Co$^{2+}$ are based on the total amount of the respective cation added to an assay mixture. When used as the sole divalent cation, a portion of this supply would be used in complexing the nucleotide substrate. Thus, the reported $K_m$ values for Mn$^{2+}$ and Co$^{2+}$ derived from such assays were understandably higher.

The value for the apparent $V_m$ offered by Mn$^{2+}$ (serving singly) was higher than that obtained with this cation in the presence of 2 mM Mg$^{2+}$ (Table I). This apparent inhibition of activity by Mg$^{2+}$ has been documented with chicken GTP-PCK-M as well (52). However, our data showed that, in the absence of DTT, rMsmPKC could perform well with either Mn$^{2+}$ or Co$^{2+}$, with Co$^{2+}$ being superior (Table I), whereas the chicken liver mitochondrial enzyme prefers Mn$^{2+}$ over Co$^{2+}$ (52).

Similar to the mitochondrial GTP-PCKs, rMsmPKC was greatly stimulated by the reducing agents containing sulfhydryl groups, although many other GTP-PCKs are inhibited by these reagents (49). The following $K_m$ values for GDP show another similarity between the mycobacterial enzyme and the eukaryotic GTP-PCKs: rMsmPKC, 66 μM (this work); A. suum, 22 μM (11); chicken liver mitochondria, 51 μM (49); *R. flavefaciens*, 9.8 mM (3).

By having a more favorable apparent $K_m$ value and by offering higher activity and catalytic efficiency (Table I), GDP appeared to be a more physiologically relevant nucleotide substrate for MsmPKC than IDP. The observed very high value of the apparent $K_m$ for bicarbonate (8.3 mM, Table I) reinforced the conclusion (see above) that MsmPKC may operate in the PEP synthesis direction. It could also indicate that with MsmPKC the actual substrate for the carboxylation reaction was CO$_2$ and not HCO$_3^−$. Such is the case with other PCKs (1). The dissociation constant for the reaction CO$_2$ (aqueous) + H$_2$O $\leftrightarrow$ H$^+$ + HCO$_3^−$ at 37 °C is about 4.97 × 10$^{-7}$ mol liter$^{-1}$ (57). Thus, for the addition of bicarbonate to a final concentration of 8.3 mM (the apparent $K_m$ value for bicarbonate; Table I), the concentration for CO$_2$ (aqueous) in the assay mixture (pH 7.2) would be at the most 1.05 mM; this estimate does not consider the loss of CO$_2$ to the atmosphere where the partial pressure of CO$_2$ is only 0.003 atm or 303 pascals. Hence, the $K_m$ value for the real carboxyl source might not be that high.

The apparent monomeric nature and the molecular mass (72 kDa) of rMsmPKC matched those of all known eukaryotic GTP-PCKs (67–80 kDa; Refs. 8, 9, 11, and 49); the subunit molecular mass for *R. flavefaciens* enzyme is 66.3 kDa (3). However, it should be noted that our high performance liquid chromatography (HPLC)-based gel filtration experiment led to an about 17% overestimate for the apparent native molecular mass of rMsmPKC (over the subunit molecular mass). This observation contrasted with the data on chicken liver PCK-M (50); a conventional molecular exclusion chromatography experiment yields a 1.8–3.4-fold lower estimate for the apparent molecular mass value, and an HPLC-based system provides a value comparable with the corresponding SDS-PAGE-derived subunit molecular mass.

Similar to the vertebrate GTP-PCKs, rMsmPKC was inhibited by oxalate (Fig. 5B), which is a structural analog of the enolate of pyruvate (a putative reaction intermediate for the PCKs; Refs. 1, 30, and 58). The $K_m$ value for oxalate was similar to the apparent $K_m$ value for PEP. Thus, oxalate acted as a potent inhibitor for the mycobacterial enzyme. Results from similar studies with chicken liver PCK-M have been presented in two reports. One of these reports describes a competitive pattern with $K_i$ for oxalate being comparable with the $K_m$ value for PEP (30), and the other documents a noncompetitive pattern with a $K_i$ value 30 times higher than the $K_m$ for PEP (58). It has been shown for rat liver PCK-C that α-KG is a competitive inhibitor with respect to PEP or OAA and that the $K_i$ value for α-KG is about 10 times higher than the $K_m$ for PEP (59). In contrast, the inhibition of rMsmPKC by α-KG was much less severe and of a mixed type (Fig. 5A); the $K_a$ and $K_i$ values were, respectively, about 25- and 200-fold higher than the $K_m$ value for PEP.

The His-tagged and the non-His-tagged versions of the recombinant mycobacterial enzyme behaved very similarly. Thus, the His-tagged version, which could be purified easily and rapidly, would allow a ready (preliminary or final) screening of the mutant proteins for key functional properties. The primary structure comparison (Fig. 6) showed many ready targets for site-targeted mutagenesis studies. For example, the lack of universal conservation or a high degree of conservation in the two proposed Cys-Lys pairs (46), raised the question whether such pairs are essential for the GTP-PCK activity, although they could have roles specific to the enzyme from a particular source. Similar to the vertebrate and nematode enzymes (11, 52), MsmPKC used both Co$^{2+}$ and Mn$^{2+}$ for activating PEP, whereas the GTP-PCK from the bacterium *R. flavefaciens* does not use Co$^{2+}$ (3). The question of whether this difference arises from certain changes in the metal binding sites (Fig. 6) can now be answered directly by site-directed mutagenesis of rMsmPKC. Interestingly, in the putative bacterial GTP-PCKs of *C. pneumoniae* and *T. pallidum* (40, 41), the positions equivalent to the Asp$^{75}$ of MsmPKC (a metal binding residue; Fig. 6) are occupied by Ser and Glu, respectively. Such a change could be introduced in the rMsmPKC, and the resulting mutant enzyme could be tested for the ability to use Co$^{2+}$.

The determination of the crystal structure for a GTP-PCK is much awaited (1). The ease of obtaining large amounts of highly active and stable rMsmPKC would help to intensify this effort. Since in the primary structure and kinetic properties MsmPKC is very similar to the vertebrate-liver PCKs, it could act as a model for this group.

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