Roles of Asp<sup>75</sup>, Asp<sup>78</sup>, and Glu<sup>83</sup> of GTP-dependent Phosphoenolpyruvate Carboxykinase from Mycobacterium smegmatis<sup>*</sup>

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The roles of Asp<sup>75</sup>, Asp<sup>78</sup>, and Glu<sup>83</sup> of the <sup>75</sup>DPSDVARVE<sup>83</sup> element of Mycobacterium smegmatis GTP-dependent phosphoenolpyruvate (PEP) carboxykinase (GTP-PEPCK) were investigated. Asp<sup>78</sup> and Glu<sup>83</sup> are fully conserved in GTP-PEPCKs. The human PEPCK crystal structure suggests that Asp<sup>78</sup> influences Tyr<sup>220</sup>; Tyr<sup>220</sup> helps to position bound PEP, and Glu<sup>83</sup> interacts with Arg<sup>81</sup>. Experimental data on other PEPCKs indicate that Arg<sup>81</sup> binds PEP, and the phosphate of PEP interacts with Mn<sup>2+</sup> of metal site 1 for catalysis. We found that D78A and E83A replacements severely reduced activity. E83A substitution raised the apparent <i>K<sub>m</sub></i> value for Mn<sup>2+</sup> 170-fold. In contrast, Asp<sup>75</sup> is highly but not fully conserved; natural substitutions are Ala, Asn, Gln, or Ser. Such substitutions, when engineered, in <i>M. smegmatis</i> enzyme caused the following. 1) For oxaloacetate synthesis, <i>V<sub>max</sub></i> decreased 1.4–4-fold. <i>K<sub>m</sub></i> values for PEP and Mn<sup>2+</sup> increased 3–9- and 1.2–10-fold, respectively. <i>K<sub>m</sub></i> values for GDP and bicarbonate changed little. 2) For PEP formation, <i>V<sub>max</sub></i> increased 1.5–2.7-fold. <i>K<sub>m</sub></i> values for oxaloacetate increased 2–2.8-fold. The substitutions did not change the secondary structure of protein significantly. The kinetic effects are rationalized as follows. In E83A the loss of Glu<sup>83</sup>–Arg<sup>81</sup> interaction affected Arg<sup>81</sup>–PEP association. D78A change altered the Tyr<sup>220</sup>–PEP interaction. These events perturbed PEP-Mn<sup>2+</sup> interaction and consequently affected catalysis severely. In contrast, substitutions at Asp<sup>75</sup>, a site far from bound PEP, brought subtle effects, lowering oxaloacetate formation rate but enhancing PEP formation rate. It is likely that Asp<sup>75</sup> substitutions affected PEP-Mn<sup>2+</sup> interaction by changing the positions of Asp<sup>78</sup>, Arg<sup>81</sup>, and Glu<sup>83</sup>, which translated to differential effects on two directions.

Most GTP-PEPCKs kinetically prefer the PEP-forming direction (2, 5–11). Consequently, in <i>vivo</i> it often catalyzes the formation of PEP from OAA, the first committed and rate-controlling step in gluconeogenesis and glycogenesis (6). Only rarely will an organism use this enzyme for anaplerosis or OAA formation (12–14). Human liver, kidney, and small intestine use GTP-PEPCK activity for the production of glucose during fasting (15–18). By virtue of this activity, GTP-PEPCK plays a role in the development of type 2 diabetes, a disorder where the gluconeogenic organs produce glucose even when the blood glucose level is high (19). One way to alleviate this problem would be to lower the activity of the enzyme by use of a therapeutic agent. This rationale has been the basis for ongoing structure-function studies on GTP-PEPCKs (19).

In this study, we have examined whether two Asp residues and a Glu residue, which belong to a well conserved sequence element of GTP-PEPCKs (Table 1), influence the enzymatic activity. The position numbers for these residues in the enzymes from three sources relevant to our work are as follows: human liver cytosolic (Asp<sup>81</sup>, Asp<sup>84</sup>, and Glu<sup>89</sup>) (20, 21); chicken liver mitochondria (Asp<sup>86</sup>, Asp<sup>89</sup>, and Glu<sup>74</sup>; of the mature enzyme) (22); and <i>Mycobacterium smegmatis</i>, the model for our work (Asp<sup>75</sup>, Asp<sup>78</sup>, and Glu<sup>83</sup>) (2). In the chicken GTP-PEPCK, the aforementioned conserved element has been described as the second metal-binding site (23). It has been proposed that in the avian enzyme Asp<sup>84</sup>, Asp<sup>89</sup>, and Glu<sup>74</sup> act as ligands for a Mn<sup>2+</sup> ion (23), and in turn the latter mediates phosphoryl group transfer from or to the nucleotide substrate (23). Interestingly, the crystal structure data for human cytos-
lic PEPCK indicate that these acidic amino acid residues are not involved in Mn\(^{2+}\) binding but are located near the PEP-binding pocket (Fig. 1). The same observation has been made in a report on the crystal structure of the chicken GTP-PEPCK (24), which was published while this manuscript was under review. Therefore, we have considered an alternative role for these residues.

Asp\(^{75}\) and Asp\(^{78}\) of the avian enzyme are fully conserved in the PEPCKs (Table 1) (2, 3, 22, 23, 25–35). In contrast, Asp\(^{66}\) is conserved in most, but not all, archaeal, bacterial, and eukaryotic GTP-dependent PEPCKs (2, 3, 22, 23, 25–35). In certain

