Inhibitors of Pyruvate Carboxylase

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Abstract: This review aims to discuss the varied types of inhibitors of biotin-dependent carboxylases, with an emphasis on the inhibitors of pyruvate carboxylase. Some of these inhibitors are physiologically relevant, in that they provide ways of regulating the cellular activities of the enzymes e.g. aspartate and prohibitin inhibition of pyruvate carboxylase. Most of the inhibitors that will be discussed have been used to probe various aspects of the structure and function of these enzymes. They target particular parts of the structure e.g. avidin – biotin, FTP – ATP binding site, oxamate – pyruvate binding site, phosphonoacetate – binding site of the putative carboxyphosphate intermediate.

Keywords: Pyruvate carboxylase, biotin-dependent enzyme, avidin, biotin, nucleotide inhibitors, acetyl coenzyme A, allosteric inhibitors, chlorothricin.

INTRODUCTION

Pyruvate carboxylase (PC, EC 6.4.1.1), a regulatory metabolic enzyme responsible for replenishing the intermediates of the TCA cycle and catalyzing the first committed step in gluconeogenesis, is found in a wide variety of organisms including bacteria, fungi, plants, invertebrates and vertebrates [1]. Eukaryotic PC is generally located in the mitochondria with the exception of yeast, where the two isoforms of PC (Pyc1 and Pyc2) are encoded by different genes, and fungal PC, both of which are localized in the cytoplasm [2].

Abnormalities in PC activity and regulation have been associated with the occurrence of Type II diabetes [3] resulting in impaired-glucose tolerance and insulin insensitivity [4]. In pre-diabetic patients, pancreatic islets compensate for the escalating insulin-resistance by increasing glucose-stimulated insulin secretion (GSIS) [3, 5]. Studies performed by Jensen and co-workers [6] proposed that GSIS activity was tightly correlated with the PC-catalyzed anaplerotic flux through the TCA cycle and subsequent cycling of pyruvate through the malate-pyruvate shuttle. Recently, the use of small interfering RNA (si-RNA) to partially suppress PC activity in INS-1 83/13-derived cell lines by Hasan *et al.* [7] demonstrated that decreases in GSIS were directly proportional to decreases in PC activity. Furthermore, Liu *et al.* [8] previously observed a 2-fold increase in *in vivo* PC β-cell activity and GSIS in obese, non-diabetic, insulin-resistant Zucker fatty rats as compared to non-insulin resistant Zucker lean rats. Inhibition of PC by the addition of phenylacetate resulted in not only a marked decrease in PC activity, but also a corresponding decrease in GSIS. The decreased PC activity and expression observed in the islets of diabetic rats, which are insulin resistant and show little GSIS [9, 10] further suggests that PC plays an important role in both GSIS and β-cell adaptation to insulin resistance in fully functioning pancreatic cells. While the mechanism by which PC activity regulates and enhances GSIS is not completely understood, it has been proposed by MacDonald [11] and others [12] that the metabolic cycling of pyruvate through PC and the subsequent formation of anaplerotic by-products, including NADPH, aids in modulating GSIS in pancreatic islets.

Abnormally, high hepatic PC activity was initially observed in diabetic rats [5, 9, 10]. Deterioration of the GSIS pathway, due in part to chronic exposure to fatty acids, decreases the ability of the β-cells to secrete insulin, and can lead to the development of Type II diabetes [5, 13]. Metabolic flux through hepatic PC is normally attenuated by the insulin-signaling pathway [14], but is increased in Type II diabetics resulting in raised hepatic glucose production [3, 13]. The metabolic abnormalities in the regulation and activities of PC associated with Type II diabetes make PC an attractive molecular target for the development of new therapeutic agents for the treatment of this progressive disease.

Fan and co-workers [15] have recently established a connection between PC activity and the "mitochondria dysfunction" observed in malignant lung cancer [16]. ^{13}C isotopomer analysis by NMR revealed direct evidence for increased glycolytic activity in malignant tumor cells and further demonstrated that both PC expression and activity, *in vivo*, were activated in human lung cancer. Previously, a nearly 100-fold increase in PC's anaplerotic activity was observed in *in vitro* studies of breast cancer cells [17] and PC's gluconeogenic activity was found to be elevated in hepatic

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tumors in rats [18], demonstrating that increased PC activity correlates with the uncontroled proliferation of tumor cells. Therefore, the selective inhibition of PC activities in tumor cells may prove to be a viable, alternative target for newly emerging antiproliferative cancer treatments.

The connection between abnormal PC activity, Type II diabetes and cancer substantiates the importance of understanding the structure, mechanism and inhibition of this regulatory enzyme. The native structure of PC from most sources is an $(\alpha)_4$ tetramer, where the biotin carboxylase (BC), carboxyl transferase (CT), biotin-carboxyl carrier protein (BCCP) and allosteric, or tetramerization, domains are contained on a single polypeptide chain, although it has been shown that some bacterial PCs have an $(\alpha\beta)_4$ structure with the BC domain forming the α subunit and the CT and BCCP domains forming the β subunit. Recently, the structures of PC holoenzymes from *Rhizobium etli* [19] and *Staphylococcus aureus* [20, 21] have been determined. These structures, along with site directed mutagenic studies [19], revealed that the covalently attached biotin moves between the BC domain of one subunit to the CT domain of a neighbouring subunit located on an opposing polypeptide chain [19] thus giving rise to a distinctive form of intersubunit catalysis. Furthermore, acetyl CoA, an allosteric activator of PC from several sources, binds in the allosteric domain [19] and appears to facilitate the interdomain movement of the BCCP and covalently attached biotin. While the activity of most vertebrate PCs is highly regulated by acetyl CoA, PC from some bacterial and fungi sources have no acetyl CoA dependence [1].

Similar to other biotin-dependent carboxylases, including acetyl CoA carboxylase, propionyl CoA carboxylase and methyl malonyl CoA carboxylase, PC catalyses the carboxylation of pyruvate in two distinct steps which occur at discrete active sites (Fig. **1A**). Biotin, which is covalently attached to the ε-NH_2 of a strictly conserved lysine residue located at the C-terminal end of the BCCP, is carboxylated at the N-1 position in the BC domain *via* the ATP-dependent activation of bicarbonate and formation of a putative carboxyphosphate intermediate [22]. Acting as a mobile carboxyl carrier, carboxybiotin is then translocated from the BC domain to a neighbouring CT domain where it is decarboxylated (Fig. **1B**). Prior to carboxylation, the transfer of a proton from pyruvate to biotin, facilitated by a strictly conserved Thr residue, is proposed to aid in the formation the nucleophilic enol-pyruvate [23]. The carboxyl group is then transferred to the nucleophilic substrate, forming oxaloacetate (Fig. **1B**).

A wealth of structural and kinetic data has been reported for PC, contributing greatly to a detailed description of the PC mechanism. The aim of this review is to focus specifically on the inhibition of PC as it relates to both the development of the current mechanistic model of PC activity and the physiological regulation of the enzyme activity (Table **1**).

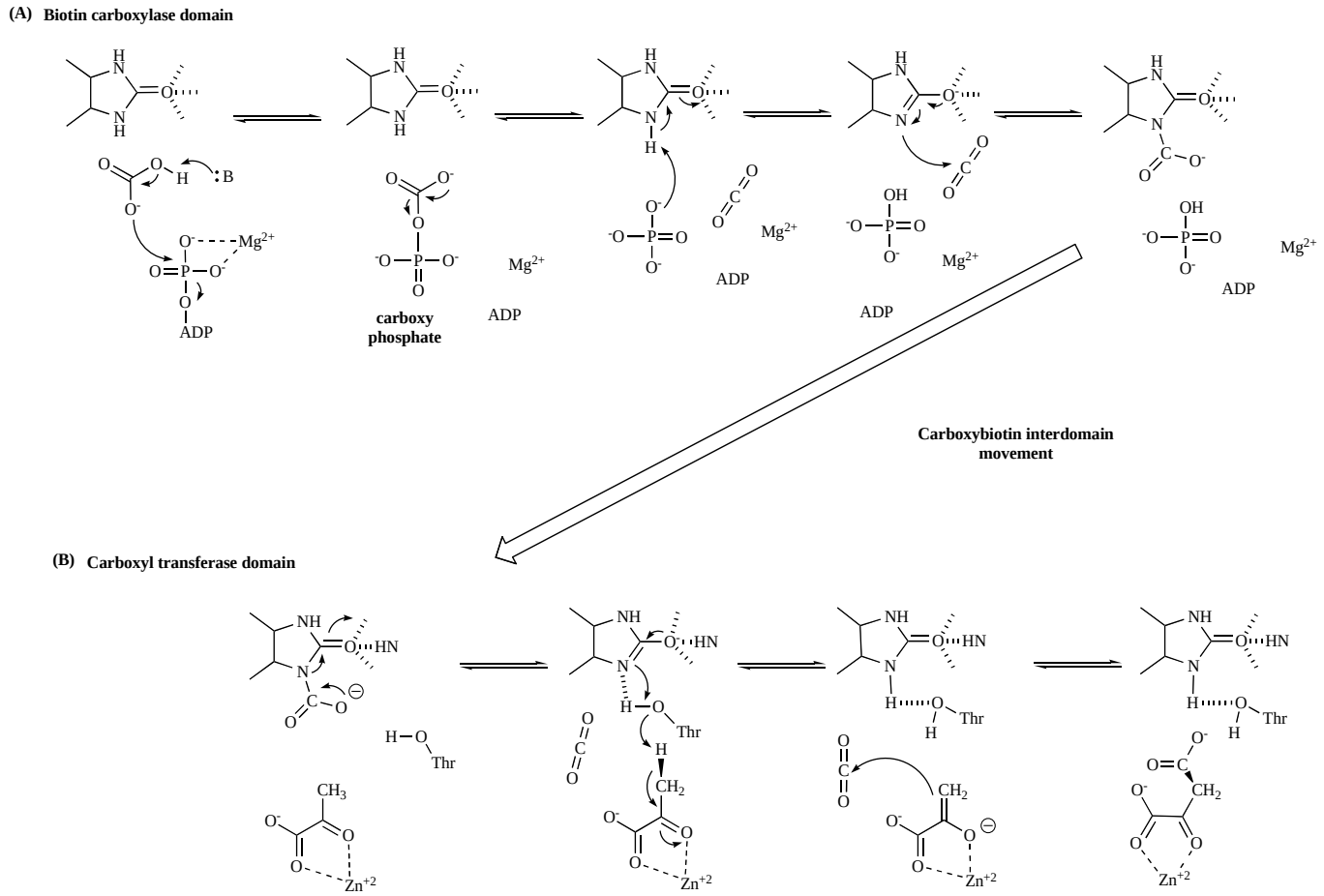


Fig. (1). Overall PC-catalysed reaction. **A)** Biotin carboxylation occurring in the BC domain. **B)** Carboxyl transfer step occurring in the CT domain.

