Introduction

Mammalian phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the reversible formation of phosphoenolpyruvate (PEP) from oxalacetate (OAA) and GTP (or ITP) in a divalent cation-dependent reaction (Scheme 1), as was elegantly discussed in the first of these three Minireviews on PEPCK (1). The recent structures of PEPCK from human, rat and chicken (2-5), the enzymes from Trypanosoma cruzi (6), Anaerobiospirillum succiniciproducens (7), and Corynebacterium glutamicum (8) in conjunction with earlier work on the isozyme from E.coli (9-13), illustrate that the active site residues and architecture are well conserved, despite what is rather poor overall sequence homology when comparing members of the ATP- and GTP-dependent families1. As detailed in this review, the cationic environment of the active site, dominated by the juxtaposition of two divalent metal ions and the positioning of lysine and arginine residues, is well suited to allow for the stabilization of the enolate intermediate discussed above and to facilitate phosphoryl transfer.

An informative aspect of the PEPCK catalyzed reaction revealed by the recent structural data on the GTP-dependent isozyme from rat is the illumination of the previously unappreciated role of conformational changes occurring in the active site during the catalytic cycle (5). The most prevalent mobile feature illustrated by the structural work is a 10-residue Ω-loop lid corresponding keto-form; in contrast, by stabilizing the enolate, PEPCK could prevent its energetically favorable protonation and tautomerization, allowing phosphoryl transfer to occur. Thus, by stabilizing this intermediate in a high-energy state, the PEPCK reaction would be energetically rendered freely reversible; the crystal structures that will be described indicate that PEPCK does, in fact, stabilize the enolate intermediate. Based solely upon this thermodynamic view, it can be argued that the enzyme-mediated stabilization of the high-energy enolate state, preventing its collapse into the deep energetic well of the keto state (i.e. pyruvate) that renders the PK reaction irreversible, could account for a large amount of PEPCK’s catalytic power.
domain whose closure is potentially capable of protecting the enolate intermediate (Fig. 2) (2-5). A similar domain is present in ATP-dependent PEPCK, as represented by the enzyme from *E. coli*, which was the first PEPCK to be structurally characterized (9). The structural data on PEPCK demonstrate that only upon closure of the lid domain are the substrates positioned correctly for catalysis to occur (5). Furthermore, another loop domain, the ubiquitous P-loop or kinase-1a motif in the GTP-dependent PEPCKs, also shows dynamic behavior, adapting various conformations correlated with substrate binding. The potential role of the dynamic P-loop in catalysis is of interest since it contains a reactive cysteine residue that is conserved in all GTP-dependent PEPCKs and whose specific modification has been known for two decades to result in the inactivation of the enzyme (14,15). As described below, recent structural work characterizing the low energy conformational states that define the reaction coordinate of the enzyme-catalyzed reaction (2-5,16) considered together with previous biochemical studies have allowed a relatively detailed picture of the mechanism of catalysis utilized by PEPCK to emerge. In the discussion that follows, both the role of the positively charged active site and the important conformational changes occurring within that site are discussed in the context of an integrated mechanism for PEPCK-mediated catalysis.

**Structural Snapshots of a Stepwise Mechanism for PEPCK**

**A. Formation of the Michaelis Complex**

The crystallographic structures of PEPCK in complex with either OAA or its analogue 3-sulfopyruvate (β-SP) demonstrate that OAA binds to the enzyme by directly coordinating to the M1$^{2+}$ manganese ion with its C3$^{\gamma}$ and C4 carbonyl oxygens displacing two of the three water molecules coordinated to that cation in the structure of PEPCK-Mn$^{2+}$ (Fig. 2A). Structure-function studies on rat cytosolic PEPCK indicate that the cis planar arrangement of the metal coordinating atoms is essential in substrate/inhibitor recognition by PEPCK, which explains the poor inhibition observed in prior kinetic studies by OAA analogues lacking this feature (16-18). In addition to the favorable interactions between OAA and the M1 Mn$^{2+}$ ion, the binding of OAA is facilitated through interactions with R87$^4$ and S286 (19). GTP binds to PEPCK within the unique nucleotide-binding site of GTP-dependent PEPCKs (Fig. 3), with the β- and γ-phosphoryl groups coordinating to the M2 metal (Fig. 2A). These two coordinating atoms from GTP, in conjunction with the Oγ of the P-loop T291 side chain and three water molecules, complete the octahedral coordination geometry of the M2 metal ion. Upon GTP binding to the PEPCK-OAA complex, forming the Michaelis complex, the remaining water molecule coordinated to the M1 metal ion is displaced fulfilling its octahedral coordination geometry. This coordination of the γ-phosphate allows it to act as a bridging ligand between the M1 and M2 metal ions (Figs. 2A-C). Further, the coordination of OAA and GTP to the M1 and M2 metal ions places the C3 carbonyl oxygen of OAA and the γ-phosphate of GTP in the perfect orientation, albeit at a distance that is too great (see Section B below), for direct inline phosphoryl transfer, consistent with the observed stereochemistry of the reaction (20,21). Thus in the fully ligated state, the M1 metal is octahedrally coordinated by three protein ligands (K244, H264 and D311) and three oxygen atoms of the OAA and GTP substrates. In a role consistent with its known function in kinases, the P-loop lysine (K290) bridges the β- and γ-phosphoryl groups, facilitating the leaving group ability of the γ-phosphate (22). Further, the importance of R405 in forming a bi-dentate salt bridge with the phosphoryl group being transferred is illustrated in the complexes representing the two Michaelis states for the reversible reaction (Figs. 2B,D). The bi-dentate interaction of R405 with the γ-phosphate of GTP suggests a role in stabilizing the phosphoryl group undergoing transfer in a higher energy eclipsed conformation relative to the β-phosphoryl group, again making the γ-phosphate a better leaving group. The importance of N403 in orienting R405 to fulfill this role is also apparent from the structures (Fig. 2).