### Table 1

Primary structure alignment for the PEP-binding site of GTP-dependent PEPCKs

| Source | PEP binding site |
|--------|------------------|
| **Eubacteria** | |
| *Mycobacterium smegmatis** | ALSDPSTDVARESTRT | 86 |
| *Mycobacterium tuberculosis* | ALSDPSTDVARESRFT | 86 |
| *Geobacter metallireducens* | LCSDPIDVARESRFT | 64 |
| *Chlorobium tepidum* | LANSDPSTDVARESRFT | 84 |
| *Acinetobacter sp. ADP1* | FRSNDPSSTDVARESRFT | 68 |
| *Clostridium thermocellum* | RSNPSSTDVARESRFT | 84 |
| *Corynebacterium glutamicum* | LSNPSSTDVARESRFT | 87 |
| *Corynebacterium efficiens* | LSNPSSTDVARESRFT | 87 |
| *Chlamydia pneumoniae* | VRSAPDVSARESRFT | 79 |
| *Chlamydia muridarum* | VRSAPDVSARESRFT | 81 |
| *Kineococcus radiotolerans* | LSNPSSTDVARESRFT | 95 |
| *Treponema pallidum* | LFSQPSSTDVARESRFT | 76 |
| **Eukarya** | |
| *Homo sapiens cytosolic* | ALTDPRDVARIESKT | 92 |
| *Homo sapiens mitochondria* | LCRTDPKVARIESKT | 109 |
| *Caenorhabditis elegans* | LARTDPKVARIESKT | 109 |
| *Gallus gallus mitochondria* | LARTDPKVARIESKT | 77 |
| *Gallus gallus cytosolic* | LALTNPDRVARIESKT | 92 |
| *Neocallimastix frontalis* | LARTNPAVARESKCT | 80 |
| *Drosophila melanogaster* | LARTNPAVARESKCT | 117 |
| **Archaea** | |
| *Ferroglossarius acidarmanus* | LYNFSDDVARTEKDT | 93 |
| *Thermoplasma volcanium* | LYNASNATVAREEKT | 80 |

* Residues investigated in this paper.
** The enzyme studied in this work.
† PEP-binding arginine (21).
enzymes it has been replaced with Ala, Asn, Gln, or Ser. It is possible that in the avian PEPCk Asp75 and Glu78 play important roles in catalysis, whereas Asp66 serves a regulatory function and therefore is more prone to evolutionary changes (Table 1). We have tested these hypotheses by using the *M. smegmatis* enzyme as the model. We have shown previously that this mycobacterial enzyme kinetically prefers the gluconeogenic direction (2). Also, it has been expressed with activity in *Escherichia coli* from a plasmid-based construct (2). This heterologous expression system is suitable for site-directed mutagenesis experiments and generation of variant enzymes. *M. smegmatis* PEPCk is highly similar to the human cytosolic and chicken mitochondrial enzymes (2). Therefore, it is a suitable model for the GTP-PEPCks. The results from our work support our hypotheses concerning the roles of the above-mentioned acidic residues in the PEPCk reaction.

### EXPERIMENTAL PROCEDURES

**Strains and Growth Conditions**—*E. coli* DH5α (36) was used as a cloning host, and *E. coli* C41(DE3) (37) was used for the overexpression of PEPCk. 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/ml.

**DNA Techniques**—Standard techniques were used for the manipulation of the DNA (38). Qiagen QIAprep spin miniprep kit (Qiagen Inc., Valencia, CA) was used for the purification of plasmids. The sequence for the mutagenized DNA was verified by determining the sequence for both strands. Site-directed mutagenesis experiments were performed by using the QuikChange® kit from Stratagene (La Jolla, CA) and a pair of mutagenic oligonucleotides. The oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

**Site-directed Mutagenesis of *M. smegmatis* PEPCk**—The desired alterations were introduced via two sets of experiments. In the first set, Asp75 was the target and the plasmid pBE129-19b, an expression vector for *M. smegmatis* PEPCk (2), was used as the template. pBE129-19b was constructed by cloning the *pck* coding sequence as an Ndel-BamHI fragment into pET19b (2). The primer pairs used in this first set of experiments (Table 2) were designed to cause the following two changes in the *pck* coding sequence: 1) replacement of the codon representing Asp75 with that for Ala, Asn, Gln, or Ser; 2) introduction of a PmlI site at nucleotide position 239–244 without causing any change in the amino acid sequence. pBE129-19b does not contain a PmlI site. The newly introduced PmlI site provided a rapid screening method for plasmids containing the designed mutations. Upon digestion with BamHI and PmlI, a mutant plasmid produced two fragments of 1.5 and 6 kb, whereas this treatment generated only a 7.5-kb fragment from pBE129-19b. From these experiments the plasmids pBE129–19bD75A, pBE129–19bD75N, pBE129–19bD75Q, and pBE129–19bD75S were obtained. In the second set of mutagenesis experiments, either Asp78 or Glu83 of the PEPCk protein was targeted. For this work the template was pBE129–19bD75A, which carried a PmlI site and encoded an Ala residue at the 75th amino acid sequence position of the enzyme (Table 2). Each primer pair used in the second round restored the wild-type sequences that were altered in the first round and replaced the codon for either Asp78 or Glu83 with that for Ala (Table 2). A loss of the PmlI site was taken as a preliminary indication for the success of a mutagenesis experiment. The plasmids pBE129–19bD78A and pBE129–19bE83A were generated from this round. For each of six mutant clones, the DNA sequence for both strands of the entire *pck* gene was determined. The data showed that in every case the mutant gene carried only the desired alterations, and the rest of the DNA sequence remained unchanged.

**Expression and Purification of Recombinant Enzymes**—From the above-mentioned constructs, the wild-type and variant *M.
**TABLE 2**

| Template for PCR | Variant Pek enzyme generated | Oligonucleotide sequence*
|------------------|-----------------------------|-------------------------|
| pBE129-19b (wild-type)* (first round of mutagenesis)** | D75N | 5′-CTTTCGCCACCGTCGACGTGACGATTCGCCACCTTC-3′ |
| | D75Q | 5′-CTTTCGCCACCGTCGACGTGACGATTCGCCACCTTC-3′ |
| | D75S | 5′-CTTTCGCCACCGTCGACGTGACGATTCGCCACCTTC-3′ |
| | D75A | 5′-CTTTCGCCACCGTCGACGTGACGATTCGCCACCTTC-3′ |
| pBE129-19bD75A (second round of mutagenesis)** | D78A | 5′-CTTTCGCCACCGTCGACGTGACGATTCGCCACCTTC-3′ |
| | E83A | 5′-CTTTCGCCACCGTCGACGTGACGATTCGCCACCTTC-3′ |

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*Only forward primers are listed.