Table 1. Summary of the Different Intermediates and Inhibitors of Pyruvate Carboxylase Activities

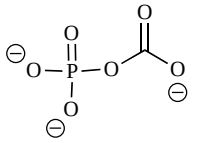
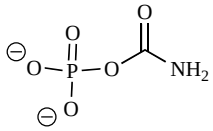
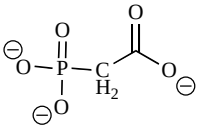
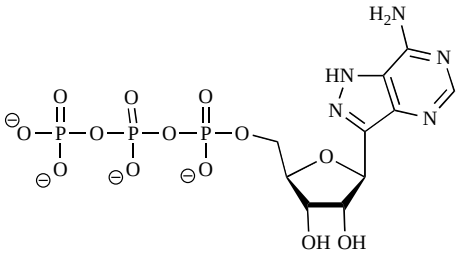
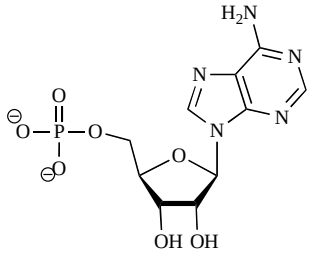
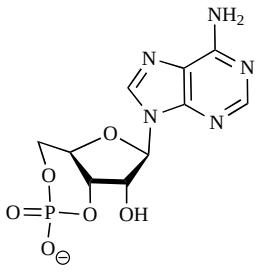
Inhibition of PC through Interactions with Other Proteins			
Name	**Structure**	**Function/Mode of Inhibition**	**References**
Avidin	• Tetrameric protein	• Binds to the biotin covalently attached to the BCCP of PC with high affinity	[31-36]
Prohibitin (Phb1)	• Mitochondrial chaperone protein	• Inhibition of anaplerotic activities of PC • Mechanism of Phb1 inhibition unclear	[48-50]
Inhibition of PC through Interactions with the Biotin Carboxylase (BC) Domain			
Name	**Structure**	**Function/Mode of Inhibition**	**References**
Carboxyphosphate		• Putative intermediate formed during the ATP-dependent carboxylation of biotin by bicarbonate	[19, 22, 23]
Carbamoyl phosphate		• Structural analogue of carboxyphosphate • Substrate for ADP phosphorylation reaction	[23, 38, 65]
Phosphonoacetate		• Competitive inhibitor with respect to ATP • Inhibition results from the increased strength of the C-CH_2-P bond	[58, 59]
Formycin A-5'-triphosphate (FTP)		• Fluorescent analogue of ATP used in structure-function studies • Competitive inhibitor with respect to ATP for chicken liver PC • Can act as substrate for PC from some sources albeit at a greatly reduced rate	[64-66]
5'-adenosine mono-phosphate (5'-AMP)		• Competitive inhibitor with respect to ATP • Less effective inhibitor than adenosine possibly due to active site interactions with negatively charged phosphoryl group	[33, 62]
3', 5'-cyclic adenosine monophosphate (3', 5'- cyclic AMP)		• Competitive inhibitor with respect to ATP • Similar to 5'-AMP, number of phosphoryl groups has greater effect on inhibition/nucleotide binding than positioning of α-phosphate	[33, 62]
Nucleotides and nucleosides (GTP, UTP, CTP, TTP, ITP)		• See Figure 4 for complete structures • Competitive inhibitors with respect to ATP	[33, 62]

Table 1. contd….

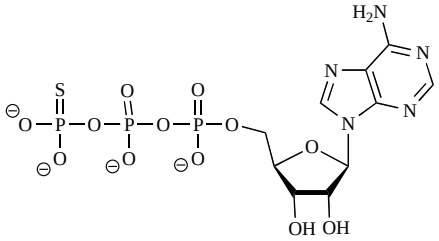
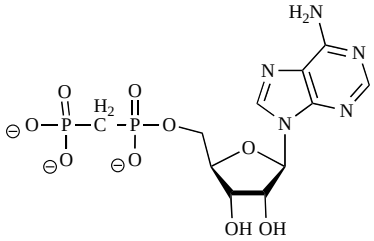
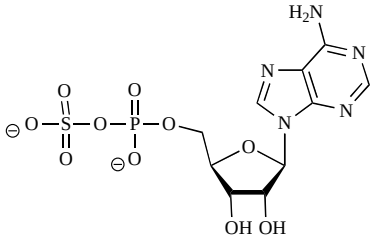
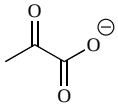
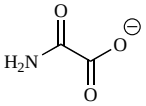
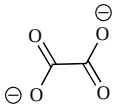
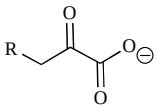
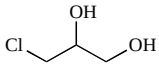
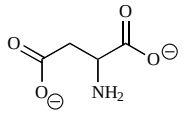
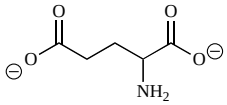
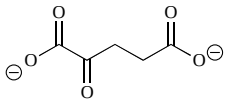
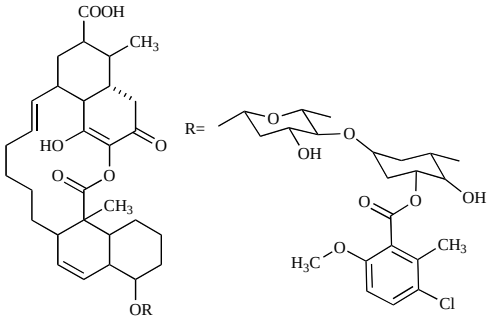
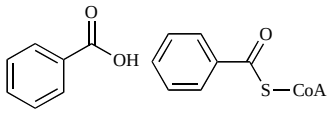
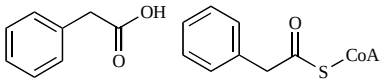
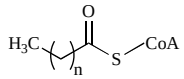
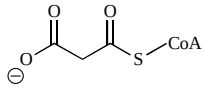
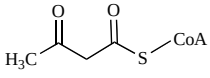
Inhibition of PC through Interactions with the Biotin Carboxylase (BC) Domain			
Name	**Structure**	**Function/Mode of Inhibition**	**References**
ATP-γ-S		• Non-hydrolysable analogue of ATP • Used extensively in structure-function studies	[19]
α,β-methylene diphosphate (Ap(CH$_2$)p)		• Analogue of ADP, competitive inhibitor with respect to ATP • Enzyme has higher affinity for analogue as compared to ADP • Inhibition similar to ATP analogues	[58, 62]
adenosine 5'-phosphosulfate (APS)		• Analogue of ADP, competitive inhibitor with respect to ATP • Not phosphorylated in reverse reaction of BC domain	[58, 62]
Inhibition of PC through Interactions with the Carboxyl Transferase (CT) Domain			
Name	**Structure**	**Function/Mode of Inhibition**	**References**
Pyruvate		• Substrate for the carboxyl transfer reaction • Enolisation of pyruvate promotes carboxylation	[19, 22, 23]
Oxamate		• Structural analogue of pyruvate • Coordinates to Mn^{2+} or Zn^{2+} metal center in the • CT domain active site • Physiologically inhibits gluconeogensis	[35, 69-71]
Oxalate		• Strong metal chealator • Binds to metal center similar to oxamate • Physiological inhibition of anaplerosis	[35, 62, 74-80]
Pyruvate derivatives		• R = -F, -Cl, -Ph, -OH • Competitive with respect to pyruvate • Some derivatives can act as carboxyl acceptors	[35, 81-84]
3-chloro-1,2-propanediol (CPD)		• Carcinogenic choloropropanol • Inhibits gluconeogenesis through direct inhibition of PC	[82]
Univalent and divalent cations		• Replaces active metals in both the CT and BC domains • Physiological regulation of PC activity through-feedback inhibition	[69, 85-89]

Table 1. contd….

Inhibition of PC through Interactions with the Allosteric Domain			
Name	**Structure**	**Function/Mode of Inhibition**	**References**
L-aspartate		• Allosteric inhibitor of microbial and fungal PC • Physiological regulatory feedback inhibition in response to TCA intermediates • Competitive inhibitor with respect to acetyl CoA	[90-109]
L-glutamate		• Allosteric inhibitor of PC from vertebrates • Non-competitive inhibitor with respect to acetyl CoA and competitive with respect to pyruvate • Similar physiological function as aspartate	[90, 110-111]
α-ketoglutarate		• PC from various sources inhibited • Physiological ratios of glutamate, acetyl CoA and α-ketoglutarate to regulate mitochondrial activities	[90, 93, 105, 112-115]
chlorothricin		• Macrolide-type antibiotic • Inhibits PC activity through antagonistic interactions with acetyl CoA • No inhibition observed in the absence of acetyl CoA	[116-122]
benzoate/ benzoyl CoA		• Conversion of benzoate to benzoyl CoA reduces availability of acetyl CoA	[113, 123, 124]
phenylacetate		• Conversion of phenylacetate to phenylacetyl CoA reduces acetyl CoA • Phenyl acetyl CoA directly inhibits PC activity	[8, 93, 100, 125-127]
acyl CoA derivatives		• Inhibition occurs when n = 4-10 in some sources	[110]
methylmalonyl CoA		• Strong competitive inhibitor with respect to acetyl CoA in chicken and rat liver PC	[36, 110]
acetoacetyl CoA		• Binds at a unique allosteric site • Noncompetitive inhibition with respect to all substrates and acetyl CoA	[115, 129]

While most of the inhibitors discussed herein have been used to probe various aspects of PC structure and function, the physiological relevance of several of these inhibitors have also been examined in hopes of further understanding PC's role in the pathogenesis of Type II diabetes.

INHIBITION THROUGH INTERACTION WITH OTHER PROTEINS

Inhibition by Avidin

Avidin, a protein abundant in egg whites, binds biotin with a K_D on the order of 10^{-15} M [24]; therefore, egg white-rich diets have been used to induce experimental biotin deficiencies in animals [25]. A tetrameric protein (MW 63 kDa), avidin is approximately cuboid in shape and contains one biotin-binding site per subunit, with the biotin-binding sites arranged in pairs on opposite faces of the cuboid structure [26, 27] (Fig. **2**). The potential use of avidin for the purification of biotinylated enzymes was quickly recognized; however, the affinity of avidin for biotin was so high that elution of the enzymes was problematic. By dissociating avidin into its constituent monomers before immobilizing them on CNBR-Sepharose, thereby effectively reducing the affinity

of avidin for biotin, avidin could be used for the selective isolation and purification of biotin-containing enzymes [28-30].