In the lid-open PEPCK-GTP (4) and PEPCK-OAA-GTP (Fig. 2A) complexes, the bound conformation of GTP shows the orientation of the nucleotide ribose ring and α-phosphate in a non-canonical conformation. The structural studies suggest that this uncommon nucleotide orientation in the lid open state holds the nucleotide away from the M1 manganese ion, perhaps minimizing GTPase activity in the absence of a phosphoryl acceptor.
B. Activation of Catalysis Upon Lid Closure.

While all the substrates bind to the lid-open form of the enzyme (Fig 2A), in a clear example of a classical induced-fit mechanism, the structural studies demonstrate that only upon lid closure are the substrates positioned correctly to allow for catalysis to proceed (3). This begs the question as to what is the energetic driving force for lid closure. The structural data support a model in which modulation of the free energy profile for the enzyme occurs as ligands bind during PEPCK’s progression toward formation of the Michaelis complex (5). This model suggests that the thermodynamic favorability of the enzyme adapting the closed lid (active) state increases as ligands add to the enzyme, with a portion of their Gibbs free energy of binding being partitioned to the protein, offsetting the entropic unfavorability of the lid assuming an ordered closed conformation rather than a conformationally dynamic open (inactive) state. Therefore, after formation of the lid-open Michaelis complex, the next step in the catalyzed reaction is the sampling of the closed lid state due to a thermodynamic shift in the favorability of its formation. Upon lid closure after formation of the Michaelis complex, the ribose ring and phosphate chain of GTP assume the canonical conformation stabilized by a shift in the interaction between R436 and the ribose ring of the C3-hydroxyl group to the lactam oxygen (compare Fig. 2A to 2B). This change in R436-ribose ring interactions results in the movement of the nucleotide forward in the active site towards the M1 ion, shortening the potential phosphoryl transfer distance by ~0.5 Å. This shift in nucleotide position also results in a shift in the position of the M2 metal ion, such that the M1-M2 metal distance decreases by ~0.5 Å between the identically ligated lid open and lid closed complexes. The movement of the nucleotide and its associated metal ion appears to result from the shift in the position of the P-loop that is correlated with the transition of the lid from an open to a closed state. The interactions between the backbone amides of the P-loop and the β-phosphate of GTP move the nucleotide towards OAA and the M1 metal as the P-loop shifts towards the M1 metal. Based upon the structural studies, the closure of the active site lid, the movement of the P-loop toward the M1 metal ion (closed P-loop conformation), and the transition of the nucleotide from the unusual conformation observed in the lid open complexes to the canonical conformation observed in the lid closed state represent three interdependent processes. Formation of the closed lid conformation is associated with the release of two potential steric constraints: (1) adaption by the P-loop of the closed conformation, which appears to be mediated via a hydrogen bond being established between S286 and the C4 oxygen of OAA; and (2) a change in the rotomeric conformation of R436 upon nucleotide association (compare Figs. 2A and 2B). The experimental evidence clearly demonstrates a correlation between the motions of the P-loop and the active site lid, with both being essential for catalysis (3-5). Unfortunately, the current data are insufficient to analyze these interdependent motions temporally; thus a mechanism of cause and effect cannot yet be established. Nevertheless, the necessity for dynamic behavior of the P-loop indicated by the recent studies does provide a possible explanation for the inactivation of PEPCKs from many sources by chemical modification of C288, which resides on the P-loop (14,15).