**Wild-type sequence of the relevant region of *M. smegmatis* PEPCK with the areas targeted for mutagenesis are underlined is as follows: 5′-CTTTCGCCACCGTCGACGTGACGATTCGCCACCTTC-3′.

**First round of mutagenesis is as follows: underlined bases show a PnuI restriction site that was introduced by changing one base (G to A, shown in boldface) without altering the amino acid sequence; white letters on a black background represent the changes providing desired alterations in the amino acid sequence.

**Second round of mutagenesis is as follows: white letters on a gray background indicate restoration of the wild-type sequence; white letters on a black background represent the changes providing desired alterations in the amino acid sequence.

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*Asp*<sup>75</sup>, *Asp*<sup>78</sup>, and *Glu*<sup>83</sup> of *M. smegmatis* PEPCK

**smegmatis** PEPCK proteins were overexpressed in *E. coli* C41(DE3) (2, 37). The recombinant proteins were purified as described previously (2), except the steps beyond nickel-nitrotriacetic acid chromatography were eliminated. As judged from SDS-PAGE analysis with Coomassie Blue staining (39), the protein preparations obtained from this modified method were homogeneous.

**Enzyme and Protein Assays and Analysis of the Enzyme Kinetics Data**—Protein was assayed according to Bradford (40) using the dye reagent from Bio-Rad. PEPCK activity was determined as described previously (2), but with modifications as indicated in the tables and figures, and the following are some of the details. The OAA forming or anaplerotic activity was measured by coupling it with the malate dehydrogenase (MDH) reaction and monitoring NADH oxidation spectrophotometrically at 340 nm (11). The assays for the PEP forming or gluconeogenic activity were conducted in a mixture where OAA generated in situ from l-malate by MDH served as the substrate (41, 42). As OAA was consumed by PEPCK, MDH produced more OAA from l-malate to maintain equilibrium (41, 42). The consequent formation of NADH was monitored spectrophotometrically. The standard assay mixture for measuring the OAA-forming activity contained 100 mm HEPES-NaOH buffer, pH 7.2, 200 mm KHCO<sub>3</sub>, 10 mm PEP, 2 mm GDP, 2 mm MgCl<sub>2</sub>, 0.2 mm MnCl<sub>2</sub>, 37 mm dithiothreitol, 0.25 mm NAD<sub>H</sub>, and 2 units of MDH in a total volume of 1 ml. For the PEP-forming reaction, the standard assay mixture contained 100 mm HEPES-NaOH buffer, pH 7.2, 3 mm l-malate, 0.2 mm GTP, 3 mm NAD<sup>+</sup>, 2 mm MgCl<sub>2</sub>, 0.2 mm MnCl<sub>2</sub>, 37 mm dithiothreitol, and 6 units of MDH in a total volume of 1 ml. The l-malate consistently provided an initial OAA concentration of 14 μM. The assay temperature for both directions was 37 °C. All initial rate data were analyzed according to Cleland (43) using the KinDist, a PC graphics program obtained from Prof. Bryce V. Plapp, University of Iowa (Iowa City). Each data set fit to the standard Henri-Michaelis-Menten equation well. The *k*<sub>cat</sub> values were calculated from *V*<sub>max</sub> data, considering that the subunit molecular mass of the monomeric enzyme is 71.2 kDa as calculated from the nucleic acid sequence-derived amino acid sequence (2).

**CD Spectroscopy**—The CD spectra of purified PEPCK proteins were collected between 190 and 260 nm at 0.5-nm intervals by using a Jasco J710 spectropolarimeter (Easton, MD) and a 0.1-cm cylindrical quartz cuvette. In each case, a solution containing 2.8 μM PEPCK protein (calculated based on a subunit molecular mass of 71.2 kDa, which was derived as indicated in the preceding paragraph (2) and 10 mm potassium phosphate buffer, pH 7, was analyzed, and the data from nine acquisitions were averaged. Each averaged spectrum was corrected for the contribution by the buffer. The measured ellipticity data (θ values in millidegrees) were converted into mean residue ellipticity or [θ]<sub>MRE</sub> values (degrees square centimeters/dmol) according to the following relationship: [θ]<sub>MRE</sub> = θ/10μCl, where *l* is the cuvette path length in centimeters; *C* is the molar concentration of monomeric PEPCK subunits, and *n* is the number of residues per PEPCK subunit (644 amino acids, including the His tag).

**Amino Acid Sequence Alignment and Analysis of Crystal Structure**—A multiple alignment of the primary amino acid sequences for GTP-dependent PEPCKs (Table 1) was generated using the ClustalW program (44) at the European Bioinformatics Institute site on the World Wide Web. The crystal structure for human liver cytosolic PEPCK (21) was viewed and manipulated by using DeepView/SwissPdbViewer 3.7 (45). The structure coordinates (Protein Data Bank codes 1KHF and 1KHB) were retrieved from the Research Collaboratory for Structural Bioinformatics site on the World Wide Web (46).

**RESULTS**

Alanine-scanning Mutagenesis of Asp<sup>75</sup>, Asp<sup>78</sup>, and Glu<sup>83</sup> in *M. smegmatis* PEPCK—Assays for PEPCK activity were conducted at about saturating concentrations of all substrates, except OAA, which was at a subsaturating concentration of 14 μM. Mn<sup>2+</sup> was supplied at a concentration of either 0.2 or 2.0...
**Asp**<sup>75</sup>, Asp<sup>78</sup>, and Glu<sup>83</sup> of *M. smegmatis* PEPCK

