Structure and Mechanism of Phosphoenolpyruvate Carboxykinase*

Allan Mattei§, Leslie W. Tari§, Hughes Goldie, and Louis T. J. Delbaere**

From the Departments of §Biochemistry and |Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E5, Canada

Phosphoenolpyruvate carboxykinase (ATP-oxaloacetate carboxylase) (transphosphorylating), EC 4.1.1.49; for the ATP- or GTP-dependent enzyme, EC 4.1.1.32; PCK, also known widely as PEPC (PEPCK) catalyzes the decarboxylation and mononucleotide-dependent phosphorylation of oxaloacetate to form phosphoenolpyruvate (Scheme 1; $M^{2+}$ represents Mg$^{2+}$ or Mn$^{2+}$).

$$
\begin{align*}
\text{CO}_2 & \quad \text{CO}_2 \\
\text{C} & \quad \text{Mg}^{2+} \\
\text{C} & \quad \text{O} + \text{NTP} \quad \text{C} & \quad \text{OPO}_3^2^- + \text{NAD} + \text{CO}_2 \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CO}_2 & 
\end{align*}
$$

**Scheme 1**

This conversion is the first committed step of gluconeogenesis in Escherichia coli and is part of the gluconeogenic pathway in virtually all organisms. In bacteria, such as E. coli, PCK is utilized during gluconeogenic growth when sugar levels are low (1).

PCK is also an important enzyme in the glycolytic pathways of some organisms, such as Ascaris suum (2) and Trypanosoma cruzi (3), in which it forms OAA from PEP, which in turn enters the citric acid cycle. In humans and other mammals, PCK is a central enzyme in carbohydrate metabolism, helping to regulate the blood glucose level. Gluconeogenic tissues, such as kidney and liver, convert lactate and other non-carbohydrate molecules to glucose, which in turn is released into the blood. The importance of PCK to carbohydrate metabolism in humans is such that it has been suggested as a potential drug target in the treatment of non-insulin-dependent diabetes mellitus (4).

PCKs have been traditionally classified according to nucleotide specificity, with the ATP-dependent enzymes found in bacteria, yeast, Trypanosomatid parasites and plants, and GTP-dependent PCKs in a variety of other eukaryotes and mammals (5). There are both important differences and similarities between ATP- and GTP-dependent PCKs. With the exception of bacterial PCKs, which are monomorphic (6), most enzymes of the ATP-dependent class are multimeric, with two (7), four (8, 9), or six (10) subunits per enzyme, while known members of the GTP-dependent class are exclusively monomorphic. While enzymes of either the ATP- or GTP-dependent classes show significant (40–80%) amino acid sequence identity within their respective groups (11, 12), there is no significant overall sequence homology between the two classes of enzyme. Despite this lack of overall homology, both groups of PCKs contain similar NTP and oxaloacetate binding “consensus motifs” in their active sites, which likely play similar roles in substrate binding (which will be described in this review). Also, both GTP-dependent and ATP-dependent PCKs have been shown to possess lysyl (13–17), arginyl (18, 19), and histidinyl (20, 21) residues at or near their active sites. The differences in nucleotide specificity and kinetic properties between ATP- and GTP-dependent PCKs have led to the suggestion that they may be potential therapeutic targets in parasitic nematodes (22) and in Trypanosomatid parasites such as T. cruzi (11).

This minireview will focus on new structural results derived from the recent crystal structure determinations of native E. coli PCK (23, 24), its complex with ATP-Mg$^{2+}$-oxalate (25), and the implications for the active site residues and catalytic mechanism of E. coli and other ATP- and GTP-dependent PCKs. We also suggest revised nucleotide-binding sites and possible active site residues for the GTP-dependent PCK family. Other aspects of GTP-dependent PCK enzymology and genetics have been recently reviewed (26).

Crystal Structure of E. coli PCK

Tertiary Structure—X-ray quality crystals of E. coli PCK were originally obtained in 1991 (27) and led to the structure determination using multiple isomorphous replacement and electron density modification techniques, followed by refinement against 1.9-Å resolution synchrotron data (23). The structure of the ATP-Mg$^{2+}$-oxalate ternary complex of E. coli PCK was subsequently solved at 1.8-Å resolution by molecular replacement techniques (25). Like several other mono- and dinucleotide-binding proteins, E. coli PCK has an open faced, mixed a/b structure. The protein is bivalent and consists of a 275-residue N-terminal domain and a more compact 265-residue C-terminal or mononucleotide-binding domain. The two domains are separated by a deep cleft of approximately 25 Å in which the active site is located (Fig. 1). Each domain is organized such that a central a-sheet is flanked by a-helices on either side, forming an a+b-a topology. A search of representative protein structures from the Protein Data Bank using the C$^\alpha$ coordinates of E. coli PCK with the program DALI (28) did not reveal any proteins with a significant degree of overall structural homology.