The modeled position of CO₂ in Figs. 2C&D, which is based upon the positions of the carboxylate of OAA and the sulfate of β-SP (4,5), mirrors the observed position of CO₂ bound in the E.coli ATP-dependent form of PEPCK (23). Based upon the structural information, decarboxylation of OAA to form the enolate intermediate is facilitated by polarization of the carboxylate between N403 and R87 (Fig. 2C). In addition, the rotation forward of Y235 provides further interactions with CO₂, as does aromatic stabilization by the ring of F333, neither of which is available in the complexes with OAA (compare Figs. 2B and 2C). As previously described, phosphorylation of the resultant enolate occurs by positioning and stabilization of the phosphoryl group undergoing transfer through electrostatic interactions with R405, K290 and the two metal ions (Fig. 2C).

C. Lid Opening and Product Release

In the reverse of the process described above, the formation of products again results in modulation of the free energy profile for the enzyme, such that the chemical transformation of the enolate intermediate to PEP results in a shift in the thermodynamic favorability of the closed lid state decreasing, allowing the enzyme
once again to sample the lid open conformation. Upon sampling of the lid open state, the PEP product shifts away from direct coordination to the M1 and M2 metal ions (Fig. 2D-E). For this transition to occur, Y235 must shift to its rearward orientation, and in the process, the changing rotomeric states of Y235 and F333 lessen the enzyme-CO$_2$ interactions and allow for the release of the CO$_2$ product. The loss of direct M1 coordination by PEP, which is consistent with NMR studies suggesting outer-sphere coordination of PEP in the PEPCK-Mn$^{2+}$PEP complex (24), results in water filling the three open coordination sites to the active site metal and the one open site on the nucleotide metal (Fig. 2E). The interactions with S286 on the P-loop are lost as PEP moves to an outer sphere coordination geometry, allowing for an opening of the P-loop and the resultant shift of the nucleotide and M2 metals away from the M1 metal as S286 orients towards solvent (Fig. 2E). The interactions between GDP and K290 on the P-loop are minimized with K290 populating two conformations in the PEPCK-PEP-GDP open complex (Fig. 2E) as suggested by the analogous crystal structure with the PEP analogue 2-phosphoglycolic acid (5). The shift of PEP to the outer sphere complex, which has been observed in both human (2) and rat (Holyoak, unpublished data) cytosolic and chicken (3) mitochondrial PEPCKs, is facilitated through a number of interactions unique to this conformation that are depicted in Figure 2E. The shift to outer-sphere coordination and P-loop and lid opening allow for PEP and GDP release and the subsequent transition back to the beginning of the catalytic cycle.

### D. Stabilization of the Enolate Intermediate

All of the data, both structural and biochemical, are consistent with the PEPCK catalyzed reaction proceeding through an enolate intermediate. As discussed herein, due to the enolate’s reactivity, this chemical mechanism requires PEPCK to protect this intermediate from alternative chemistries, especially its protonation that results in the formation of pyruvate. The structural data indicate that the closed lid state becomes more highly populated upon transition of the enzyme from the Michaelis complex to the intermediate complex (5), which is consistent with a role for the active site lid in protecting the enolate intermediate from protonation and subsequent tautomerization to pyruvate. Through the closed lid conformation of the enzyme being the most stable conformational state for the protein upon formation of the intermediate complex (Fig. 2C), the dynamics of the lid element become an essential component of the reaction pathway. The extrapolation of these results suggests the intriguing possibility that by carrying out the reaction in a stepwise fashion, the individual chemical steps of this pathway are more favorable and have lower transition state barriers than other mechanistic possibilities. Intrinsically to this hypothesis is the requirement that the enzyme stabilize the enolate intermediate from alternative chemistries that make this chemical mechanism impossible for the uncatalyzed reaction in solution. In the case of PEPCK catalysis, by using this chemical mechanism, the enzyme has potentially evolved to stabilize the enolate intermediate more than either of the two transition states (Fig. 1B), a general possibility originally suggested by Fersht (25).