### TABLE 3
Specific activities of *M. smegmatis* PEPCK variants generated from alanine scanning mutagenesis of Asp<sup>75</sup>, Asp<sup>78</sup>, and Glu<sup>83</sup>

| Enzyme       | [Mn<sup>2+</sup>] | OAA-forming activity<sup>a,b</sup> | PEP-forming activity<sup>a,b</sup> |
|--------------|-------------------|----------------------------------|----------------------------------|
|              | mU                | μmol min<sup>-1</sup> mg<sup>-1</sup> | % activity | μmol min<sup>-1</sup> mg<sup>-1</sup> | % activity |
| Wild type    | 0.2               | 43.0 ± 1.0                        | 100       | 12.3 ± 0.9                       | 100       |
|              | 2.0               | 29.4 ± 1.3                        | 100       | 10.6 ± 0.1                       | 126       |
| D75A         | 0.2               | 10.6 ± 0.3                        | 25        | 15.5 ± 1.1                       | 126       |
| D78A         | 0.2               | 0.13 ± 0.08                       | 0.3       | 0.06 ± 0.01                      | 0.4       |
|              | 2.0               | 0.15 ± 0.09                       | 0.5       |                                 |           |
| E83A         | 0.2               | 0.40 ± 0.02                       | 0.9       | 0.36 ± 0.03                      | 2.3       |
|              | 2.0               | 1.0 ± 0.1                         | 3.4       |                                 |           |

<sup>a</sup> Average of data from three assays ± S.E. are shown.

<sup>b</sup> Assays were performed in standard reaction mixtures, but with Mn<sup>2+</sup> at levels as shown.

The D75A enzyme was active, and the values of the corresponding specific OAA-forming and PEP-forming activities at a Mn<sup>2+</sup> concentration of 0.2 mM were about 25 and 126% of that of the wild-type or Asp<sup>75</sup> enzyme, respectively (Table 3). A more detailed study on the kinetic properties of the D75A variant is presented below. The D78A and E83A variants exhibited very low activity in either the OAA- or the PEP-forming direction (Table 3). With Mn<sup>2+</sup> at 0.2 mM, the specific OAA-forming activities of D78A and E83A were 0.3 and 0.9% that of the wild-type enzyme, respectively (Table 3). These values increased to 0.5 and 3.4%, respectively, when the Mn<sup>2+</sup> concentration in the assay was raised to 2 mM (Table 3). The D78A and E83A enzymes were impaired in the PEP forming activity as well, and the corresponding specific activities at a Mn<sup>2+</sup> concentration of 0.2 mM were 0.4 and 2.3% that of the wild type, respectively (Table 3). An increase in Mn<sup>2+</sup> concentration beyond 0.2 mM did not improve the PEP forming activities of D78A and E83A PEPCK (data not shown).

**Substrate Kinetics for the OAA and PEP Forming Activities of E83A Variant of *M. smegmatis* PEPCK—**Assays for OAA forming activity were run at approximately saturating conditions for the substrates that were held at fixed concentrations. Assay mixtures were standard except for Mn<sup>2+</sup>, which was provided at a concentration of 2.0 mM. For the PEP-forming assays, two sets of conditions were used. Because the assay used did not allow for establishing a saturating concentration for OAA, the GTP kinetics were performed at a subsaturating GTP level of 14 μM. The initial velocity data at varying concentrations of OAA (1–14 mM) were collected at a GTP concentration of 0.2 mM. Each initial rate data set fit the standard Henri-Michaelis-Menten kinetics equation (Fig. 2), and the values of the kinetic constants obtained from the fits are shown in Table 4. The apparent *Kₘ* values for PEP and GDP of E83A were not significantly different from that of Glu<sup>83</sup> enzyme. A similar conclusion could also be made for the *Kₘ* value for bicarbonate (Table 4), if one considers the fact that the measured values had large errors because of low activities. The apparent *Vₘₐₓ*
values for the OAA forming activity of the variant enzyme, as measured by varying the concentration of Mn$^{2+}$ or one of the substrates, were 23–39-fold lower than that of the wild-type or Glu$^{83}$ enzyme (Fig. 2, A–D, and Table 4). As a result, E83A had substantially lower apparent catalytic efficiency ($k_{cat}/K_m$) values with respect to Mn$^{2+}$, PEP, GDP, and bicarbonate, compared with the wild-type enzyme (Table 4). In the PEP-forming assay, E83A exhibited 40-fold lower $V_{max}$ than Glu$^{83}$ enzyme, although the apparent $K_m$ values of these enzymes for OAA were not significantly different from each other (Fig. 2E and Table 4). Correspondingly, the catalytic efficiency of E83A in the PEP-forming direction with respect to OAA was 69-fold lower than that of the wild type. The $K_m$ value for GTP of E83A determined at an OAA concentration of 14 $\mu$M was 21.4 ± 2.6 $\mu$M and that for the wild type was 29.2 ± 6.5 $\mu$M.

**Effect of Mg$^{2+}$ on the Apparent $K_m$ Values for Mn$^{2+}$ and GDP of the E83A Variant**—Assays were conducted in the OAA-forming direction. Under standard assay conditions (2 mM MgCl$_2$ and 2 mM GDP), the apparent $K_m$ value of E83A for Mn$^{2+}$ (560 ± 80 $\mu$M) was 170-fold higher than that for the wild-type (Glu$^{83}$) PEPPCK (3.3 ± 0.5 $\mu$M) (Table 4). When the MgCl$_2$ concentration was raised from 2 to 5 mM, the apparent $K_m$ of E83A for Mn$^{2+}$ dropped by 17-fold to 33 ± 3 $\mu$M. In contrast, there was no change in the apparent $K_m$ value of wild-type PEPPCK for this rise in Mg$^{2+}$ concentration. An increase in the MgCl$_2$ level from 5 to 7.5 mM did not change the apparent $K_m$ value for Mn$^{2+}$ of either E83A or wild-type PEPPCK significantly. Also, a change in the Mg$^{2+}$ concentration from 2 to 5 or 7.5 mM did not alter the $V_{max}$ values for E83A or Glu$^{83}$ enzyme. Similarly, the apparent $K_m$ value for GDP was determined at fixed MgCl$_2$ concentrations of 5 and 7.5 mM and compared with that obtained at 2 mM MgCl$_2$. For these studies, the MnCl$_2$ concentration was held constant at a saturating level of 0.8 mM. The apparent $K_m$ value of E83A for GDP at 5 mM MgCl$_2$ (82 ± 6 $\mu$M) was 1.5-fold lower than at 2 mM MgCl$_2$ (121 ± 12 $\mu$M). The corresponding drop for the wild-type enzyme was 1.4-fold (from 69 ± 9 to 49 ± 5 $\mu$M). An increase in the MgCl$_2$ concentration from 5 to 7.5 mM raised the apparent $K_m$ value for GDP of the Glu$^{83}$ and E83A enzymes 1.1- and 1.08-fold (to 55 ± 4 and 89 ± 8 $\mu$M), respectively.