A Unique Mononucleotide-binding Fold—Comparative analysis of the derived E. coli PCK amino acid sequence with sequences from other ATP- and GTP-binding proteins suggested a typical Walker A-like consensus sequence, $^{248}$GXXGXXGK(T/S)255, most likely represented the ATP-binding site (29). We have adopted the recently described nomenclature of Traut (30) for the designation of peptide sequences within proteins that interact with mononucleotides. Under this scheme, the Walker-A motif (31), representing the phosphoryl-binding sequence, is now designated Kinase-1a. Within the PCK crystal structure, the Kinase-1a sequence forms a phosphoryl-binding loop or P-loop motif, common to many ATP- and GTP-binding proteins (32). This P-loop is contained within the mononucleotide-binding domain and lines one face of the active site cleft.

Analysis of the P-loop motif itself shows that it is homologous in structure with P-loops from a variety of other proteins, including adenylyl kinase (33), p21$^{Ha}$-ras (34), and RecA protein (35). Despite this, the structural context within which the P-loop is found appears to be different than for other proteins possessing this motif. Schulz (36) has described the classical mononucleotide-binding fold, common to a number of P-loop-containing ATP- and GTP-binding proteins, which consists of a core parallel b-sheet, flanked by a-helices. In the Structural Classification of Proteins data base (37), proteins having the P-loop motif are organized into a common fold and superfamily and subgrouped into four distinct families based on the ordering of b-strands within the b-sheet of the mono-
Minireview: Structure and Mechanism of PCK

Crystallographic Studies of Substrate Binding

ATP-induced Conformational Changes—Analysis of the crystal structure with bound ATP-Mg\(^{2+}\)-oxalate revealed a hinge-like movement of the two domains toward one another (Fig. 1) through an angle of about 20° (25). This movement of the two domains is not accompanied by large conformational changes within the interdomain linker regions; rather, it occurs through relatively small changes in polypeptide torsion angles in the three regions that connect the two domains. No detectable changes in secondary structure occur upon substrate binding, a result consistent with CD and Fourier transform infrared spectroscopy studies on ATP-dependent PCKs (38).

As summarized recently (39), substrate-induced domain movements in enzymes are not uncommon and can serve to exclude water, correctly orient active site residues for catalysis, trap substrates, and prevent the escape of reaction intermediates. All of these factors potentially contribute to increasing the rate of catalysis. The exclusion of bulk solvent may be particularly important for kinases, such as PCK, as water can potentially act as a nucleophile, resulting in ATP hydrolysis rather than phosphotransfer. Similar large substrate-induced domain movements have also been observed in hexokinase (40) and adenylyl kinase (41).

ATP-Mg\(^{2+}\)-binding Site—There are three main regions of the PCK structure that interact with ATP-Mg\(^{2+}\) (25). The Kinase-1a sequence, or P-loop motif, interacts with the phosphoryl oxygen atoms of ATP through electrostatic interactions and hydrogen bonds. Lysine 254 is a key residue within this sequence, interacting with both \(\beta\) and \(\gamma\)-phosphoryl groups of ATP. As well, the polar side chain following the conserved lysine, Thr-255, contributes an oxygen atom to the Mg\(^{2+}\) coordination sphere. This mode of interaction is similar to that found with several other proteins that exhibit the P-loop motif.

The second important region is the Kinase-2 sequence (30), which functions specifically to bind the Mg\(^{2+}\) cation. Both Asp-268 and Asp-269 form hydrogen bonds with two water molecules, which in turn form part of the Mg\(^{2+}\) coordination sphere. A third water molecule, oxygens from the \(\beta\) and \(\gamma\)-phosphoryl groups of ATP, and the \(\gamma\)-oxygen atom of Thr-255 make up the remainder of the octahedral environment about the Mg\(^{2+}\).

The third region of the PCK structure is that which interacts with the adenine base and ribose sugar. Unlike many other mononucleotide-binding proteins, PCK does not possess a Kinase-3 motif to bind these portions of ATP. Rather, residues primarily from the sequence 442RISIKDT450 are used. Here, Arg-449, Ile-452, and Thr-455 interact with the adenine base through hydrogen bonding and non-polar interactions. The preceding sequence, 442GWNHTG447, forms a flexible loop in the enzyme’s structure, which undergoes a major conformational change (primarily in Gly-447) upon ATP-Mg\(^{2+}\) binding (25). This in turn leads to the residues from Arg-449 to Thr-455 moving toward the active site cleft, with Arg-449 and Ile-452 sandwiching the hydrophobic surface of adenine. An additional consequence of this conformational change is that Thr-443 becomes more solvent exposed when ATP-Mg\(^{2+}\) is bound by the enzyme, a finding consistent with fluorescence studies in solution (42). An additional unique aspect of ATP binding in E. coli PCK is that it is bound in a syn rather than anti conformation, with a sugar-base torsion angle, \(\chi\), of 57.5° (25).