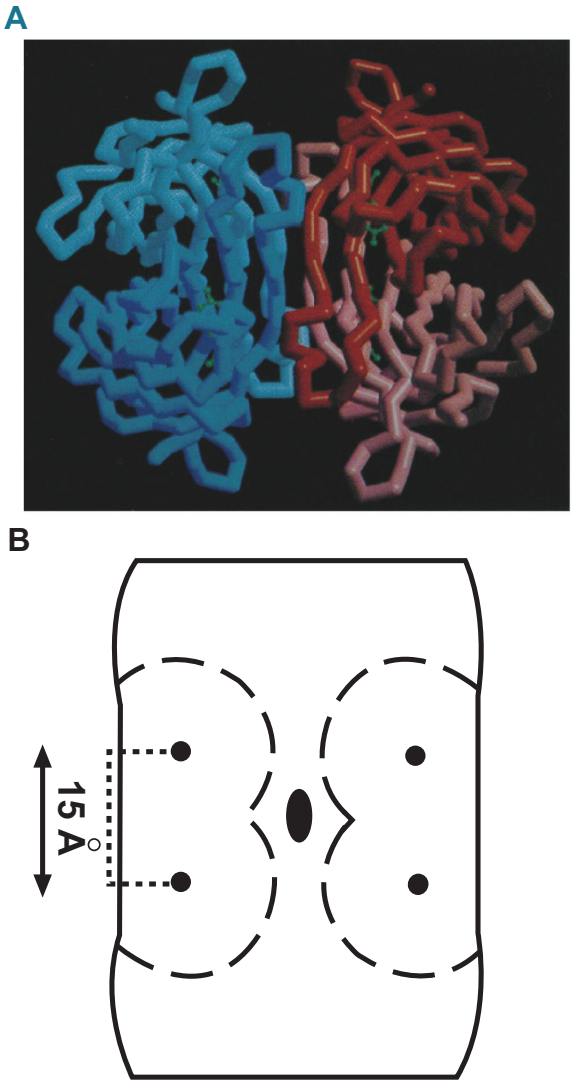


Fig. (2)(A). Model of the quaternary structure of tetrameric avidin, with one biotin molecule (in green) bound to each monomer. The polypeptide backbones of the four monomers are each coloured differently. Reproduced from [27] **B)** Diagramatic representation of the structure of tetrameric avidin, showing the arrangement of the biotin-binding sites in pairs on opposite sides of the cuboid structure. The pair of biotin-binding sites on the opposite 55 Å x 55 Å faces of the molecule are shown as overlapping dotted circles. Reproduced with permission from Green, N.M., Konieczny, L., Toms, E.J. and Valentine, R.C. (1971) Biochem J. 125: 781-791 [26] and the Biochemical Society (http://www.biochemj.org).

The extremely high affinity of avidin for biotin also makes it a potent inhibitor of biotin-dependent enzymes. The first report of inhibition of PC by avidin dates back to 1960 [31] and, subsequently, Scrutton and co-workers used avidin as a probe of the enzymatic mechanism [32, 33]. In these experiments, incubation of the enzyme with avidin was shown to completely inhibit both [^{14}C]pyruvate:oxaloacetate and $^{32}P_i$:ATP isotopic exchange reactions [32], demonstrating the direct participation of biotin in these reactions. Avidin only inhibits the [^{14}C]ADP:ATP isotope exchange by about 10%, indicating that biotin is not directly involved in this reaction [33].

Assuming the avidin-inhibition of PC to be irreversible, Scrutton and Utter [33] also performed the first reported kinetic investigation of the inhibition. Under pseudo-first order conditions, analysis of the reaction order gave a stoichiometry of avidin: PC binding of 1.4, indicating that approximately one avidin molecule binds per enzymic PC tetramer [33]. In an extension of these early kinetic studies, Duggleby *et al.* [34] examined the kinetics of avidin-PC inhibition using methodologies developed for the study of slow, tight-binding inhibitors. Consequently, PC was added to reaction mixtures containing fixed, saturating concentrations of the substrates for pyruvate carboxylation, acetyl CoA and varying concentrations of avidin. By using the coupling enzyme malate dehydrogenase and monitoring the reduction of NADH [34], the development of the inhibition of the reaction was directly determined from the decrease in the initial rates over the duration of the assay. The mode of inhibition of PC by avidin could be sufficiently explained using a scheme where the slow development of the inhibition was due to the binding of avidin to PC, rather than the enzyme undergoing a slow conformational change [34]. The second order rate constant for avidin binding was determined to be 1.49×10^5 $M^{-1}s^{-1}$, which is several orders of magnitude lower than the calculated diffusion-controled constant of 1.7×10^8 $M^{-1}s^{-1}$ and about 500 times lower than that for the binding of free biotin to avidin [24], suggesting that strong steric factors govern the rate of avidin binding to the covalently attached biotin in PC. In the structures of *Staphylococcus aureus* PC where the position of biotin has been determined [20, 21], it is bound either in the active site of the CT domain or in a pocket outside of the CT domain. Its other possible position is in the active site of the BC domain. In any case, the biotin does not appear to be easily accessible to the binding sites of avidin.

The inhibition of PC by avidin was shown to be at least partially reversible, as determined by the ability to reactivate the enzyme initially incubated with a 2-5 fold molar excess of avidin through the addition of 3000-5000 fold molar excess of free biotin [34]. Owing to the very slow nature of the reactivation process, the time course of these experiments was 16 hours during which there was an approximately 75% loss of enzymic activity in control solutions of PC that did not contain avidin. However, this loss of activity did not account for the small degree of reactivation that was observed in the experimental sample and, thus, it was proposed that PC in complex with avidin also underwent an inactivation reaction [34]. Based on kinetic analysis of the reactivation of the PC-avidin complex in the presence of free biotin, the reactivation occurred at a rate ($6\text{-}10 \times 10^{-5}$ s^{-1}) that was about 4-8 fold greater than that for the inactivation of the free enzyme (7.6×10^{-6} s^{-1}) and for the dissociation of the avidin from PC (1.42×10^{-5} s^{-1}) [34]. This dissociation rate constant of avidin from the tethered biotin is about 160 times greater

than that for the dissociation of avidin and free biotin [24]. Thus the dissociation constant for the avidin-PC complex is 10^{-10} M, which is five orders of magnitude greater than that determined for the avidin-biotin complex. One of several factors contributing to the lower affinity of the biotinylated enzyme for avidin, as compared to free biotin, may be the covalent attachment of biotin to the side-chain of a conserved lysine residue in the BCCP domain of PC *via* the formation of an amide bond with the carboxyl group of biotin. In free biotin, the carboxyl oxygen forms two hydrogen bonds with residues in the biotin-binding site of avidin [26], which could partially account for some of the decreased affinity. Even so, the large steric effect introduced by the attachment of the biotin to the enzyme, which subsequently reduces the ability of avidin to bind to the covalently attached biotin as tightly as free biotin, is most likely responsible for increasing the overall dissociation constant determined for the PC-avidin complex.

In addition to measuring the simple kinetics of avidin-inhibition of PC, Scrutton and co-workers also examined the effect various nucleotides had on the inhibition. It was found that saturating concentrations of not only ATP, but also ADP, 3'-AMP and 5'-AMP completely protected PC from avidin inhibition [33]. The protective effect of these latter two nucleotides, which do not promote biotin carboxylation, strongly suggests that nucleotide binding to PC is liable to reduce the accessibility of the tethered biotin to avidin by inducing conformational changes which place biotin in the BC domain. On the other hand, substrates and products of the CT domain active site enhance the rate of avidin inhibition of PC by approximately 33% and 78% with saturating pyruvate and oxaloacetate, respectively [35], indicating the possibility that the binding of these molecules to PC increases the exposure of biotin to avidin by inducing the movement of the tethered biotin from the BC domain to the CT domain.

The effect of the allosteric activator, acetyl coenzyme A (acetyl CoA), on the avidin inhibition of PC is more complicated. The rate of PC inactivation due to avidin- inhibition is increased approximately 8-fold when acetyl CoA concentrations were kept between 10 and 200 μM, but at concentrations of above 200 μM, the rate of inactivation decreased [36]. It is interesting to note that the acetyl CoA concentrations which resulted in an increased rate of PC inactivation by avidin correspond to the same concentrations where acetyl CoA is most potent as an activator [36]. These results further support the previous conjecture that a conformational change in the PC structure, induced by the binding of the allosteric activator and CT domain substrates, increases the exposure of the tethered biotin to avidin. At higher concentrations of acetyl CoA, there is a counteracting conformational change, possibly due to the non-productive, partial occupation of the nucleotide binding site [36], which imparts a protective effect against avidin-inhibition, similar to that observed in the presence of the various nucleotides. These early avidin- inhibition studies were the first indication that the movement of the BCCP and tethered biotin is dependent on the presence of substrates and activators. Later kinetic studies [23, 37, 38] and crystal structures [19, 20] confirmed that the presence of BC and CT domain substrates and analogues dictate the location of the tethered biotin.

The stoichiometry of the avidin-PC binding was determined to be 1 mol /mol from both the kinetic measurements of Scrutton and Utter [33] and titration experiments which measured the displacement of 2-(4'-hydroxyazobenzene) benzoic acid from the biotin binding sites in avidin by PC [34]. Electron microscopy studies of PC revealed that the enzyme subunits were arranged such that there appeared to be an active site cleft running along the long axes with the four subunits arranged in a tetrahedron-like configuration, as shown in Fig. (**3A**) [39]. It was difficult to envisage how one tetrameric avidin molecule could bind the four biotins of one tetrameric PC molecule, leading to the conclusion that hetero-oligomers of avidin and PC may form. When Johannssen *et al.* [40] examined the complexes of chicken liver PC and avidin formed from varying ratios of the two molecules under the electron microscope, they observed the formation of chain-like hetero-oligomers when the avidin: PC ratio was between 2:1 and 1:2 in the presence of acetyl CoA (Fig. **3B**). Rohde *et al.* [41] obtained similar results using yeast PC. A proposed model of these hetero-oligomers indicated that avidin may act as a "glue," thereby joining two PC tetramers, with the biotin-binding sites on one face of the avidin tetramer binding two biotins from one PC molecule and the binding sites on the opposite face of the avidin molecule binding two biotins from another PC tetramer (Fig. **3C**) [40]. When the avidin:PC ratio was 9:1, PC-avidin complexes which contained two avidin tetramers for every PC tetramer were observed to be bound in a similar arrangement to those previously described (Figs. **3D** and **E**) [40]. X-ray crystal structures of the PC holoenzyme from a variety of sources have confirmed the arrangement of pairs of biotins on opposite faces of the PC tetramers [19-21].

Attwood *et al.* [42] showed that when PC was incubated with avidin in a 1:1 ratio in the absence of acetyl CoA, only a small number of shorter hetero-oligomers and few intact PC tetramers were formed. In addition, the complexation of PC with avidin was shown to stabilise the tetrameric structure of the enzyme, protecting it against dilution- dissociation [43]. This stabilization was further enhanced by the presence of acetyl CoA, suggesting that the binding of the activator induces PC to adopt a conformation that not only facilitates the binding of avidin, thereby forming the hetero-oligomers, but also aids in the stabilization of the PC tetramer. The proposed conformational and catalytic effects of acetyl CoA binding to PC have been recently demonstrated in the X-ray crystallographic structure determined for the PC holoenzyme from *Rhizobium etli*, which is activated in the presence of acetyl CoA [19]. The *R. etli* PC structure suggests the possibility that the binding of the allosteric activator promotes critical, global domain rearrangements which facilitate inter-subunit catalysis *via* the movement of the tethered biotin from the BC domain of one subunit to the CT domain of the neighbouring subunit, [19]. Subunits without acetyl CoA bound in the allosteric domain appear to adopt a different conformation such that the inter-subunit catalysis would be difficult [19]. These large conformational changes induced by acetyl CoA binding could, in part, explain the effect that acetyl CoA has on the formation of the PC-avidin hetero-oligomers. Interestingly, high concentrations of pyruvate were also determined to have a similar effect on hetero-oligomer formation [42], although no structure of PC has yet been obtained at these concentrations of pyruvate.