**Mn$^{2+}$ Requirements of M. smegmatis PEPPCK Asp$^{75}$ Variants**—The assays were conducted in the OAA-forming direction. The concentration of Mn$^{2+}$ was varied although that of PEP, GDP, and added bicarbonate was held constant at saturating levels of 10, 2, and 200 mM, respectively. The apparent values of $K_m$, $V_{max}$, and $k_{cat}/K_m$ for Mn$^{2+}$ derived from the Henri-Michaelis-Menten fits (Fig. 3A) are shown in Table 5. The apparent $K_m$ value for Mn$^{2+}$ of the Asp$^{75}$ or wild-type PEPPCK was 2.4 ± 0.3 $\mu$M and a 3.5-fold higher value was observed for the D75N and D75S variants. It increased 9-fold when Asp$^{75}$ was replaced with Ala, but a substitution with Glu did not cause a significant change. The engineered alterations reduced the catalytic efficiency of the enzyme significantly (Table 5).

**Substrate Kinetics for the OAA Forming Activity of M. smegmatis PEPPCK Asp$^{75}$ Variants**—The replacement of Asp$^{75}$ with Ala, Asn, Gln, or Ser significantly reduced the apparent maximum specific activity or $V_{max}$ of M. smegmatis PEPPCK in the anaplerotic or OAA-forming direction (Fig. 3, B–D, and Table 5). In studies where the concentrations of Mn$^{2+}$, GDP, and added bicarbonate were held constant at the saturating concentrations of 0.2, 2, and 200 mM, respectively, and the PEP concentration was varied in the 0.025–10 mM range, the apparent $V_{max}$ values for the altered enzymes were found to be 1.4–4-fold lower than that of the wild type (Table 5). The order of $V_{max}$ values was Asp$^{75}$ > D75N > D75S > D75Q > D75A (Table 5). The apparent $K_m$ values for PEP of the D75A, D75N, and D75S variants were 3-fold higher than that of the wild-type enzyme (Table 5). For D75Q, the apparent $K_m$ values for PEP was 9-fold higher than that of Asp$^{75}$. Consequently, the catalytic efficiencies for D75A, D75N, D75Q, and D75S were 13-, 4-, 26-, and 5-fold lower than that of Asp$^{75}$ enzyme, respectively (Table 5). In contrast, the apparent $K_m$ values for GDP of the variant enzymes were similar to that of the wild type (Table 5). A similar observation was made for bicarbonate (Table 5), except the apparent $K_m$ value for bicarbonate with D75N was 2-fold higher than that of Asp$^{75}$.

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**TABLE 4**

Apparent kinetic values of the wild-type and E83A variant of M. smegmatis PEPPCK

The data are from the fits shown in corresponding figures.

| Variable substrate (concentration range) | Enzyme         | $K_m$ (mM) | $V_{max}$ ($\mu$mol min$^{-1}$ mg$^{-1}$) | $k_{cat}/K_m$ ($\times 10^6$ M$^{-1}$ s$^{-1}$) | Figure for the plot |
|------------------------------------------|----------------|------------|------------------------------------------|---------------------------------------------|--------------------|
| OAA forming activities$^a$               |                |            |                                          |                                             |                    |
| Mn$^{2+}$ (0.001–2 mM)                   | Glu$^{83}$ (wild type) | 3.2 ± 0.5 | 32.0 ± 0.5                                | 116                                         | 2A                 |
|                                            | E83A           | 560 ± 80   | 1.4 ± 0.1                                 | 0.050                                       | 2A                 |
|                                            | PEP (0.1–4 mM) | 471 ± 25   | 38.2 ± 1.2                                | 0.97                                        | 2B                 |
|                                            | Glu$^{83}$ (wild type) | 373 ± 46  | 1.3 ± 0.1                                 | 0.042                                       | 2B                 |
|                                            | E83A           | 69 ± 9     | 33.6 ± 0.9                                | 5.8                                         | 2C                 |
|                                            | E83A           | 121 ± 12   | 1.2 ± 0.1                                 | 0.12                                        | 2C                 |
|                                            | HCO$_3^-$ (1–200 mM) | 7,580 ± 550 | 33.7 ± 0.5                                | 0.053                                       | 2D                 |
|                                            | E83A           | 27,500 ± 6,200 | 1.3 ± 0.1                                   | 0.001                                       | 2D                 |
| PEP forming activities$^a$               |                |            |                                          |                                             |                    |
| OAA (1–14 $\mu$M)                       | Glu$^{83}$ (wild type) | 6.7 ± 1.8 | 21.0 ± 2.4                                | 37                                          | 2B                 |
|                                            | E83A           | 11.5 ± 2.4 | 0.52 ± 0.06                               | 0.54                                        | 2E                 |

$^a$ Data are based on a subunit molecular mass of 71.2 kDa, which was calculated from the nucleic acid sequence-derived amino acid sequence for the monomeric PEPCK (2).

$^b$ Assays were performed in standard reaction mixtures, except the concentrations of the variable substrates were as indicated, and for the OAA-forming assays the concentrations of PEP and MnCl$_2$ were 4 and 2 mM, respectively.