Oxalate-binding Site—Oxalate (\(\text{C}_2\text{O}_4^{2-}\)) is a structural analog of the enolate of pyruvate, the reaction intermediate in the conversion of oxaloacetate to phosphoenolpyruvate. While oxalate has not been characterized as an inhibitor of ATP-dependent PCKs, some data suggest it is a good competitive inhibitor with respect to OAA [\(K_i = 3 \text{mM}\)] in chicken mitochondrial and rat liver cytosolic PCKs (43), while other experiments have indicated weak, non-competitive inhibition with \(K_i\) values in the low millimolar range (44). In the enzyme-substrate complex, oxalate binds to the side chains of Arg-65, Tyr-207, Lys-213, and Arg-333.

Implications for Other PCKs

Active Site Organization and Structure of ATP-dependent PCKs—Comparative amino acid sequence alignment of ATP-dependent PCKs from E. coli, Staphylococcus aureus, Rhizobium spp., Saccharomyces cerevisiae, Trypanosoma spp., and plants shows that all of the putative active site residues are identical among this group of enzymes (Fig. 2) (23). A sequence, designated the PCK-specific domain (residues 204–213 of E. coli PCK), has been found to be conserved in both ATP- and GTP-dependent PCKs (11, 45). The PCK-specific domain contains Tyr-207 and Lys-213, residues that are identical among all ATP- and GTP-dependent sequences and that have been shown to bind the inhibitor oxalate in E. coli PCK. On this basis, we have suggested (23) that the PCK-specific domain represents a portion of the PCK active site, which has become structurally conserved among both groups of PCKs, perhaps as a result of convergent evolution (46). In addition to the PCK-specific domain, both the Kinase-1a and Kinase-2 motifs are essentially identical among all ATP-dependent PCKs (23). There is more variability in the adenine-binding region among the sequences, although the critical residues are either identical or show conservative substitutions (25) (Fig. 2).

Conserved Functional Sequences Between ATP- and GTP-dependent PCKs—Despite the lack of overall sequence homology between the two families of PCKs, careful analysis of multiple se-
sequence alignments of GTP-dependent PCKs in light of the structural results obtained with the E. coli enzyme suggests that there are motifs of common functional importance to both groups of enzymes. The PCK-specific domain has already been mentioned as an example. A portion of this sequence had previously been suggested to represent a possible phosphoryl-binding sequence common to other ATP- or GTP-binding proteins (11, 47–50). A more likely candidate for the phosphoryl-binding motif in GTP-dependent PCKs is shown in Fig. 2. Although this sequence shows some deviation from the classical Kinase-1a motif, due to the absence of the first glycine, all of the side chains critical for interacting with the phosphoribosyl group and nucleotide-associated metal (Mg$^{2+}$) are identical.

Similarly, the Kinase-2 motif is highly conserved among both classes of enzymes. Biochemical experiments with avian PCK have recently demonstrated that Asp-295 and Asp-296 of this enzyme represent a divalent metal-binding site (51), a finding consistent with this region representing the Kinase-2 motif. No Kinase-3 motif corresponding to the guanine-binding region could be found, reflecting both the absence of this motif in E. coli and other ATP-dependent PCKs (25), as well as differences between guanine- and adenine-binding sites (30). It is noteworthy that the separation between the putative Kinase-1a and Kinase-2 motifs (16 residues in all but Chlorobium limnicola PCK) is similar to that found with the ATP-dependent PCKs (10 residues), suggesting that they exist in a similar spatial arrangement within the tertiary structure.