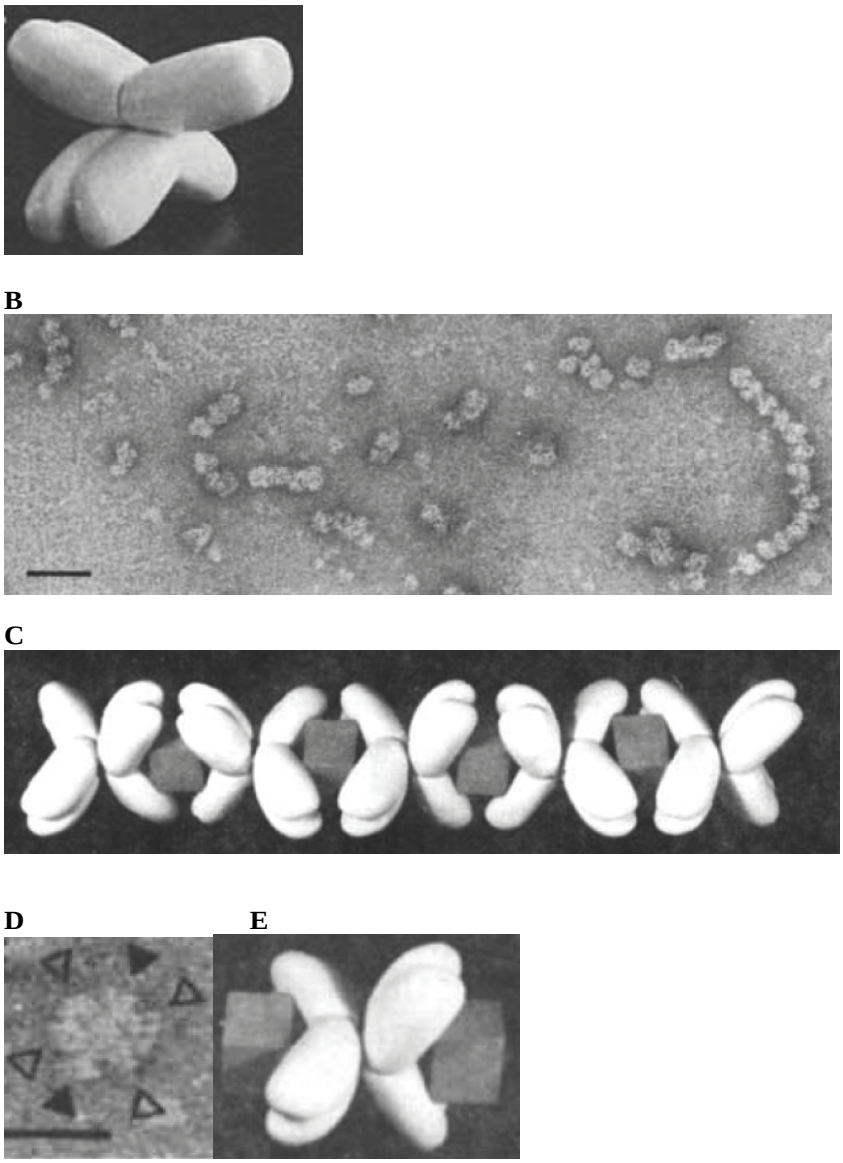


Fig. (3)(A). Model of the tetrahedron-like arrangement of the subunits of PC derived from electron microscopic studies of the enzyme. Reproduced with permission from Mayer, F., Wallace, J.C., Keech, D.B. Further electron microscope studies on pyruvate carboxylase. Eur. J. Biochem. 1980; 112: 265-272 [39] (Wiley-Blackwell). **B)** Electron micrograph of hetro-oligomers formed between chicken liver PC and avidin at a ratio of avidin:PC of 1:2, in the presence of 0.25 mM acetyl CoA. The bar represents 40 nm. Reproduced with permission from Johannssen, W., Attwood, P.V., Wallace, J.C., Keech, D.B. Localization of the active site of pyruvate carboxylase by electron microscopic examination of avidin-enzyme complexes. Eur. J. Biochem. 1983; 133: 201-206 [40] (Wiley-Blackwell). **C)** Model of a hetero-oligomer of avidin and PC, with the PC subunits shown in white and the avidin tetramers shown as grey cuboids, linking two PC tetramers together. Johannssen, W., Attwood, P.V., Wallace, J.C., Keech, D.B. Localisation of the active site of pyruvate carboxylase by electron microscopic examination of avidin-enzyme complexes. Eur. J. Biochem. 1983; 133: 201-206 [40] (Wiley-Blackwell). **D)** Electron micrograph of complex between a chicken liver PC tetramer and two avidin molecules, at a ratio of avidin:PC of 9:1. The avidin molecules are indicated by filled triangles and the PC subunits by open triangles. The bar represents 16 nm. Johannssen, W., Attwood, P.V., Wallace, J.C., Keech, D.B.. Localisation of the active site of pyruvate carboxylase by electron microscopic examination of avidin-enzyme complexes. Eur. J. Biochem. 1983; 133: 201-206 [40] (Wiley-Blackwell). **E)** Model of the complex between a PC tetramer and two avidin molecules with the PC subunits shown in white and the avidin tetramers shown as grey cuboids. Johannssen, W., Attwood, P.V., Wallace, J.C., Keech, D.B.. Localization of the active site of pyruvate carboxylase by electron microscopic examination of avidin-enzyme complexes. Eur. J. Biochem. 1983; 133: 201-206 [40] (Wiley-Blackwell).

Avidin has proven to be an extremely useful tool for the examination of the biotin-dependence of the PC mechanism. It has permitted the investigation of tethered biotin movement induced by the binding of substrates and inhibitors and has suggested a possible structural role for the activator in the stabilization of the quaternary enzyme structure. In addition, the avidin-inhibition studies of PC enabled the approximate determination of structural aspects of the tetrameric enzyme, including the positioning of the biotins on the subunits, which was especially important prior to X-ray crystal structure determination.

Inhibition by Prohibitin

The prohibitins, Phb1 and Phb2, belong to a family of highly conserved membrane-associated proteins which had initially been identified as having putative regulatory roles in eukaryotic cell proliferation, apoptosis, tumor suppression and transcriptional activities [44, 45]. Furthermore, the localization and abundance of Phb1 at the inner mitochondrial membrane [46] suggests its importance as a membrane-bound chaperone protein which aids in stabilizing newly synthesized or imported mitochondrial proteins [47]. Sequence homology with other chaperone proteins, such as HSP60, and computational predictions suggest the N-terminal, transmembrane-spanning helix in Phb1 may anchor the protein to the inner-mitochondrial membrane [48]. Within the mitochondria, Phb1 and Phb2 form high-molecular weight, ring-shaped Phb1/Phb2 complexes which further facilitate their function as chaperone proteins [45].

Phb1 and PC were identified as two of several mitochondrial proteins isolated from lipolytically stimulated 3T3-L1 adipocyte lipid droplets [49], demonstrating that adipocytes contain lipid metabolic enzymes and proteins. *In vivo* and *in vitro* studies using mice adipocytes revealed that Phb1 is a potent, specific inhibitor of PC and, through this inhibition, attenuates both fatty acid and insulin-stimulated glucose oxidation [50]. The insulin-stimulated release of $^{14}CO_2$ from uniformly labeled ^{14}C-glucose was markedly inhibited in the presence of 2 nM prohibitin and a K_i of approximately 4 nM for Phb1 inhibition of PC *in vivo* was determined. Inhibition of fatty acid oxidation in the presence of Phb1 in the mouse adipocytes was most likely due to the lack of available oxaloacetate resulting from the almost complete inhibition of PC. By monitoring the incorporation of $NaH^{14}CO_3$ into oxaloacetate, Vessal and co-workers further established that Phb1 potently inhibited ($K_i \approx 5$ nM) PC isolated from the adipocyte lysate [50]. While the mechanism of PC inhibition by Phb1 is not clearly understood, both *in vivo* and *in vitro* studies indicate that Phb1 that has been translocated into the mitochondria effectively inhibits anaplerosis through interactions with PC. Whether this inhibition has any physiological relevance, or if Phb2 and the Phb1/Phb2 complex exhibit the same inhibitory effects on PC remains uncertain [48].

INHIBITION OF THE BIOTIN CARBOXYLASE DOMAIN

The reaction catalysed in the BC domain, namely the ATP-dependent carboxylation of biotin by bicarbonate, is common to biotin-dependent carboxylases, with the exception of transcarboxylase [22]. Recent studies have focused on the inhibition of the biotin carboxylase subunit of acetyl CoA carboxylase from *E. coli*, which exists as a homodimer in solution and utilizes free biotin as a substrate [51]. Reaction-based intermediate analogues [52, 53] and small molecules, determined from a fragment-based screening approach [54-56], have been shown to successfully inhibit *E. coli* biotin carboxylase. Since many of the examples of biotin carboxylase inhibition have been extensively discussed in a previous review [57], only those studies which involve the inhibition of the biotin carboxylase domain in the context of the PC holoenzyme will be discussed here.

Intermediate and Substrate Analogue Inhibitors

Ashman and Keech [58] used analogues of the putative carboxyphosphate intermediate to probe the reaction mechanism of the BC domain in PC. In steady-state kinetic experiments, phosphonoacetic acid exhibited noncompetitive inhibition with respect to MgATP ($K_i = 0.5$ mM) when the initial rates of oxaloacetate formation were determined using PC from sheep kidney. Similarly, phosphonoacetate was a competitive inhibitor with respect to MgATP ($K_i = 7.8 \pm 1.8$ mM) and a noncompetitive inhibitor with respect to HCO_3^- for the biotin carboxylation reaction catalysed by *E. coli* biotin carboxylase [59]. It was proposed that the inhibition was due, in part, to the replacement of the highly labile C-O-P bond in carboxyphosphate with a stable C-CH_2-P bond. Since phosphonoacetic acid is an isosteric and isoelectronic analogue of carboxyphosphate, these inhibition studies lent further support to the proposed mechanism whereby biotin carboxylation occurs *via* the reversible formation of the unstable, mixed-anhydride intermediate.