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**Asp$^{75}$, Asp$^{78}$, and Glu$^{83}$ of M. smegmatis PEPPCK**

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Asp$^{75}$, Asp$^{78}$, and Glu$^{83}$ of M. smegmatis PEPCK

Variants—The apparent $K_m$ values for GTP of the wild-type, D75A, D75N, D75Q, and D75S enzymes, determined at a subsaturating OAA concentration of 14 μM, were 29.2 ± 6.5, 26.4 ± 5.0, 30.8 ± 6.2, 25.3 ± 5.7, and 24.5 ± 4.1 μM, respectively. The initial velocity data at varying concentrations of OAA (1–14 μM) and a GTP concentration of 0.2 mM showed that the apparent $V_{\text{max}}$ values for the altered enzymes were 1.5–2.7-fold higher than that of the wild-type enzyme (Fig. 3E and Table 6). The order for the apparent $V_{\text{max}}$ values was Asp$^{75}$ < D75Q < D75N < D75A < D75S. However, the substitutions increased the apparent $K_m$ values for OAA by about 2–2.8-fold and the order was Asp$^{75}$ < D75N < D75Q = D75A = D75S. The corresponding changes in the $k_{\text{cat}}/K_m$ value fell in the range of almost none to 1.8-fold.

**CD Spectra of Wild-type and Variant PEPCKs**—The CD spectrum of each protein showed minima at 208 and 220 nm (Fig. 4). These data were analyzed via CDSSTR (47, 48) at the Dichroweb website (49) to obtain quantitative information on the $\alpha$-helical, $\beta$-sheet, and $\beta$-turn content of each protein (Table 7).

**DISCUSSION**

We have investigated the functions of two Asp residues and one Glu residue of a well conserved sequence element of a GTP-PEPCK (Table 1), which have been called the second Mn$^{2+}$-binding site for the enzyme (23). The Mn$^{2+}$-binding role for these residues was proposed based on the data from binding studies (23). It was thought that the Mn$^{2+}$ bound to this site facilitates phosphoryl transfer between PEP and the nucleotide substrate (23). However, the GTP-PEPCK crystallographic data show that the aforementioned residues are situated near the PEP-binding pocket and do not serve as ligands for the enzyme-bound Mn$^{2+}$ (21, 24). Therefore, it is necessary to re-evaluate the roles of these residues. We had reported previously that these Asp and Glu residues show different degrees of conservation in the GTP-PEPCK amino acid sequences (2). Consequently, they all might not play the same role in a PEPCK reaction. To investigate this possibility, we performed a more extensive sequence comparison (Table 1), site-directed mutagenesis (Table 2) and kinetic studies (Figs. 1 and 2 and Tables 3–6), and we analyzed the results in light of the x-ray crystallographic structure of human cytosolic GTP-PEPCK (21). We used the M. smegmatis GTP-PEPCK (2) for our site-directed mutagenesis and kinetic studies. The three-dimensional structural information for M. smegmatis PEPCK is not available. The percentage identity and percentage strong similarity between the primary structures of the mycobacterial and human PEPCKs are 50 and 19%, respectively (2). The identity values are much higher for the PEP-binding pocket. Almost every residue of this site of the human enzyme is conserved in the M. smegmatis PEPCK. Therefore, inferences on the structural aspects of the bacterial enzyme could be made from the structure data for the human enzyme. Our results show that two of the above-mentioned residues (an Asp and one Glu) are important for catalysis. Interestingly, the other residue (an Asp) influences the activity of the enzyme in a subtle manner. Our analysis suggested that through long range structural interactions this Asp residue influences the optimal position of one or
more catalytically active or important residues. Interestingly, a replacement of this Asp with Ala, Asn, Gln, or Ser changed the $V_{\text{max}}$ value for two directions of the PEPC reaction differently. We discuss these findings below.

Secondary Structure Changes in M. smegmatis PEPC Variants—Far-UV CD spectra showed that the engineered substitutions did not alter the secondary structure of the protein significantly, although some small changes occurred because of D75N and D75Q substitutions (Fig. 4). A CDDSSTR analysis (47–49) of the CD data identified minor changes in all variants except D75A and E83A (Table 7).

Asp$^{75}$ and Glu$^{83}$ of M. smegmatis PEPC—These residues are fully conserved in the GTP-PEPCks (Table 1). Therefore, they are expected to play important roles in catalysis. Our experimental data supported this conclusion. A replacement of either Asp$^{75}$ or Glu$^{83}$ with Ala severely diminished activity of the enzyme in both anaplerotic and gluconeogenic directions (Table 3). The x-ray crystallographic data for the human cyto- 

colic PEPC (21) helped to rationalize our kinetic data. Asp$^{75}$ of M. smegmatis PEPC is equivalent to Asp$^{84}$ of the human enzyme (2, 21). The Asp$^{84}$ -COO$^-$ interacts directly with the backbone amino group of Tyr$^{235}$, for they are only 2.65 Å apart from each other. Tyr$^{235}$ (underlined in sequence below) is one of the fully conserved active site residues of GTP-dependent PEPCks (21). It belongs to the highly conserved 235SF(Y)SGYGNNLGGKC$^{243}$ element (2, 21) (Fig. 1, A and B). The binding of PEP to the enzyme causes displacement of the aromatic group of Tyr$^{235}$ from its original location by about 2.1 Å (Fig.

**TABLE 5**

Apparent kinetic values for the OAA-forming reaction of the wild-type and Asp$^{75}$ variants of *M. smegmatis* PEPC