In addition to the presence of similar Kinase motifs in ATP- and GTP-dependent PCKs, other residues that are functionally important in ATP-dependent PCKs appear at similar relative locations along the polypeptide chains of the GTP-dependent enzymes. In the crystal structure of the ATP-Mg$^{2+}$-oxalate ternary complex of E. coli PCK, His-232 and Arg-333 are associated with substrate binding and appear to be important catalytically (25). His-232 is involved in direct and water-mediated interactions with the γ-phosphate of ATP, while Arg-333 bridges oxalate and the ATP γ-phosphate. Homologous residues to both His-232 and Arg-333 are found in all ATP-dependent PCKs (23). Conserved histidinyl and argininyl residues also appear in the GTP-dependent enzymes at similar relative positions along the polypeptide chain. A single histidinyl residue is found within a highly conserved region in all GTP-dependent PCKs, 20 residues toward the N terminus from the putative Kinase-1a motif, compared with 16 residues in the ATP-dependent enzymes. A histidinyl residue proximal to the active site in chicken liver mitochondrial PCK was demonstrated in chemical modification studies using histidinyl-specific reagents (20). It is possible that this conserved histidine in the GTP-dependent class of enzymes serves the same function as its counterpart in the ATP-dependent PCKs. A residue corresponding to Arg-333 of E. coli PCK also appears within a conserved region in the GTP-dependent enzymes, 92–94 residues on the C-terminal side along the polypeptide chain from the Kinase-2 motif. This compares with 64 residues in the ATP-dependent enzymes. The presence of similarly disposed histidinyl and argininyl residues, as well as similar Kinase motifs and OAA-binding sequences in both classes of PCK, suggests that ATP- and GTP-dependent PCKs bind their respective substrates in a similar manner and share a common catalytic mechanism.

**A Unified Catalytic Mechanism for ATP- and GTP-dependent PCKs**

Despite the differences in overall amino acid sequence homology and nucleotide specificity, the similar active site organization between ATP- and GTP-dependent PCKs in terms of both homologous sequences associated with OAA binding (the PCK-specific enzyme) and NTP-Mg$^{2+}$ binding (Kinase-1a and Kinase-2 motifs, respectively) suggests that they catalytically operate in a similar fashion. Chemical modification studies on both groups of enzymes, as well as structural analysis of the ATP-Mg$^{2+}$-oxalate ternary complex of E. coli PCK, clearly display a role for lysinyl, argininyl, and histidinyl residues in substrate binding and catalysis. The highly reactive cysteinyl residues of some PCKs from either group can be explained by their proximity to nucleotide-binding motifs or other active site residues, without actually being essential for either substrate binding or catalysis. One outstanding difference is the current evidence for nucleotide-dependent conformational changes in monomeric but not multimeric PCKs. The evolution of PCKs having a quaternary structure may be specifically related to its metabolic regulation in some species, such as covalent phosphorylation of the enzymes from *S. cerevisiae* (52) and cucumber (53).

It is not entirely clear whether the conversion of OAA to PEP consists of a two-step process (the decarboxylation of OAA to form the pyruvyl enolate anion intermediate, followed by transfer of the γ-phosphoryl group from ATP or GTP) or if it is a single, concerted reaction (54–57). However, we favor the former possibility, both in terms of the expected chemistry of the reaction and the available structural data obtained with the *E. coli* enzyme. Through electrostatic interactions, Arg-333 may promote decarboxylation while at the same time contributing to the stabilization of enolate anion (25) (Scheme 2). The possibility of argininyl residues from both ATP-dependent (58) and GTP-dependent (19) PCKs participating in the carboxylation or decarboxylation reactions has been suggested previously. The available experimental data indicate that CO$_2$, and not HCO$_3^-$, is the enzyme substrate (59, 60).
Insight into the decarboxylation reaction should be forthcoming from detailed analysis of the Asp-268 → Asn mutant of E. coli PCK, in which OAA decarboxylation is maintained but phosphoryl transfer is abolished (61). The two steps of the reaction are effectively uncoupled in this mutant, making it a valuable tool for further investigation of the catalytic mechanism. Several GTP-dependent PCKs also possess an irreversible OAA decarboxylase activity of unknown physiological relevance (62).

Essentially all PCKs require a second metal ion, in addition to that which is associated with the nucleotide (Mg²⁺), for optimal activity. A possible function for this second metal, often a transition metal such as Mn²⁺, is in the decarboxylation or carboxylation steps, although a role in phosphotransfer has also been suggested (55, 66, 67). Metals such as Mn²⁺ can promote decarboxylation of OAA in enzymes such as OAA decarboxylase and PCK by forming complexes with OAA and may also stabilize the enolate anion during catalysis in either the forward or reverse directions (reviewed in Ref. 68). In GTP-dependent PCKs, this second metal ion may bind the phosphoryl group of both PEP and the β- or γ-phosphate of nucleotide substrates through either direct (54) or water-mediated (66, 67) interactions; this likely helps to position substrates for catalysis.