Carbamoyl phosphate, a structural analogue of carboxyphosphate, will directly phosphorylate ADP in reactions catalyzed by acetyl CoA carboxylase from *E. coli* [60] and PC [58], allowing for the examination of the partial reverse reaction of the BC domain without complications due to biotin carboxylation. Inhibition of the PC-catalyzed phosphorylation of ADP with carbamoyl phosphate [38] and the bicarbonate-dependent ATP cleavage [61] was observed in the presence of oxamate, a substrate analogue of pyruvate (see inhibition of the CT domain reactions). Even though oxamate has been shown to bind in the CT domain, a hyperbolic, non-competitive inhibition with respect to carbamoyl phosphate was observed in those reactions catalyzed by PC from chicken liver [38]. Correspondingly, the addition of 20 mM of oxamate resulted in a 98% reduction in the rate of the PC-catalyzed bicarbonate-dependent ATP cleavage [61]. The non-competitive nature of the oxamate inhibition of the BC domain reactions further confirmed initial avidin inhibition studies [34] and later kinetic studies by Goodall *et al.* [37], which suggested that the binding of oxamate in the CT domain active site induces the translocation of biotin from the BC domain to the CT domain. While not directly involved, the presence of free biotin has been shown to have a stimulatory effect on both the ADP phosphorylation and bicarbonate-dependent ATP cleavage reactions. Consequently, the oxamate-induced removal of the tethered biotin from the BC domain explains the apparent inhibition by oxamate.

Nucleoside and Nucleotide Inhibitors

While MgATP is the physiological nucleotide substrate for the pyruvate carboxylation reaction of PC (K_m of the order of 40-60 μM), MgdATP is also an effective substrate, with a K_m of approximately 70 μM [33, 62]. MgGTP, MgUTP, MgCTP, MgTTP and MgITP (Fig. **4**) have all been reported to act as competitive inhibitors of pyruvate carboxylation with respect to MgATP, giving K_i values in the range of 0.7-0.9 mM [62]. However, Scrutton and Utter [33] reported them to be non-competitive inhibitors of the [^{32}P]ATP:ADP isotope exchange reactions with chicken liver PC. As McClure *et al.* [62] pointed out, the non-competitive nature of the inhibition may have been due to the fact that free nucleotides, rather than those complexed with Mg^{2+},

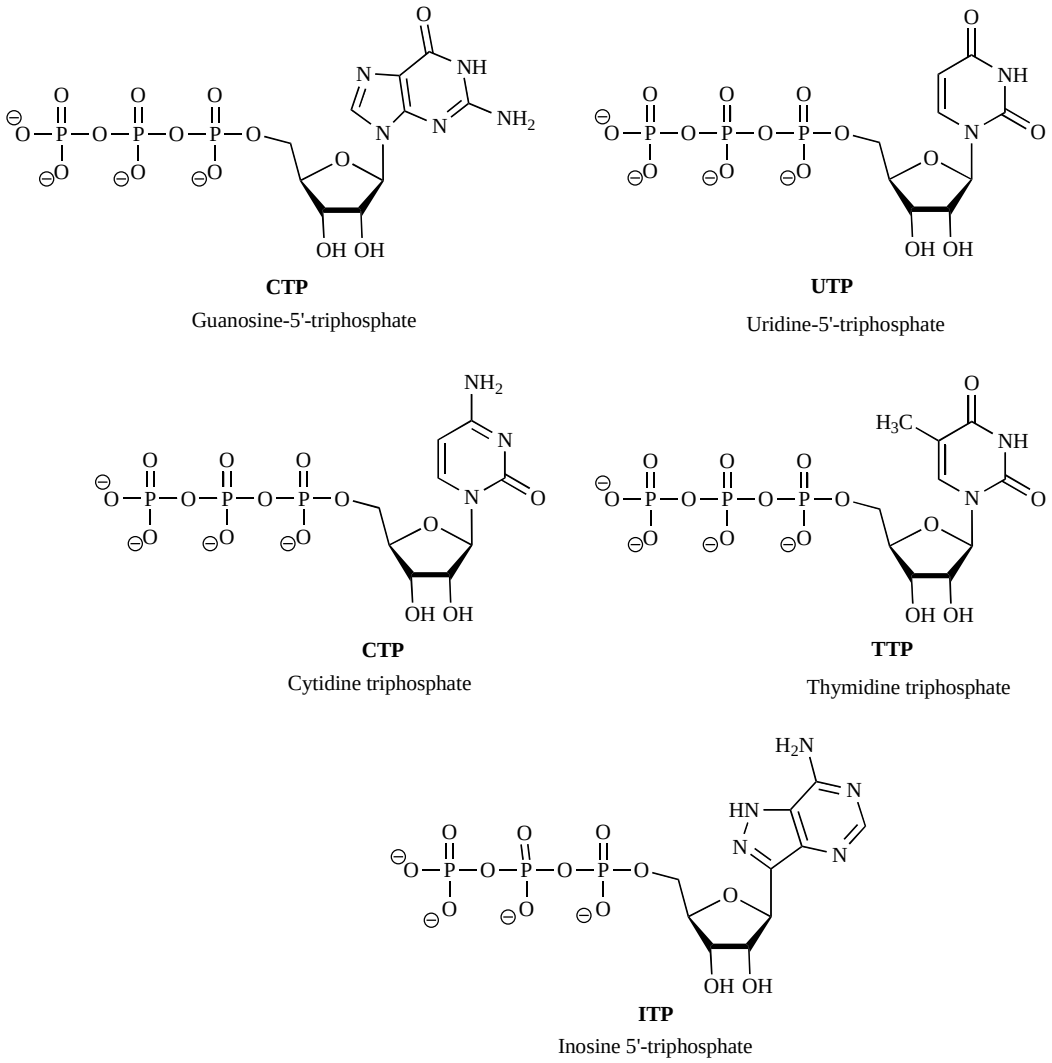


Fig. (4). Structures of the various nucleotide and nucleoside inhibitors of PC.

were used in the inhibition studies. Interestingly, the K_i values reported by Scrutton and Utter [33] are approximately an order of magnitude lower than those reported by McClure *et al.* [62], suggesting that un-complexed nucleotides may bind with a higher affinity than the MgNTP complexes.

Both 5'-AMP and 3', 5'-cyclic AMP have been reported to be competitive inhibitors with respect to MgATP [62] with PC isolated from rat liver (K_i = 1.3 mM and 1.0 mM, respectively). Based on these steady-state kinetic studies, it was proposed that that the reduction in the number of phosphoryl groups, as compared to ATP, had a greater effect on the nucleotide binding affinity than the positioning of the α-phosphate did. In light of these results, it is somewhat surprising that adenosine is an extremely potent competitive inhibitor with respect to MgATP [62, 63] (K_i = 0.08-0.09 mM). In the *R. etli* PC holoenzyme structure with ATP-γ-S bound in the BC domain, there are seven potential hydrogen-bonding interactions between the adenosine portion of ATP-γ-S and residues in the active site (Fig. **5**) [19]. These observed interactions, combined with the adenosine inhibition kinetics, support the proposal that a significant amount of the nucleotide binding energy in the BC domain comes from interactions between the active site residues and the adenosine moiety of MgATP. Therefore, it can be argued that the negatively charged phosphoryl group of 5'-AMP or 3'-AMP introduces unfavorable electrostatic interactions within the active site that result in the decreased affinity of PC for these molecules as compared to adenosine. In another study, 5'-AMP (K_i = 9.3 mM) and 3'-AMP (K_i = 13 mM) were both found to be non-competitive inhibitors of chicken liver PC [33], suggesting that in this case the nucleotides were binding with low affinity to a site outside the normal substrate binding site, possibly part of the acetyl CoA binding site.

Analogues of ATP, such as ATP-γ-S which cannot act as a substrate, have been used in structure-function studies of PC [19]. The Mg^{2+}-complex of the fluorescent ATP analogue, formycin A-5'-triphosphate (MgFTP), is a competitive inhibitor of chicken liver PC with respect to MgATP (K_i = 50 μM) [64]. However, sheep liver PC can utilize MgFTP as a substrate for pyruvate carboxylation, albeit with a k_{cat} that is one-twentieth of that with MgATP as a substrate and a K_m one-third of that for MgATP [64]. Since binding to PC enhances the fluorescence of MgFTP approximately 2-fold, FTP and FDP have been extensively used to study the pre-steady state kinetics of nucleotide binding to PC in stopped-flow experiments [65, 66]. The second-order binding rate

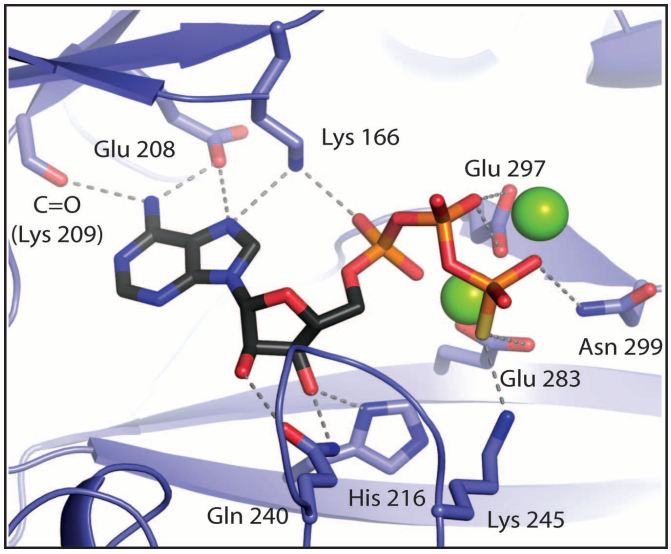


Fig. (5). Model of part of the active site of the CT domain of Rhizobium *etli* PC showing the potential binding interactions between ATP-γ-S and residues in the active site. Reproduced with from [19].

constant of MgFTP was determined to be 2.6×10^6 M^{-1} s^{-1} and the dissociation rate constant was 82 s^{-1} in experiments using chicken liver PC [66]. The fluorescence of PC-bound MgFTP has also been used to measure the effects of other substrates and activators on nucleotide binding [66].

Analogues of ADP have also been used to probe the mechanism of the BC domain. Ashman and Keech [58] found that α, β-methylene adenosine diphosphate ($Ap(CH_2)p$) and adenosine 5'-phosphosulfate (APS) were competitive inhibitors for pyruvate carboxylation with respect to MgATP in reactions catalysed by sheep kidney PC. The K_i (0.58 mM) determined for $Ap(CH_2)p$ was nearly three times lower than that of MgADP, indicating that the enzyme had a high affinity for this analogue. While APS also exhibited competitive inhibition with respect to MgATP, the K_i value of 3 mM signified the enzyme had a much lower affinity for the analogue. Additionally, it was determined that PC would not catalyse the phosphorylation of these analogues in the full reverse reaction. Similar to conclusions drawn by McClure [62] these data strongly suggest that substitution at the α, β position of ADP is less critical for catalysis than substitutions at the position of bond cleavage/formation.

INHIBITION OF THE CARBOXYL TRANSFERASE DOMAIN

In the CT domain, carboxybiotin is decarboxylated and a strictly conserved Thr residue is proposed to facilitate the transfer of a proton from pyruvate to biotin, thereby forming the highly nucleophilic enol-pyruvate [23]. Pyruvate analogues and several dicarboxylic acids and their derivatives have been shown to be specific, reversible inhibitors of the carboxyl transfer step, usually through interactions with the Mn^{2+}- or Zn^{2+}-metal centre contained in the CT domain active site.