| Variable substrate* (concentration range) | Enzyme     | $K_m$ $\mu M$ | $V_{\text{max}}$ $\mu$mol min$^{-1}$ mg$^{-1}$ | $k_{\text{cat}}/K_m$ $\times 10^6$ s$^{-1}$ | Figure for the plot |
|----------------------------------------|------------|--------------|---------------------------------|---------------------------------|-----------------|
| Mn$^{2+}$ (0.5–100 µM)                 | Asp$^{75}$ (wild type) | 2.4 $\pm$ 0.3 | 38.4 $\pm$ 0.3 | 189 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75A       | 21.2 $\pm$ 3.2 | 11.4 $\pm$ 0.5 | 6.5 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75N       | 8.4 $\pm$ 0.7 | 27.5 $\pm$ 0.6 | 39.1 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75Q       | 3.0 $\pm$ 0.40 | 14.4 $\pm$ 0.3 | 58.4 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75S       | 8.4 $\pm$ 0.6 | 17.1 $\pm$ 0.3 | 24.5 $\times 10^6$ s$^{-1}$ | A                |
| PEP (0.025–10 mM)                      | Asp$^{75}$ (wild type) | 352 $\pm$ 48 | 41.3 $\pm$ 1.4 | 1.41 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75A       | 1,130 $\pm$ 76 | 10.3 $\pm$ 0.2 | 0.11 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75N       | 1,010 $\pm$ 91 | 29.9 $\pm$ 0.8 | 0.35 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75Q       | 3,220 $\pm$ 410 | 13.8 $\pm$ 0.6 | 0.05 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75S       | 980 $\pm$ 67 | 21.3 $\pm$ 0.4 | 0.26 $\times 10^6$ s$^{-1}$ | A                |
| GDP (0.005–1 mM)                       | Asp$^{75}$ (wild type) | 92.0 $\pm$ 8.2 | 40.8 $\pm$ 0.9 | 5.3 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75A       | 64.1 $\pm$ 8.1 | 7.8 $\pm$ 0.2 | 1.5 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75N       | 87.4 $\pm$ 7.9 | 30.0 $\pm$ 0.8 | 4.1 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75Q       | 86.6 $\pm$ 6.2 | 8.1 $\pm$ 0.2 | 1.1 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75S       | 79.1 $\pm$ 4.9 | 18.4 $\pm$ 0.3 | 2.8 $\times 10^6$ s$^{-1}$ | A                |
| HCO$_3^-$ (1–300 mM)                   | Asp$^{75}$ (wild type) | 16,400 $\pm$ 1,300 | 38.5 $\pm$ 0.7 | 0.03 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75A       | 21,100 $\pm$ 2,500 | 11.1 $\pm$ 0.3 | 0.006 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75N       | 32,700 $\pm$ 8,700 | 25.3 $\pm$ 1.9 | 0.009 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75Q       | 17,100 $\pm$ 3,400 | 11.4 $\pm$ 0.6 | 0.008 $\times 10^6$ s$^{-1}$ | A                |
|                                        | D75S       | 18,900 $\pm$ 3,000 | 19.8 $\pm$ 0.6 | 0.013 $\times 10^6$ s$^{-1}$ | A                |

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*The assay mixture was standard, except the concentrations of the variable substrates were as indicated.

*Data are based on a subunit molecular mass of 71.2 kDa, which was calculated from the nucleic acid sequence-derived amino acid sequence for the monomeric PEPC (2).

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Asp<sup>75</sup>, Asp<sup>78</sup>, and Glu<sup>83</sup> of M. smegmatis PEPCK

| TABLE 7 | Secondary structure estimations of wild-type and variant M. smegmatis PEPCK enzymes |
|-----------------|---------------------------------|
| Enzyme          | α-Helix | β-Sheet | β-Turn | Other | NRMSD<sup>a</sup> |
| WT              | 30      | 34      | 16     | 20    | 0.001 |
| D75A            | 29      | 32      | 17     | 22    | 0.001 |
| D75N            | 32      | 27      | 20     | 21    | 0.001 |
| D75Q            | 32      | 29      | 17     | 22    | 0.001 |
| D75S            | 31      | 27      | 19     | 24    | 0.001 |
| D78A            | 32      | 29      | 18     | 21    | 0.001 |
| E83A            | 31      | 34      | 15     | 20    | 0.001 |

<sup>a</sup> NRMSD indicates normalized root mean square deviation.

1A) (21). After this movement, the aromatic side chain of tyrosine and the carboxylate group of PEP is separated by about 3.5 Å (21). As a result, an energetically favorable, edge-on interaction between the carboxylate group of PEP and the aromatic side chain of Tyr<sup>235</sup> is established (21). We hypothesize that for this role of Tyr<sup>235</sup> and the above-described interactions between Tyr<sup>235</sup> and Asp<sup>84</sup>, the latter residue influences catalysis. The activity of the D78A enzyme was too low to allow meaningful kinetic studies. In contrast, E83A PEPCK was stimulated by Mn<sup>2+</sup>. As a result, we were able to perform a detailed kinetic study with this enzyme (Fig. 2). The results were analyzed with respect to the following structure information. Glu<sup>83</sup> of the M. smegmatis enzyme is equivalent to Glu<sup>88</sup> of the human PEPCK (2, 21). We found that in the PEP-free state, Glu<sup>88</sup> appears to bind Arg<sup>87</sup>, which is equivalent to Arg<sup>81</sup> of the M. smegmatis enzyme (2, 21). In the PEP-bound state, the positively charged side chain of Arg<sup>87</sup> interacts with the negatively charged phosphate group of PEP (21) (Fig. 1, A and B). From the structure data (21) we found that Glu<sup>89</sup> is displaced by over 2 Å when PEP binds to the enzyme (Fig. 1A). We hypothesize that in the absence of PEP, Glu<sup>89</sup> stabilizes Arg<sup>84</sup> through charge interactions and maintains the latter ready for establishing a similar interaction with the phosphate group of PEP. Arg<sup>87</sup> is fully conserved in all GTP-PEPCKs (Table 1). Therefore, Arg<sup>87</sup> is a catalytically important residue in the human PEPCK. The equivalent residue in an ATP-PEPCK (Arg<sup>70</sup> in the S. cerevisiae PEP carboxykinase) is necessary for the complete reaction (50). Our data on the K<sub>m</sub> values provided insight on how the role of this Arg residue is linked to the observed substantial loss of enzymatic activity caused by the E83A replacement in M. smegmatis PEPCK. The E83A residue change did not affect the apparent K<sub>m</sub> values for PEP, GDP, and HCO<sub>3</sub> significantly, but increased the apparent K<sub>m</sub> value for Mn<sup>2+</sup> by more than 2 orders of magnitude (Table 4). Therefore, we conclude that in the E83A enzyme, Arg<sup>81</sup> remained efficient in binding PEP, but the environment of Mn<sup>2+</sup> bound at the metal-binding site 1 (shown in purple in Fig. 1) changed. We hypothesize that a loss of the favorable charge-charge interaction between Glu<sup>83</sup> and Arg<sup>81</sup> because of the E83A substitution caused a perturbation in the Arg<sup>81</sup>-PEP interaction, which in turn changed the location of bound PEP with respect to site 1 Mn<sup>2+</sup> (Fig. 1A) (21). The result was a substantial rise in the K<sub>m</sub> value for Mn<sup>2+</sup> and a severe loss of the enzymatic activity. We examined whether the observed increase in the K<sub>m</sub> value for Mn<sup>2+</sup> was a mere reflection of a rise in the requirement of the variants for Mg<sup>2+</sup>. When the Mg<sup>2+</sup> concentration was raised to 5 mM, the apparent K<sub>m</sub> value for Mn<sup>2+</sup> of the E83A enzyme dropped substantially, but still was 1 order of magnitude higher than that of the wild-type enzyme. The apparent K<sub>m</sub> value for Mn<sup>2+</sup> of the wild-type enzyme did not change because of such a change in the Mg<sup>2+</sup> concentration. The K<sub>m</sub> value for GDP of both the Glu<sup>83</sup> and E83A enzymes decreased following an elevation of the Mg<sup>2+</sup> concentration, but these effects were small in magnitude. These observations can be explained as follows. At an elevated Mg<sup>2+</sup> concentration, more nucleotide was complexed with this cation, and less Mn<sup>2+</sup> was combined with the nucleotide. As a result, more free Mn<sup>2+</sup> was available to bind at site 1, and the enzyme was saturated at a lower concentration of total Mn<sup>2+</sup>. However, this change could not restore the wild-type interactions between PEP and Mn<sup>2+</sup> in the variant enzyme. This conclusion is supported by the fact that a rise in the Mg<sup>2+</sup> level did not improve the activity of the enzyme significantly. In summary, our results suggest a critical role for PEP and site 1 Mn<sup>2+</sup> interaction in the OAA synthesis. However, the available data do not provide any clue to exactly how a change in the location of PEP affected the rate-determining step for the OAA formation activity of PEPCK. In fact, the identity of the rate-determining step for this direction of the PEPCK reaction remains unknown. For the PEP synthesis activity of chicken liver mitochondria PEPCK, the catalytic cleavage of the γ-phosphate bond of GTP has been argued to be the rate-limiting step (51).