#### Structural Explanations for Nucleotide Specificity

The reasons that mammalian PEPCK utilizes GTP or ITP, but not ATP, as a substrate are suggested by the crystal structures of PEPCK-nucleotide complexes (Fig. 3) (2-5). Structurally, adenosine and guanosine nucleotides differ at the two and six positions of the purine ring. The structural data in combination with binding studies of inosine (lacking the C-2 amino group) and guanosine nucleotides show that the C2-amino group of GTP does play a role in nucleotide binding, likely mediated through its interaction with F525 (Fig. 3) (2). However, the ability of the mammalian PEPCKs to utilize inosine and guanosine nucleotides with similar kinetic efficiency indicates that the absence of a C2 amino group in ATP is not the basis for PEPCK’s selectivity. In GTP and ITP the C6 carbonyl functions as a hydrogen bond acceptor, which is of course not possible for the C6-amino group of ATP. Consistent with this distinguishing feature, structures of PEPCK complexes show that the C6 carbonyl of GTP forms hydrogen bonds with the backbone amide of F530 and the side chain amide nitrogen of N533 (Fig. 3). Further, the amide of N533 is held in position by hydrogen bonds with the indole nitrogen of W527 and the backbone carbonyl of F530 (Figure 3). These interactions prevent N533 from assuming an alternative rotomeric state that could allow it to position its
amide group to accept a hydrogen bond from the C6 amino group of ATP. The other distinguishing feature of guanosine and inosine nucleotides is the ability of the purine ring to tautomerize. While many studies demonstrate that in solution these bases populate the keto form, the possibility exists that the physicochemical characteristics of the nucleotide binding pocket of the mammalian PEPCKs, which sandwiches the purine ring between F333 and F517 (Figure 3), may preferentially impose selectivity for the enol tautomer unique to inosine and guanosine nucleotide bases. Unfortunately, unlike the structural comparison leading to the successful conversion of guanylate cyclase into adenylate cyclase (26), a direct structural comparison of the ATP- and GTP-dependent PEPCKs may not be relevant, as their nucleotide binding sites are substantially different. Further studies will be necessary to firmly establish in detail the structural bases for the different nucleotide specificities of the two general classes of PEPCK isozymes.

Summary

Taken as a whole, the structural data indicate that the active site of PEPCK creates an electrostatic environment that is tailored to stabilize the large amount of localized negative charge that results from the close juxtaposition of its multiple anionic substrates. This environment facilitates the stabilization of the two transition states as well as the enolate intermediate and allows for efficient catalysis. In concert with this electrostatic environment, the unique dynamic properties associated with the reaction pathway allow for the enzyme to minimize off pathway reactions, such as nucleotide hydrolysis and OAA decarboxylation, that would decrease catalytic fidelity and lead to metabolic cycling in vivo.

References

Footnotes

1 Both ATP- and GTP-dependent PEPCK enzymes exist in nature. The ATP-class has been identified in bacteria, trypanosomatids, C4 plants and yeast, while the GTP-dependent isozymes are found in mammals and other eukaryotes (excluding yeast) and some bacteria (*Corynebacterium* sp., *Mycobacterium* sp.) (8,27,28).

2 In the absence of substrates, PEPCK binds a single divalent cation cofactor (M1). Manganese is the most activating cation and is thought to be the physiologically relevant cofactor due to this property in combination with PEPCK’s higher affinity for manganese relative to magnesium. An additional divalent cation (M2) is bound in the presence of nucleotide, as the catalytically competent form of the nucleotide is the M2+-nucleotide complex.

3 The atom numbering utilized is consistent with that used previously (4). While this is contrary to IUPAC nomenclature, it is consistent with the atom numbering utilized for the molecules in the PDB.

4 The residue numbering utilized is that of the rat cytosolic isozyme.
Figure 1: Diagram representing the reaction coordinates for the pyruvate kinase (A) and phosphoenolpyruvate carboxykinase (B) catalyzed reactions. The standard free energy values given are approximate values based upon the average values from a number of literature sources. The heights of the transition state barriers for the reactions are arbitrary and are used for illustrative purposes only.
**Figure 2:** Crystallographic snapshots defining the chemical reaction path of PEPCK mediated conversion of OAA to PEP. A schematic drawing to aid in the interpretation of the structural data is presented on the right hand side of each panel. In the left hand figures, the substrates/products are rendered as stick models colored by molecule type: GTP (magenta), GDP (purple), OAA (blue), PEP (burgundy), CO$_2$ (cyan) and the enolate intermediate (green). The active site Ω-loop lid and P-loop motif are rendered in yellow and red, respectively. The amino acids involved in important substrate/product interactions are rendered as grey ball-and-stick models colored by atom type and labeled adjacent to their respective α-carbon atom. Dashed lines illustrate important protein-substrate/product interactions. The positions of the PEP, OAA, enolate, and CO$_2$ molecules are based upon the authentic binary complexes of the enzyme with those substrates as well as ternary complexes with the substrate analogues 2-phosphoglycolic acid, 3-sulfopyruvate and oxalate and the corresponding GDP or GTP nucleotide.
Figure 3: The unique nucleotide-base binding pocket found in mammalian PEPCK. Those residues discussed in the text are illustrated and labeled.