Oxamate

A potent inhibitor of lactate dehydrogenase [67], oxamate is a structural analogue of pyruvate which binds in the CT domain and induces the movement of the tethered biotin from the BC domain to the CT domain [37]. Furthermore, oxamate stimulates the decarboxylation of carboxybiotin, allowing for the examination of the steady-state kinetics of the reverse reaction of the CT domain in isolation from reactions occurring in the BC domain [23, 68].

Using chicken liver PC and monitoring the steady-state kinetics of pyruvate carboxylation with the malate dehydrogenase coupled assay system, Scrutton *et al.* [69] determined that oxamate was a non-competitive inhibitor with respect to pyruvate in the overall pyruvate carboxylation reaction (K_i = 1.6 mM). Previous pulsed NMR experiments examined the effect of oxamate on the relaxation rate of water protons coordinated to the paramagnetic Mn^{2+}-metal centre in the CT domain of PC from chicken liver mitochondria [35]. In this way, the coordination of substrates, inhibitors and products to the paramagnetic metal-center could be determined, allowing for the direct examination of the inhibition kinetics. Based on the decreased enhancement of the water protons relaxation rate, due to their displacement by the substrate analogue inhibitors, oxamate was proposed to chelate to the Mn^{2+}-metal centre in the CT domain. Uncompetitive oxamate inhibition with respect to pyruvate was observed for the pyruvate carboxylation reaction, but the inhibition was competitive when determined as a function of oxaloacetate for the reverse reaction. From the analysis of the proton relaxation data and the effect of oxamate on PC inactivation by avidin, a dissociation constant of K_D = 1.45 mM was determined for the oxamate-enzyme complex. Direct coordination of oxamate to the Mn^{2+}-metal centre, as suggested by the pulsed NMR experiments, would be expected to give competitive inhibition patterns with respect to pyruvate, as oxamate would presumably prevent the coordination and subsequent enolization of pyruvate. The uncompetitive nature of the inhibition could possibly be explained if oxamate forms an enzyme-inhibitor complex where the carboxylation of biotin is kinetically insignificant and pyruvate will only interact appreciably with the enzyme once biotin is carboxylated. Although it is difficult to envision how oxamate prevents the carboxylation of the biotin at low concentrations, the movement and requisition of the tethered biotin to the CT

domain, induced by the coordination of oxamate to Mn^{2+}, may be faster than the partially rate-limiting carboxylation reaction in the BC domain.

To assess the physiological relevance of oxamate inhibition of PC on the rates of hepatic gluconeogenesis, Martin-Requero and co-workers analysed the effect of added oxamate on the gluconeogenic flux and PC activity in perfused rat livers, isolated hepatocyte suspensions [70] and isolated rat liver mitochondria [71]. In these studies, the resulting concentrations of metabolic intermediates were used to determine the effect of oxamate on gluconeogenesis, while the rate of pyruvate carboxylation was approximated from a determination of $H^{14}CO_3^-$ incorporation into the metabolites isolated from mitochondrial extracts. In perfused rat livers and isolated hepatocytes [70], the presence of oxamate resulted in a decrease in the concentrations of all metabolites after pyruvate, indicating that the inhibitory effect on gluconeogenesis was due, in part, to the inhibition of PC. Oxamate was determined to be a competitive inhibitor of gluconeogenesis at concentrations of pyruvate less than 0.4 mM and had little effect on glucose production at concentrations of pyruvate greater than 0.4 mM. These results are somewhat surprising in light of studies performed with the isolated rat liver [62] and chicken liver enzymes (above), both of which showed an uncompetitive oxamate inhibition when pyruvate was the varied substrate. The discrepancy between the type of inhibition exerted by oxamate in the isolated enzymes and in the perfused liver studies suggested that oxamate may be inhibiting not only PC but also another enzyme whose mitochondrial function is directly related to pyruvate utilization in gluconeogenesis.

In an extension of these initial studies, oxamate was determined to exhibit a mixed-type inhibition pattern with respect to pyruvate in isolated mitochondria from rat liver [71]. Similar to the previous study, oxamate inhibited pyruvate carboxylation by approximately 40% at low pyruvate concentrations, but had no discernible effect on the net gluconeogenic flux at concentrations of pyruvate greater than 0.4 mM. Varying HCO_3^- and determining the rates of pyruvate carboxylation in the presence of oxamate resulted in a non-competitive inhibition pattern. The lack of substantial inhibition at high concentrations of pyruvate and competitive inhibition with respect to pyruvate at low concentrations strongly suggested that oxamate inhibition of gluconeogenesis in the isolated mitochondria resulted from the non-competitive inhibition of PC and the competitive inhibition of the mitochondrial pyruvate translocator protein, which facilitates pyruvate transport across the mitochondrial membrane [71].

Oxalate

Oxalate, an inhibitor of both lactate dehydrogenase [72] and transcarboxylase [73], was also found to be an effective inhibitor for PC from a variety of sources. For example, oxalate was a non-competitive inhibitor with respect to HCO_3^- (K_i = 60-300 μM [62]) in rat liver PC and a non-competitive inhibitor with respect to pyruvate (K_i = 70 μM) for PC isolated from yeast [74] as well as PC from rat liver (K_i = 50-130 μM [62]) and chicken liver (K_i = 12 μM) [69]. Barden and co-workers [75] extended the initial kinetic studies of oxalate inhibition of chicken liver PC. Steady-state kinetic analysis determined that, while the inhibition was uncompetitive with respect to pyruvate for the pyruvate carboxylation reaction, oxalate inhibition was competitive with respect to oxaloacetate for the decarboxylation reaction, confirming results previously obtained from pulsed NMR experiments [35]. Avidin inactivation studies in the presence of oxalate [35] provided a dissociation constant of 8.9 μM for the oxalate-PC complex, nearly three orders of magnitude lower than that determined for oxamate, indicating that the bidentate chelation of oxalate to the Mn^{2+}-metal centre most likely increases the affinity of the enzyme for the inhibitor. McClure [62] probed the effect that the inhibition of the CT domain with oxalate had on the affinity for substrates in the BC domain by studying the steady-state kinetics of the pyruvate carboxylase reaction catalyzed by PC from rat liver. As expected, oxalate was also found to be a non-competitive inhibitor with respect to MgATP.

Similar to oxamate, oxalate also inhibits gluconeogenesis in isolated mitochondria and hepatocytes. Dennis *et al.* [76] determined that oxalate had no inhibitory effect on pyruvate transportation into the mitochondria; therefore the inhibition of glucose production was presumably due to the direct inhibition of PC by oxalate. In subsequent studies, both Yount and Harris [77] and Tonon and co-workers [78] found that the infusion of isolated rat hepatocytes with oxalate significantly inhibited hepatic gluconeogenesis from alanine, pyruvate and lactate as determined from the concentrations of the metabolic intermediates. In bicarbonate-deficient media, where PC activity became completely rate-limiting, the inhibition of glucose production by oxalate was even more apparent, further supporting the idea that oxalate was directly inhibiting PC activity [77]. The addition of 100 μM of oxalate reduced the steady-state levels of oxaloacetate by 48% in rat liver cells [79] and physiological concentrations of oxalate (50-100 μM) were found to partially impair the mitochondrial metabolism of pyruvate, resulting in decreased anaplerosis due to the oxalate inhibition of PC [80].

Pyruvate Derivatives

Many of the derivatives of pyruvate, including fluoro and chloropyruvate, not only act as carboxyl acceptors for the PC-catalyzed carboxylation reaction but can also act as effective, specific inhibitors of the carboxyl transfer reaction. Scrutton *et al.* [69] examined the inhibition of the carboxyl transfer reaction using fluoropyruvate (K_i = 0.17 mM) and phenylpyruvate (K_i = 0.48 mM). Both analogues were non-competitive inhibitors with respect to pyruvate when the initial rates of oxaloacetate formation were determined. Pulsed NMR studies [35] suggested that fluoropyruvate inhibited the carboxyl transfer in a mechanism similar to oxamate and oxalate where the direct coordination of the analogue to the cationic-metal centre partially prevented pyruvate coordination and subsequent enolization.

In rat brain mitochondria [81], fluoropyruvate was determined to inhibit anaplerosis by not only the direct inhibition of PC, but also by lowering the available concentration of acetyl CoA in the mitochondria. The addition of 0.5 mM of fluoropyruvate resulted in a 95% inhibition of cerebral PC activity as determined from the initial rate of oxaloacetate formation using $H^{14}CO_3^-$. In order to determine if the potent inhibition of the mitochondrial activity was due solely to the

inhibition of PC, the activities of the isolated enzyme, treated with varying concentrations of fluoropyruvate were determined. The competitive inhibition of the isolated PC by fluoropyruvate was mild; only a 47 % inhibition of PC activity was observed with the addition of 5 mM of fluoropyruvate which accounted for only a fraction of the inhibition observed in the intact cerebral mitochondria. The addition of 10 μM of acetyl CoA to the reactions completely reversed the inhibition of pyruvate carboxylation at low concentrations of fluoropyruvate. It is possible that, in the cerebral mitochondria, the inhibitory effect of fluoropyruvate manifests itself in two ways, namely through the direct, competitive inhibition of PC and through the formation of fluoroacetyl CoA, which thereby limits the availability of the potent PC activator, acetyl CoA.

The investigation of hepatic gluconeogenesis inhibition by fluoro- and chloropyruvate, chloroacetic acid and 3-chloro-1,2-propanediol (CPD), a carcinogenic chloropropanol, in rat liver slices demonstrated that chloropyruvate was a more potent inhibitor of PC activity than the other analogues examined [82]. High doses of CPD, which is proposed to be metabolized to either chloropyruvate or chloroacetic acid also inhibited gluconeogenesis. The addition of 10 mM of chloropyruvate resulted in the nearly complete inhibition of glucose production (> 95%), compared to 85% and 54% inhibition using 10 mM of fluoropyruvate and chloroacetic acid, respectively. Chloropyruvate was also a more potent inhibitor as compared to CPD, although a 45% reduction in PC activity was observed in the presence of 10 mM of CPD. Further experiments confirmed that the inhibition of gluconeogenesis was in fact due to the direct modulation of PC activity in the rat liver slices [82].

PC isolated from *Thiobacillus novellus*, a methylotrophic chemiolithautotroph, is allostericaly activated by acetyl CoA and inhibited by hydroxypyruvate [83]. Aspartate, glutamate and other dicarboxylic acids, such as α-ketoglutarate, had no effect on oxaloacetate production [84]. The addition of 2 mM of hydroxypyruvate resulted in a 35% reduction in pyruvate carboxylating ability. Initial rate studies determined that this pyruvate derivative was a non-competitive inhibitor with respect to HCO_3^- (K_i = 7.1 mM), MgATP (K_i = 5.5 mM), and, surprisingly, pyruvate (K_i = 5.4 mM), indicating that the binding of these substrates did not significantly affect the enzyme's affinity for hydroxypyruvate. It is interesting to note that hydroxypyruvate was also an uncompetitive inhibitor with respect to acetyl CoA (K_i = 3.6 mM) and reduced the Hill coefficient for acetyl CoA binding 2-fold. The inhibition patterns observed with respect to acetyl CoA suggested that the reversible binding of the activator to the enzyme increased the affinity of the inhibitor for PC. While somewhat uncommon that PC from *Thiobacillus novellus* is only mildly inhibited by hydroxypyruvate, it is further evidence that substrate derivatives, like fluoropyruvate, chloropyruvate and hydroxypyruvate, appear to down-regulate PC gluconeogenic activity in most cases, rather than completely inhibiting the enzyme.