Asp<sup>75</sup> of M. smegmatis PEPCK—As elaborated in the Introduction, this residue is not invariant (Table 1) (2). On the other hand, it is highly conserved and belongs to a sequence element that bears catalytically critical or important residues (Table 1) (Fig. 1A) (21). Therefore, we hypothesized that Asp<sup>75</sup> of M. smegmatis PEPCK is not critical for catalysis. However, it could influence the PEPCK reaction through indirect interactions with the catalytically active residues. Evolutionarily, such a role makes this residue an avenue for diversifying the nature of PEPCK activity through mutations. To explore this hypothesized role of Asp<sup>75</sup> of M. smegmatis PEPCK, we have investigated the kinetic properties of variant enzymes generated via substitutions at this position. We found that unlike the D78A and E83A variants, which were substantially less active in both directions than the wild type, D75A retained significant activity in the OAA formation direction and was more active than the wild type in the PEP formation direction (Table 3). Then a kinetic study with the variant enzymes that represented all substitutions for Asp<sup>75</sup> (Ala, Asn, Gln, and Ser) that are found in nature (Table 1) provided the following details. The engineered changes caused a moderate decrease in the specific activity and efficiency of the enzyme in the anaplerotic or OAA-forming direction (Fig. 3, A–D, and Table 5) and improved the gluconeogenic activity of the enzyme (Fig. 3E and Table 6). The apparent K<sub>m</sub> values for Mn<sup>2+</sup>, PEP, and OAA were elevated, whereas for GDP remained unchanged (Table 5). Therefore, the substitutions altered the interactions between enzyme, PEP, and Mn<sup>2+</sup>. The three-dimensional structure of the human enzyme (Fig. 1, A and B) provides an explanation for these results. Asp<sup>81</sup> of the human enzyme, which is equivalent to Asp<sup>75</sup> of M. smegmatis PEPCK, is situated near the PEP-binding pocket, but unlike Asp<sup>84</sup> and Glu<sup>88</sup> does not interact directly with an active site residue or a substrate (Table 1 and Fig. 1, A
and B) (21). As discussed above, Asp^84, Arg^87, and Glu^89 of the human enzyme, three of the fully conserved residues of PEPCk, influence the PEP-Mn^{2+} interaction. In addition, the α-carboxyl group of Val^85, another fully conserved residue, interacts with the aromatic ring of Tyr^{235} (Fig. 1B) and therefore indirectly determines the characteristics of the Tyr^{235}-PEP and PEP-Mn^{2+} interactions. A replacement of Asp^81 in human PEPCk is likely to change the positions of Asp^84, Val^85, Arg^87, and Glu^89 (Fig. 1A and B), which in turn could influence the placement of PEP, the PEP-Mn^{2+} interaction, and consequently the catalysis. Such indirect effects are expected to bring about subtle changes in catalysis, which is consistent with our observations of the Asp^79 variants of M. smegmatis PEPCk (Tables 5 and 6). Examples of an effect on the active site over long distances through multiple interactions have been presented previously (52–55). We are currently investigating exactly how the structural perturbations caused by substitutions at Asp^79 position translated into two opposite effects on catalysis in two directions without violating the Haldane’s equilibrium relationship (56) of the reaction. Nevertheless, our data show that it is possible to influence the activity of PEPCk by manipulating a residue distal to the active site. Asp^81 of human PEPCk (Asp^79 of M. smegmatis PEPCk) is not located at the active site but near the surface of the enzyme (21). Because such an approach involving a surface residue has the potential of reducing the activity of the enzyme without directly interfering with catalysis, it could be used to design a compound that can prevent PEPCk from participating in the overproduction of glucose in the liver of a person with type 2 diabetes.

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