In contrast to the decarboxylation and carboxylation reactions, much more is understood about the process of phosphoryl transfer. Both the crystal structure of the E. coli PCK ternary complex (25) and biochemical data for both ATP-dependent (70) and GTP-dependent (55–57) PCKs support a direct displacement, S₂-type mechanism of phosphoryl transfer (Scheme 2). In E. coli PCK, both His-232, which makes water-mediated interactions with the γ-phosphoryl group of ATP, and Arg-333, which bridges oxalate and ATP, are candidate residues for stabilization of the transition state (25).

It appears somewhat more difficult to reconcile the various kinetic mechanisms proposed for various PCKs into a coherent picture. Semi-ordered (55, 69) and fully random (57, 60, 70) mechanisms with either NTP or OAA adding first have been proposed, depending on the source of the enzyme. In E. coli PCK, the kinetic mechanism is semi-ordered, with a preferred pathway of substrate addition (oxaloacetate before ATP) and product release (69). Analysis of the ternary complex of E. coli PCK is consistent with an ordered mechanism, where OAA adds prior to ATP, since the ATP-induced conformational change results in exclusion of bulk solvent from the active site and completely buries the inhibitor oxaloacetate (25). Such a mechanism might also be expected for rat liver PCK, as this enzyme may undergo a similar change in structure upon binding NTPs (42).

In summary, it does appear that despite the differences between both groups of PCKs they do have a similar active site organization and likely operate with the same overall mechanism of catalysis. Structure determination of at least one member of the GTP-dependent PCK family will be eagerly awaited to compare the overall structure and mode of substrate binding with the E. coli enzyme.

**REFERENCES**

1. Goldie, H., and Sanwal, B. D. (1980) J. Bacteriol. 141, 1115–1211
2. Christie, D. A., Powell, J. W., Stables, J. N., and Watt, R. A. (1987) Mol. Biochem. Parasitol. 24, 125–139
3. Cymerung, C., Cascales, J. J., and Cannata, J. J. (1995) Mol. Biochem. Parasitol. 73, 91–101
4. Valera, A., Pujol, A., Pelegrin, M., and Bosch, F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9511–9515
5. Utter, M. F., and Kolanbrander, H. M. (1972) Enzyme (Basel) 6, 117–168
6. Goldie, H., and Sanwal, B. D. (1980) J. Biochem. Mol. Biol. 35, 1399–1405
7. Urbina, J. A. (1987) Arch. Biochem. Biophys. 256, 186–185
8. Tortera, P., Hanoez, G. M., and Guerritore, A. (1985) Anal. Biochem. 144, 179–185
9. Walker, R. P., Watanabe, S., and Leegood, R. C. (1991) Physiologia Plantarum 85, 213–217
10. Finnegan, P. M., and Burnett, J. N. (1985) Plant Mol. Biol. 5, 365–376
11. Lin, S., Goldie, H., and Cardemil, E. (1988) Biochim. Biophys. Acta 937, 207–210
12. Chen, C.-Y., Emig, F. A., Schramm, V. L., and Ash, D. E. (1991) J. Biol. Chem. 266, 16645–16652
13. Guidinger, P. F., and Nowak, T. (1991) Biochemistry 30, 8851–8861
14. Malebran, L., and Cardemil, E. (1987) Biochim. Biophys. Acta 915, 385–392
15. Cheng, K. C., and Nowak, T. (1989) J. Biol. Chem. 264, 3137–3142
16. Dunn, J. R., and Nowak, T. (1989) J. Biol. Chem. 264, 19666–19676
17. Bustos, P., Gajardo, M. I., Gomez, C., Goldie, H., Cardemil, E., and Jabalquinto, A. M. (1996) J. Protein Chem. 15, 467–472
18. Klein, D. R., Winterton, C. A., Hasenbuhl, T. N., Sha, M. H., Faurou, M. A., Naik, S. C., and Geourjon, C. (1992) Mol. Biochem. Parasitol. 56, 285–294
19. Matte, A., Goldie, H., Sweet, R. M., and Delbaere, L. T. J. (1990) J. Mol. Biol. 206, 128–143
20. Matte, A. (1996) Crystallographic Studies of E. coli Phosphoenolpyruvate Carboxykinase. Ph.D. thesis, University of Saskatchewan, Saskatoon, Canada
21. Tari, L., Matte, A., Pugazhenthi, U., Goldie, H., and Delbaere, L. T. J. (1996) Nat. Struct. Biol. 3, 355–363
22. Hansen, R. W., and Patel, Y. M. (1994) Adv. Enzymol. Relat. Areas Mol. Biol. 68, 203–281
23. Delbaere, L. T. J., Vanzonin, M., Glaceske, D., Jabs, C., and Goldie, H. (1997) J. Mol. Biol. 219, 589–594