INHIBITION BY UNIVALENT AND DIVALENT CATIONS

In the PC-catalysed pyruvate carboxylation reaction, there appears to be an absolute mechanistic requirement of two Mg^{2+} ions, which are sequestered into the BC domain active site *via* the complexation to ATP [19]. There is also a need for a divalent cation in the CT domain, generally Mn^{2+} or Zn^{2+}, which is proposed to act as a Lewis acid and promote the enolization of pyruvate [23, 69]. Although PC from various sources exhibit similar catalytic properties, there is a wide difference in responses to activation and inhibition by various cations including Ca^{2+}, Zn^{2+}, Mn^{2+}, Li^{2+} and Na^+. While this review does not attempt to be comprehensive in its discussion of the inhibition of PC by univalent and divalent cations, some of the more detailed studies of the effects of these inhibitors will be discussed.

Zn^{2+}, Cu^{2+} and Cd^{2+} are potent inhibitors of chicken liver PC [69], most likely due to the formation of inactive metal-ATP complexes. Similarly, pyruvate carboxylation catalyzed by PC from baker's yeast [85] is effectively inhibited by the addition of Sr^{2+}, Co^{2+}, Cu^{2+}, and Sn^{2+}. Interestingly, the addition of 5 μM of Na^+ resulted in a 60% decrease in the rates of the P_i:ATP exchange reaction in yeast PC, indicating that the inhibitory effect of the monovalent cation was on the biotin carboxylation reaction rather than the carboxyl transfer to pyruvate. Mn^{2+} was inhibitory in rat liver PC [86] at concentrations above 2 mM but, while appreciable concentrations of free Mn^{2+} inhibited pyruvate carboxylation, the presence of Mg^{2+} fully restored PC activity. Zn^{2+} was also a potent inhibitor of rat liver PC [86], where the addition of 0.06 mM of $ZnSO_4$ completely and irreversibly inactivated the enzyme. Further experiments suggested that even in the presence of saturating Mg^{2+}, the two Zn^{2+} ions remained coordinated to the enzyme. Unlike chicken liver PC, which is activated by the addition of Co^{2+} [87], rat liver PC is inhibited by the cation [86].

Barden and Scrutton [88] extended the initial studies of cation inhibition of PC from chicken liver. While a majority of univalent cations, including K^+, Rb^+ and Cs^+, were activators of the overall pyruvate carboxylation reaction, Li^+ inhibited not only the K^+-stimulated activity, but also PC activity in the absence of any activators. Li^+ was determined to be a non-competitive inhibitor (K_i = 42 ± 3 mM) with respect to K^+ for the K^+-stimulated pyruvate carboxylation, suggesting that Li^+ is coordinating to sites inaccessible to K^+.

Physiologically, while the inhibition of rat liver PC by Co^{2+} and Mn^{2+} appears to be of little consequence, the inhibition of PC activity by Ca^{2+} as observed in isolated rat liver [86, 88] and in intact rat liver mitochondria [89] may be a means by which the mitochondria regulates gluconeogenesis. PC activity in the intact mitochondria was determined by measuring the release of $^{14}CO_2$ from pyruvate-1-^{14}C, which allowed for the determination of both PC and pyruvate dehydrogenase activity, as well as directly measuring the concentrations of the TCA cycle intermediates. Based on this investigation, Kimmich and Rasmussen [89] determined that Ca^{2+} was a potent and specific inhibitor of PC in the intact mitochondria with the addition of 100 μM of Ca^{2+} resulting in a 75% loss of PC activity. The extent of Ca^{2+} inhibition was dependent on the Mg^{2+}/Ca^{2+} ratio, prompting further studies by Wimhurst and Manchester [86] which examined the inhibitory effect of Ca^{2+} at varying concentrations of Mg^{2+} (3.2 and 8.0 mM) using isolated rat liver PC. Ca^{2+} was a competitive inhibitor with respect to Mg^{2+} (K_i = 0.38 mM), suggesting the displacement of Mg^{2+} in the ATP binding site

with Ca^{2+}. PC was less susceptible to Ca^{2+} inhibition when Mn^{2+} was used instead of Mg^{2+}, resulting in only 20% inhibition in the presence of 1 mM of Ca^{2+} as opposed to 60% inhibition when Mg^{2+} was used as the enzyme activator [86]. The interplay between Ca^{2+} inhibition and Mg^{2+} activation of PC suggests that changes in the intracellular concentrations of these ions may have a role in regulating gluconeogenesis *via* the direct inhibition or activation of PC [89].

INHIBITORS OF THE ALLOSTERIC SITE

Acetyl Coenzyme A is an allosteric activator of many PC enzymes. Recent structural studies reveal that the acetyl CoA binding site is centered in an allosteric domain, also referred to as the tetramerization domain, which is remote from either of the two active sites [19, 20]. A number of PC inhibitors exert their influence on the enzyme through interactions in the allosteric domain, either by competing with acetyl-CoA and/or by modulating the cooperativity of acetyl CoA binding.

Dicarboxylate Allosteric Inhibitors: Aspartate, Glutamate and α-Ketoglutarate

Aspartate, α-ketoglutarate and glutamate are allosteric inhibitors of PC that, under physiological conditions, provide regulatory feedback inhibition in response to the increased production of TCA cycle intermediates. Aspartate serves as an allosteric inhibitor of microbial and fungal PC, while glutamate serves this same role in PC isolated from vertebrate sources [90-93].

Aspartate Inhibition of Microbial PC

PC from *Saccharomyces cerevisiae* is activated by acetyl CoA and inhibited by L-aspartate in an allosteric manner. In PC from *S. cerevisiae*, as in most PC enzymes inhibited by aspartate, the mode of inhibition occurs through an antagonistic effect on the activation by acyl CoA derivatives [93-97]. Aspartate increases the Hill coefficient for acetyl CoA and, conversely, acetyl CoA increases the Hill coefficient for aspartate [95-97]. The aspartate inhibition kinetics are complex, typically exhibiting non-classical behaviour. PC from *S. cerevisiae* is inhibited by aspartate in the presence of the activators acetyl CoA or palmitoyl CoA, with the addition of 10 mM aspartate resulting in a 70- to 80-fold increase in the activator concentration required for half-maximal activation [96]. In fungi, the aspartate allosteric binding site appears to be distinct from the site of acetyl CoA binding, despite the observed competitive inhibition of acetyl CoA by aspartate [95, 98]. The suggestion of independent binding sites is supported by the fact that the loss of activation by acetyl CoA upon incubation of PC with trinitrobenzene sulfonate does not correlate with any concomitant loss of inhibition by aspartate [93, 98, 99]. PC from the fungus *Aspergillus nidulans* has also been shown to be partially inhibited by aspartate [98, 100]]. Aspartate is a competitive inhibitor with respect to acetyl CoA and is non-competitive with respect to MgATP and pyruvate [98], consistent with a binding site located in the allosteric domain of the enzyme. Aspartate has an IC_{50} = 0.6 mM and displays positive cooperativity (Hill coefficient = 2.1) in the absence of acetyl-CoA. Contrary to the effect of acetyl CoA on the cooperativity of aspartate in most other PC enzymes, the Hill coefficient is *reduced* in PC from *A. nidulans* in the presence of 20 μM acetyl CoA. Both aspartate and glutamate inhibit the cytosolic isoenzyme of PC from *Rhizopus arrhizus*, though it is likely that glutamate exerts its affect by acting as an analogue of aspartate [101]. The reduced stringency in discriminating between aspartate and glutamate appears to be unique to *R. arrhizus* PC.

Bacterial PC enzymes are also reported to be inhibited by L-aspartate, though there is significant variation among the bacterial enzymes. PC from *Pseudomonas citronellis* [102, 103], *Pseudomonas fluorescens* [104], *Methanosarcina barkeri* [105] and *Azotobacter vinelandii* [106] are arranged in an $(\alpha\beta)_4$ structure where two polypeptide chains contribute to the protomer. PC with this $(\alpha\beta)_4$ structure are neither activated by acetyl CoA nor inhibited by aspartate. Conversely, bacterial PC enzymes that consist of four identical subunits $(\alpha)_4$, similar to the arrangement in the PC enzymes isolated from eukaryotic sources, are affected by the presence of acetyl CoA and aspartate. For example, the bacterial $(\alpha)_4$ enzymes isolated from thermophilic *Bacillus* [93], *Arthrobacter globiformis* [107], *Rhodobacter capsulatus* [108] and *Sinorhizobium meliloti* [109] are allosterically activated by acetyl CoA and allosterically inhibited by aspartate. This suggests that the aspartate binding site in the $(\alpha)_4$ PC enzymes is most likely located in the relatively small allosteric domain, near the acetyl CoA binding site [19, 21]. While the binding site for acetyl CoA has been identified in X-ray crystal structures of bacterial $(\alpha)_4$ enzymes [19, 21], the precise binding site for aspartate has yet to be delineated. The concentration of aspartate required for significant inhibition of PC is relatively high (~5 to 10 mM), but it has been suggested that these concentrations are, nonetheless, physiologically relevant [95].

Glutamate Inhibition of Vertebrate PC

While PC isolated from vertebrate sources is insensitive to aspartate, they are allosterically inhibited by L-glutamate [90, 110, 111] Scrutton and White [99] reported approximately 50% inhibition of the acetyl CoA activated rate of oxaloacetate synthesis in the presence of 5 mM glutamate for PC isolated from both chicken liver and rat liver. No inhibition was observed in the presence of 5 mM aspartate. Comparable concentrations of glutamate have been measured in rat liver [112, 113], suggesting that glutamate provides a physiological mechanism for the negative feedback regulation of PC activity. Glutamate appears to be a non-classical, non-competitive inhibitor with respect to acetyl CoA with an apparent K_i of 3.8 mM for chicken liver PC and 4.7 mM for rat liver PC. The Hill coefficient is insensitive to acetyl CoA and approximates to a value of 1 at all concentrations of acetyl CoA [90]. For both the chicken and rat liver PC, glutamate acts as a competitive inhibitor with respect to pyruvate, a non-competitive inhibitor with respect to bicarbonate and an uncompetitive inhibitor with respect to MgATP [90]. A similar pattern of inhibition is observed with respect to the substrates of human liver PC [111].

Inhibition by α-Ketoglutarate

Allosteric inhibition of PC by α-ketoglutarate has been characterized for PC enzymes from a number of sources, including avian liver [90], the fungi *A. nidulans* and *S. cerevisiae* [93, 98], and the prokaryotes *Bacillus* [93] and *Rhodobacter capsulatus* [108]. Modest inhibition (~15%) has also been reported in PCs isolated from the methanogens

Methanosarcina barkeri [105] and *Methanobacterium thermoautotrophicum* [114]. Unlike PC inhibition by glutamate and aspartate, inhibition by α-ketoglutarate appears to be widely distributed, but not universal. Several dicarboxylic and tricarboxylic acids are inhibitory for PC from *R. arrhizus* with 2-oxoadipate exhibiting many similar characteristics to α-ketoglutarate [101]. Inhibition properties suggest that 2-oxoadipate and aspartate inhibit the enzyme by binding to independent regulatory sites. PC from *A. nidulans* has been shown to be inhibited by the binding of α-ketoglutarate at a distinct allosteric site removed from the site of action for L-aspartate. [91, 98, 100]. Similarly, PC from avian liver is also inhibited by α-ketoglutarate binding at a distinct inhibitory site from that of glutamate [90]. The α-ketoglutarate inhibition has been extensively characterized in chicken liver PC, where a Hill coefficient of 1.5 and an apparent K_i of 3 mM has been reported [90]. As the acetyl CoA concentration is increased to saturation, the Hill coefficient for α-ketoglutarate increases to ~3. Conversely, the concentration of α-ketoglutarate has no effect on the Hill coefficient for acetyl CoA. The inhibitory site for α-ketoglutarate in chicken liver is also proposed to be distinct from the acetyl CoA binding site, since α-ketoglutarate inhibition is insensitive to reagents that eliminate acetyl CoA activation [90]. The presence of separate allosteric binding sites for acetyl CoA, α-ketoglutarate and glutamate in the chicken liver enzyme led Scrutton and White [90] to propose that the physiological ratio of these metabolites permits regulatory control over chicken liver PC activity in the mitochondria [115].

Chlorothricin

Chlorothricin is a macrolide-type antibiotic produced by *Streptomyces antibioticus* strain Tü 99 [116, 117]. It is a freely reversible competitive inhibitor of PC from *Bacillus stearothermophilus* with an apparent K_i of 173 μM that increases the Hill coefficient for acetyl CoA from 2 to 3 [118]. Chlorothricin's effect on *Bacillus stearothermophilus* PC activity is limited to its antagonistic interaction with acetyl CoA, as it does not inhibit PC in the absence of acetyl CoA [118]. However, chlorothricin does inhibit both *Azotobacter vinelandii* PC and rat liver PC in the presence and absence of acetyl CoA [119]. Chlorothricin is noncompetitive with respect to both MgATP and pyruvate and suppresses substrate activation by pyruvate while promoting MgATP substrate inhibition [118, 119]. These results lead Schindler and Zahner [118] to propose that chlorothricin binding to PC stabilizes a less active conformation of the enzyme.

Chlorothricin also reversibly inhibits PC from *Bacillus subtilis*, rat liver, chicken liver and *Azotobacter vinelandii* [118-120], with IC_{50} values ranging from 0.12 to 0.50 mM. The antibiotic has a greater affect on PC enzymes that have a more pronounced sensitivity to acetyl CoA activation. The aglycone of chlorothricin, or the chlorothricolide methyl ester, is also inhibitory against all enzymes from *B. stearothermophilus*, *A. vinelandii*, chicken liver and rat liver, though to a lesser degree [119]. The α-methyl rhamnoside portion of chlorothricin did not display any inhibitory properties, indicating that the aglycone portion of the molecule is the primary site of interaction. It is noteworthy that intact chlorothricin displays cooperative inhibition of the acetyl CoA activation in the vertebrate liver PC enzymes, while the aglycone portion of chlorothricin does not exhibit cooperativity [119]. Chlorothricin is a non-competitive inhibitor with respect to acetyl CoA in PC isolated from vertebrate liver, and is a non-competitive inhibitor with respect to all substrates [119]. Chlorothricin not only protects chicken liver PC against cold inactivation, but also protects rat liver PC against inactivation by trinitrobenzenesulfonate at the allosteric activator site, though in neither case does it confer the same degree of protection as does acetyl CoA [119, 121]. Despite these findings, Schindler and Scrutton [119] argue against chlorothricin binding in the allosteric activator binding site of vertebrate PC on the basis of its non-competitive inhibition of acetyl CoA activation and its lack of sensitivity to inhibition of rat liver PC that has an inactivated allosteric activator binding site. While there are no other reports of PC inhibition by other macrolide-type antibiotics, NMR studies probing the inhibitory effects of the polyene antibiotic, amphotericin B, on glucose metabolism in *S. cerevisiae* suggest that it may function by reducing PC activity [122].

Benzoate

Griffith *et al*. first demonstrated that PC activity is inhibited by sodium benzoate in the intact mitochondria of rat liver [123]. Even though benzoate is converted to benzoyl CoA in the rat liver [113, 123, 124] benzoyl CoA does not directly compete with acetyl CoA activation in PC from mitochondrial extracts [123, 124]. Rather, benzoate exerts its inhibitory effect on PC through its conversion to benzoyl CoA and subsequent depletion of the pool of available coenzyme A and acetyl CoA [123]. The addition of 1 mM sodium benzoate resulted in the reduction of intracellular acetyl CoA concentration from 0.5 mM (a concentration greater than K_a) to 0.1 mM (a concentration less than K_a) [113] .

Phenylacetate/Phenylacetyl CoA

Bahl *et al*. [125] first reported the suppression of liver gluconeogenesis by the inhibition of PC using phenylacetic acid. HPLC analysis of rat liver mitochondrial preparations demonstrated that phenylacetate was largely converted to phenylacetyl CoA *in vivo*. Kinetic investigations of the purified rat liver PC demonstrated that, at high acetyl CoA concentrations (> 100 μM) phenylacetyl CoA inhibits PC while the precursor phenylacetate has no inhibitory effect on the enzyme activity. At low acetyl CoA concentrations, phenylacetyl CoA inhibited the enzyme in the 5 to 50 μM range. The report of phenylacetyl CoA inhibition by Bahl *et al*. conflicts with earlier reports that both phenylacetyl CoA and benzoyl CoA are weak *activators* of PC from rat liver and thermophilic *Bacillus* [93, 110]. It is possible, therefore, that the mechanism of inhibition also includes the sequestration of mitochondrial CoA pools, similar to the proposed mode of benzoate inhibition. Phenylacetate has been used as an effective inhibitor to study the role of PC in GSIS in INS-1 cells [126, 127] and in the isolated pancreatic islets of rats [8, 127].

Acyl CoA Analogues

Several studies have revealed that a broad spectrum of acyl CoA analogues and acetyl CoA fragments will maintain low levels of allosteric activation for PC isolated from a variety of microbial and vertebrate species, indicating that the allosteric site of the enzyme is relatively tolerant to modifications of acetyl CoA [21, 36, 93, 110, 128]. In chicken liver

PC, acyl CoA analogues that have acyl chain lengths less than 4 act as allosteric activators while the longer chain acyl CoA analogues act as allosteric, competitive inhibitors with respect to acetyl CoA. Increasing the acyl chain lengths from 4 (valeryl CoA) to 10 (*n*-decanoyl CoA), resulted in increases in both the IC_{50} and Hill coefficients [110]. Contrary to chicken liver PC, acyl CoA analogues of any length are activators of the rat liver and *Bacillus* PC enzymes [36, 93]. Carboxylated acyl analogues of acetyl CoA are competitive inhibitors of rat liver PC, chicken liver PC and *Bacillus* PC with respect to acetyl CoA. Of the carboxylated analogues examined, methylmalonyl CoA exhibits the highest degree of inhibition against both chicken liver and rat liver PC [36, 110]. Furthermore, rat liver PC is also inhibited by succinyl CoA, maleyl CoA and glutaryl CoA [110].

Analogues of the diphosphoadenosine portion of acetyl CoA do not activate PC and, in fact, will act as competitive inhibitors with respect to acetyl CoA in PC from both rat and chicken liver PC [36, 110]. Unlike other acyl CoA analogues, acetoacetyl CoA is a noncompetitive inhibitor of chicken liver PC with respect to all substrates. Surprisingly, acetoacetyl CoA is also a noncompetitive inhibtor with respect to acetyl-CoA and, furthermore, the inhibition results in a decreased Hill coefficient for acetyl CoA activation in chicken liver PC [115, 129]. While acetoacetyl CoA is a much weaker inhibitor for rat liver PC and has no effect on the acetyl CoA Hill coefficient in rat liver PC [115] these data, nevertheless, suggest that acetoacetyl CoA binds at a unique allosteric site, though the physiological relevance of this effect is unclear [115].

Sulfate

The role of the sulfate ion (SO_4^{2-}) in chicken liver PC inhibition was investigated in detail by Scrutton and Fung [130]. Sulfate is a competitive inhibitor of chicken liver PC with respect to acetyl CoA, exhibiting a K_i of 1.0 to 1.3 mM at pH 7.8. The anion appears to exert its affect by interacting at the allosteric site of chicken liver PC as determined from its competitive inhibition with acetyl CoA, its noncompetitive inhibition with respect to MgATP, inorganic phosphate (oxaloacetate decarboxylation assay) and bicarbonate and its uncompetitive inhibition with respect to pyruvate. There is little cooperative effects observed for SO_4^{2-} inhibition of PC, though the Hill coefficient increases slightly with increasing concentrations of acetyl CoA. While both $(NH_4)SO_4$ and K_2SO_4 increase the rate of inactivation of chicken liver PC by *N*-ethylmaleimide, the mechanistic basis of the increased rate of inactivation remains unclear [131]. The competitive inhibition of SO_4^{2-} with respect to acetyl CoA observed in PC isolated from chicken liver appears to be a unique property of the avian enzyme since sulfate inhibition has not been observed in PC enzymes isolated from other sources, including rat liver, rat brain, *S. cerevisiae* and *Pseudomonas citronellis* [130, 132, 133].

CONCLUDING REMARKS

Much of the research focusing on the inhibition of pyruvate carboxylase that has been undertaken over the last forty or so years has greatly facilitated a preliminary understanding of the overall PC mechanism. With the recent solution of several structures of the enzyme, the results of these inhibition studies can now be fully realized and re-evaluated in light of the structure-function relationships that are currently being investigated. Since PC has been shown to be a major bottleneck in the commercial production of amino acids, the development of small molecule effectors, which regulate enzymatic activity, may also be of importance in various industrial applications. [134]. Moreover, with the vast amount of available evidence correlating PC activity and the development of debilitating metabolic diseases, such as Type II diabetes, these initial inhibitor studies may provide valuable starting points for the design of drugs that modulate the activity of the enzyme for therapeutic purposes.

ABBREVIATIONS

PC	=	Pyruvate carboxylase
BC	=	Biotin carboxylase
CT	=	Carboxyl transferase
BCCP	=	Biotin-carboxyl carrier protein
Acetyl Coenzyme A	=	Acetyl CoA

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